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CD40 Engagement on Synovial Fibroblast Up-Regulates Production of Vascular Endothelial Growth Factor

Chul-Soo Cho,*† Mi-La Cho,*† So-Youn Min,† Wan-Uk Kim,*† Do-June Min,*† Shin-Seok Lee,* Sung-Hwan Park,*† Jongseon Choe,‡ and Ho-Youn Kim2*†

We tested the impact of CD40 engagement on the production of vascular endothelial growth factor (VEGF) from rheumatoid synovial fibroblasts. Fibroblast-like synovial cells (FLS) were prepared from the synovial tissues of rheumatoid arthritis patients and cultured in the presence of CD40 ligand-transfected (CD40L+) L cells. VEGF levels were determined in the culture supernatants by ELISA. Stimulation of FLS by CD40L+ L cells increased the production of VEGF by 4.1-fold over the constitutive levels of unstimulated FLS. The CD40L on activated T cells from rheumatoid synovial fluid also up-regulated VEGF production from FLS. Neither indomethacin nor Abs to IL-1β, TNF-α, and TGF-β did affect CD40L-induced VEGF production. Stimulation of FLS with TNF-α, IL-1β, and TGF-β increased VEGF production by 1.6-, 2.0-, and 5.2-fold, respectively, and displayed an additive effect on the production of VEGF by CD40L. VEGF mRNA expression was also up-regulated by the stimulation of FLS with membranes from the CD40L+ L cells. Dexamethasone completely abrogated CD40L-induced VEGF production. In addition, pyrrolidine dithiocarbamate partially down-regulated CD40L-induced VEGF production, showing that the NF-κB pathway was partly involved in the signaling of CD40L leading to VEGF production. Collectively, these results suggest that the interaction between CD40 on synovial fibroblasts and CD40L expressed on activated T lymphocytes may be directly involved in the neovascularization in rheumatoid synovitis by enhancing the production of VEGF. The Journal of Immunology, 2000, 164: 5055–5061.

Rheumatoid arthritis (RA)3 is characterized by a pronounced tumor-like expansion of the synovium composed of proliferated synoviocytes and blood vessels (1). Neovascularization, the formation of new blood vessels, plays an important role in the perpetuation and exacerbation of rheumatoid synovitis, because the extensive migration of mononuclear cells into the synovium as well as the overgrowth of rheumatoid pannus is largely dependent on the presence of a rich vascular bed. Angiogenesis in the inflamed joints represents the net balance between the effects of angiogenic and anti-angiogenic factors. A variety of positive regulators of angiogenesis have been described in the normal and the inflamed synovium, including acidic and basic fibroblast growth factors, platelet-derived endothelial cell growth factors, TGF-α, TGF-β, angiogenin, and vascular endothelial growth factor (VEGF) (as reviewed in Refs. 2 and 3).

VEGF is a heparin-binding, dimeric glycoprotein that induces endothelial cell proliferation, angiogenesis, and capillary permeability (4, 5). VEGF plays a pivotal role in both normal and pathologic processes such as embryonic development (6), wound healing (7), solid tumor growth, and ascites formation (8). Recently, it has been documented that VEGF may be involved in the pathogenesis of RA. Significantly greater quantities of VEGF are found in the synovial fluid of RA patients than in osteoarthritis or other forms of arthritis (9, 10). VEGF is also highly expressed in the inflamed synovium of RA, where it is produced by synovial fibroblasts and activated macrophages (9, 11). An important stimulus for VEGF release is hypoxia, which up-regulates VEGF protein and mRNA expression in rheumatoid synovial cells (12–14). In addition, inflammatory mediators which play an important role in the pathogenesis of RA, including PG (15), IL-1, IL-6 (15, 16), and TGF-β (13, 17), have been described to induce VEGF.

CD40 is a 50-kDa membrane-bound type I glycoprotein described initially on B lymphocytes, but also expressed on monocytes, thymic epithelium, dendritic cells, endothelial cells, and fibroblasts (as reviewed in Refs. 18 and 19). CD40 ligand (CD40L), a member of the TNF superfamily, is a 30- to 33-kDa type II transmembrane protein expressed on activated T cells, mast cells, basophils, and eosinophils (as reviewed in Refs. 18 and 19). It has been reported that stimulation with CD40L-expressing cells or purified recombinant CD40L induces the secretion of proinflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α from monocytes, dendritic cells, epithelial cells, and fibroblasts, and augments the expression of adhesion molecules and metalloproteinase (20–24). However, the effect of CD40L on the production of VEGF by synovial cells has not been addressed to date. Before this study, we hypothesized that the interaction of CD40L on T cells with CD40 on synovial fibroblasts could stimulate neovascularization at the site of synovitis. To investigate this hypothesis, we examined whether CD40 ligation could induce VEGF production from rheumatoid synovial cells. We demonstrate herein that the ligation of CD40 upon synovial fibroblasts directly enhances VEGF in both protein and mRNA levels. Moreover, the combined stimulation of synovial fibroblasts with CD40L and cytokines, including IL-1β, TNF-α, and TGF-β, has an additive effect on VEGF production.
Dexamethasone (DEX) abrogates CD40L-induced VEGF production in a dose-dependent manner and pyrroline dithiocarbamate (PDTC) partially down-regulates CD40L-induced VEGF production, which show that the NF-κB pathway is partly involved in VEGF production by CD40 ligation.

Materials and Methods

Reagents, cell lines, and mAb

Recombinant TNF-α and IL-1β were purchased from Endogen (Woburn, MA). Recombinant IL-10 and TGF-β were purchased from R&D Systems (Minneapolis, MN). Indomethacin, DEX, and PDTC were obtained from Sigma (St. Louis, MA). Mouse fibroblastic L cells transfected with the human CD40L (CD40L+ L cells), as described previously (25), or synovial fluid (SF) T cells from patients with RA were used for CD40 activation on cultured synovial cells. Untransfected (CD40L−) L cells served as a control. mAb against human CD40 (m; mouse IgG1) obtained from Genzyme (Cambridge, MA) was used for the inhibition study. Neutralizing anti-IL-1β mAb was purchased from Endogen and anti-TNF-α mAb and anti-TGF-β mAb was obtained from R&D Systems. All isotype controls were purchased from Jackson ImmunoResearch (West Grove, PA).

Isolation and cultures of synoviocytes

Cells were isolated by enzymatic digestion of synovial tissues obtained from RA patients undergoing total joint replacement surgery. Tissues were minced into 2- to 3-mm pieces and treated for 4 h with 4 mg/ml collagenase (type I; Worthington Biochemical, Freehold, NJ) in DMEM at 37°C in 5% CO2. Dissociated cells were then centrifuged at 500 × g, resuspended in 100 ml DMEM supplemented with 10% FCS (Life Technologies, Grand Island, NY), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), and plated in 75-cm2 flasks. The cells were kept at 37°C in 5% CO2 and the culture medium was replaced every 3 days. When cells approached confluence, they were passed after diluting 1:3 with fresh medium and recultured until used.

Preparation of L cell membranes

Cell membranes were prepared from L cells as described previously (26). Briefly, cells were washed four times with PBS and suspended at a density of 3 × 106 cells/ml in lysis buffer containing 0.25 M sucrose, 10 mM Tris (pH 7.4), 10 mM NaCl, 0.1 M MgCl2, 1 mM PMSF, and 500 ng/ml polymyxin B. All manipulations were performed at 4°C. Cells were lysed by sonication three times (each burst was 90 W for 8 s) in a Braun sonicator myxin B. All manipulations were performed at 4°C. Cells were lysed by 100 ml DMEM supplemented with 10% FCS (Life Technologies, Grand Island, NY), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), and plated in 75-cm2 flasks. The cultures were kept at 37°C in 5% CO2 and the culture medium was replaced every 3 days. When cells approached confluence, they were passed after diluting 1:3 with fresh medium and recultured until used.

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VEGF production by CD40L or cytokines

A homogenous population of fibroblast-like synovial cells (FLS) from passage 4 through 8 were used for each experiment. CD40L+ L cells or CD40L− L cells were grown in RPMI 1640 supplemented with 10% FCS and irradiated with 200 Gy before use. FLS were seeded in 24-well plates at 6 × 104 cells/well in 1 ml DMEM/5% FCS and incubated at 37°C for 24 h, and medium was changed to serum-free DMEM supplemented with insulin-transferrin-selenium A (Life Technologies). After another 48-h incubation, the medium was replaced with fresh DMEM/insulin-transferrin-selenium A, and 6 × 104 CD40L+ L cells or CD40L− L cells were added to the wells at 5 × 105 cells/well. In selected wells, membranes from L cells were added instead of intact cells. As an inhibition study, anti-human CD40 mAb or unrelated isotype-matched mouse IgG1 was added to the wells in varying concentrations. Cytokines, including IL-1β, TNF-α, TGF-β, and IL-10, were added to the wells at the onset of culturing. In some experiments, FLS were stimulated with CD40L+ L cells in the presence or the absence of 1–50 μg/ml of neutralizing Abs to IL-1β, TNF-α, TGF-β, or indomethacin (10–7, 10–6, and 10–5 M) to determine whether VEGF production was indirectly mediated by IL-1, TNF-α, TGF-β, and PGE2 produced upon CD40 ligation. Various concentrations of PDTC were added to the wells at 1 h before the stimulation with CD40L+ and DEX was added at the initiation of culturing. After 24 h of incubation (unless otherwise stated), cell-free supernatants and 830 μl of cell lysates prepared from the cultures were collected and stored at −20°C until assayed. All cultures were set up in either triplicate or quadruplicate and the results are expressed as means ± SD.

Results

CD40 ligation enhances VEGF production by FLS

As shown in Fig. 1A, unstimulated FLS constitutively produced VEGF over the 24-h incubation period (206 ± 25 pg/ml). The levels of VEGF were significantly increased by the addition of CD40L+ L cells compared with those on either untreated cultures or cultures with CD40L− L cells. The VEGF levels from six separate experiments were 303 ± 54 pg/ml for CD40L+ L cells and 98 ± 52 pg/ml for CD40L− L cells. The VEGF levels from six separate experiments were 303 ± 54 pg/ml for CD40L+ L cells and 98 ± 52 pg/ml for CD40L− L cells. The VEGF levels from six separate experiments were 303 ± 54 pg/ml for CD40L+ L cells and 98 ± 52 pg/ml for CD40L− L cells. The VEGF levels from six separate experiments were 303 ± 54 pg/ml for CD40L+ L cells and 98 ± 52 pg/ml for CD40L− L cells. The VEGF levels from six separate experiments were 303 ± 54 pg/ml for CD40L+ L cells and 98 ± 52 pg/ml for CD40L− L cells. The VEGF levels from six separate experiments were 303 ± 54 pg/ml for CD40L+ L cells and 98 ± 52 pg/ml for CD40L− L cells. The VEGF levels from six separate experiments were 303 ± 54 pg/ml for CD40L+ L cells and 98 ± 52 pg/ml for CD40L− L cells. The VEGF levels from six separate experiments were 303 ± 54 pg/ml for CD40L+ L cells and 98 ± 52 pg/ml for CD40L− L cells.

To exclude the possibility that the VEGF might be produced by L cells rather than FLS, membranes were prepared from L cells and 250 μg/ml of membranes were used to stimulate FLS. The membranes prepared from CD40L+ L cells were also capable of stimulating FLS to produce VEGF. In contrast, membranes from CD40L− L cells were unable to enhance VEGF production (Fig. 1B).

The specificity of CD40L in VEGF production was demonstrated by inhibition studies using anti-CD40 mAb. Treatment of
FLS with 5 μg/ml of anti-CD40 mAb for 24 h completely abrogated the production of VEGF, whereas the equivalent concentration of isotype control mAb did not (Fig. 1C and data not shown). The increase of VEGF production by CD40 ligation was evident after only 6 h of culture and persisted up to 96 h (Fig. 2).

To determine the dose dependence of CD40L action, FLS were incubated with different numbers of L cells. As shown in Fig. 3, VEGF production was increased with increasing numbers of CD40L+ L cells in the FLS cultures, whereas CD40L- L cells did not significantly affect the production of VEGF. In all experiments, CD40L-induced VEGF production from FLS was not attributed to cellular proliferation, because the number of FLS, determined at 24 h after incubation, was the same in CD40L+ L cells and CD40L- L cells cultures (data not shown).

Induction of VEGF by activated synovial T cells from RA patients

To investigate the effect of CD40-CD40L interaction on VEGF in a physiologic condition, SF T cells from three patients with RA, rather than CD40L+ L cells, were used to induce VEGF production from FLS. Stimulation of SF T cells with PMA and ionomycin strongly increased CD40L expression on the cells analyzed by flow cytometry (~65% after 6 h stimulation; data not shown), which is consistent with an earlier report (28). When the stimulated T cells were incubated with FLS for 24 h, they significantly increased VEGF production from FLS (Fig. 4). The production of VEGF by T cells was dose dependent, as seen with the CD40L+ L cells (data not shown). Moreover, anti-CD40 mAb, but not control mAb, significantly inhibited the ability of SF T cells to produce VEGF. Together, these data demonstrate that VEGF production by CD40-CD40L interactions is physiologically relevant and

**FIGURE 1.** Effect of CD40L on VEGF production from FLS. A, FLS were cultured for 24 h with 5 × 10^5 CD40L-transfected (CD40L+) or -untransfected (CD40L-) L cells as a control. L cells were also cultured alone without FLS. B, Stimulation of FLS by CD40L-bearing membranes. Acellular preparations of membranes from CD40L+ L cells or control L cells were added to FLS. Other experimental conditions were similar to those in A. C, Inhibition test using anti-CD40 mAbs. Blocking mAbs were added to the wells at the initiation of cultures with the CD40L+ L cells and FLS. The amount of VEGF in the supernatants was determined by ELISA. Data are expressed as means ± SD of culture triplicates.

**FIGURE 2.** Time course of VEGF production by CD40 ligation. FLS were cultured with CD40L+ L cells, CD40L- L cells, or medium alone. Supernatants were collected at different periods of times, and the amount of VEGF in the supernatants was determined by ELISA. Data are expressed as means ± SD of culture triplicates.

**FIGURE 3.** Dose dependence of CD40L action. FLS were cultured in triplicate with increasing numbers of CD40L+ or CD40L- L cells, ranging from 0 to 5 × 10^5.
also up-regulated VEGF production by 1.6-fold. TGF-β (10 ng/ml) remarkably enhanced the production of VEGF by 5.2-fold over constitutive levels. VEGF production induced by IL-1β, TNF-α, and TGF-β was further increased when CD40L⁺ L cells were co-incubated, by factors of 5.9, 5.5, and 9.6, respectively, which indicated that these cytokines had an additive effect on VEGF production driven by CD40L (Fig. 5). However, IL-10 (0.1–50 ng/ml) did not affect VEGF production alone or with CD40 stimulation (Fig. 5).

**CD40L induces the expression of VEGF mRNA in FLS**

To determine whether the protein level of VEGF are reflected at the RNA level, we examined the effect of CD40L on the expression of VEGF mRNA in FLS using Northern blot analysis. Representative levels of VEGF mRNA expression in FLS cultured in the presence of membranes of CD40L⁺ and CD40L⁻ L cells are shown in Fig. 6. Unstimulated FLS or FLS stimulated with membranes of CD40L⁻ L cells showed a very low constitutive expression of VEGF mRNA (lanes 1 and 3), whereas stimulation of FLS with membranes from the CD40L⁺ L cells resulted in high levels of the VEGF mRNA (lane 2).

**DEX and PDTC inhibit VEGF production by FLS**

CD40 ligation results in the activation of transcription factors NF-κB (31, 32), and the inhibitory effect of glucocorticoids and antioxidant PDTC on NF-κB activation are well documented in other types of cells (33, 34). To verify whether the NF-κB pathway is involved in the FLS production of VEGF by CD40L⁺ L cells, we cocultured FLS and L cells for 24 h with variable concentrations of DEX and PDTC. As shown in Fig. 7A, DEX inhibited constitutive and CD40L-induced VEGF production in a dose-dependent manner; the maximum effect was achieved at a concentration of 2 μM (the highest dose tested) (Fig. 5A). In addition, the pretreatment of FLS with 400 μM PDTC 1 h before the addition of CD40L⁺ L cells also inhibited VEGF production by 55% (Fig. 5B). The inhibitory effects of DEX or PDTC were not due to nonspecific toxicity, since the viability of FLS, determined by MTT assay, was not influenced by DEX (0.1 nM-2 μM) or PDTC (10–400 μM) (data not shown).

**Discussion**

The importance of CD40-CD40L interactions in RA synovitis has been suggested because CD40 is expressed on the synovial fibroblasts. This interaction results in an increased expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin (21), which can recruit circulating leukocytes to the site of antigenic challenge. Furthermore, activation of T cells via antigenic receptors enhances CD40L expression as well as IFN-γ production, which provides an additional mechanism capable of augmenting inflammatory process. T cells expressing CD40L stimulate synovial fibroblasts and monocytes to generate a series of proinflammatory cytokines and increase

**Table I.** CD40L induction of VEGF is independent of endogenous production of PGE₂, TNF-α, IL-1β, and TGF-β

<table>
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<tr>
<th>Conc. (M)</th>
<th>Indomethacin VEGF (pg/ml)</th>
<th>Anti-TNF-α mAb VEGF (pg/ml)</th>
<th>Anti-IL-1β mAb VEGF (pg/ml)</th>
<th>Anti-TGF-β mAb VEGF (pg/ml)</th>
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<tr>
<td>0</td>
<td>599 ± 53</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>597 ± 44</td>
<td>588 ± 26</td>
<td>769 ± 44</td>
<td>723 ± 100</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>631 ± 93</td>
<td>579 ± 62</td>
<td>776 ± 12</td>
<td>732 ± 95</td>
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<tr>
<td>10⁻⁵</td>
<td>596 ± 74</td>
<td>594 ± 87</td>
<td>770 ± 18</td>
<td>717 ± 10</td>
</tr>
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</table>

* FLs were cocultured with CD40L⁺ L cells in the presence or absence of indomethacin, anti-TNF-α, anti-IL-1β, and anti-TGF-β mAb. Values are expressed as the means ± SD of culture triplicates.

* Conc., concentration.
the expression of matrix metalloproteinase (20, 21, 24). The presence of VEGF in arthritic synovium strongly suggests its participation in synovitis (9, 10), probably by promoting angiogenesis, vascular permeability (4), and microvascular stability in the synovium (35). Since VEGF appears to be very important for angiogenesis in vivo, several factors that regulate VEGF production by synovial cells are of great interest.

In this study, we investigated the role of CD40L, displayed by activated T cells, in the production of VEGF by synovial fibroblasts. The production of VEGF was markedly increased by the stimulation of FLS with either CD40L

fractions, which express high levels of CD40L, but was suppressed by anti-CD40 mAb. Moreover, SF T cells of RA patients, stimulated with PMA and ionomycin, also could up-regulate VEGF production. These observations provide strong evidence that CD40L on activated T cells is responsible for the induction of VEGF from FLS and that it induces a novel pathway of RA inflammation. At the site of synovitis, activated T cells are recruited adjacent to the resident synoviocytes by the stimulatory effect of a set of cytokines or chemokines. Consequently, it is possible that through the formation of the CD40L-CD40 bridge, infiltrating T cells induce the proliferation of synovial fibroblasts and up-regulate VEGF, which in turn, could further augment the recruitment of inflammatory cells into the synovium by promoting neovascularization. In this context, CD40L could be responsible for establishing a critical amplification loop, which leads to the persistence of synovitis.

Anti-CD40L mAb treatment is reported to suppress the development of collagen-induced arthritis, an experimental animal model of RA (36). Anti-CD40L mAb blocks the development of joint inflammation, the infiltration of inflammatory cells into synovial tissue, and the erosion of cartilage and bone. It is also documented that the prevention of collagen-induced arthritis by anti-CD40L mAb is possibly mediated by the suppression of circulating Abs to collagen and by a decrease in the production of inflammatory mediators such as NO and matrix metalloproteinase by macrophages or synovial cells (37). In this study, the finding that CD40 ligation induced VEGF production suggests that anti-CD40L mAb therapy also may block the interaction between activated T cells and synovial cells and the subsequent production of VEGF and neovascularization in vivo. The fact that angiogenesis inhibitors such as integrin αvβ3 antagonist and AGM-1470 suppress synovitis in animal models supports this idea (38, 39).

The production-enhancing effect of CD40 ligation upon inflammatory cytokines from synovial fibroblasts and monocytes (21, 22, 24, 40) has been well documented. Since IL-1 and TNF-α are also involved in the modulation of VEGF in vivo and in vitro (12, 13, 15, 17, 41), it could be expected that an increase of VEGF by
CD40 ligation would be indirectly enforced by the action of cytokines (IL-1β, TNF-α) released following CD40 ligation. Given that neither of the neutralizing Abs to IL-1β nor TNF-α affected the production of VEGF in the present study, it is unlikely that the up-regulation of VEGF may be mediated by the indirect effect of these cytokines. It also seems likely that PGE2 and TGF-β, potent inducers of VEGF, are not involved in the production of VEGF by CD40 ligation because indomethacin or anti-TGF-β Ab did not block the ability of CD40L to induce VEGF production. Together, these observations suggest that CD40L induce VEGF, independently of fibroblast-derived endogenous inducers of VEGF.

Several cytokines are able to modulate the CD40L-dependent activity in different target cells (40, 42). In the present study, IL-1β, TNF-α, and TGF-β increased the secretion of VEGF by factors of 2.0, 1.6, and 5.2, respectively, compared with the control, which is consistent with the results of previous studies (12, 13, 15, 41). Notably, the combined effect of CD40L+ L cells with these cytokines was additive and not synergistic. When one considers that the sum of stimulatory effects through independent pathways is usually additive rather than synergistic, it may be that two kinds of stimuli, CD40L and cytokines, promote VEGF production via distinct pathways. This possibility is also supported by our observation that VEGF production by CD40 ligation was not mediated by IL-1β, TNF-α, and TGF-β.

CD40L trimer induces clustering of the receptors to initiate signal transduction. Exactly how signal transduction via CD40 occurs is unknown, but multiple pathways may be involved (43, 44). CD40 ligation results in the activation of transcription factors including NF-κB (31, 32), NF-AT (45), and AP-1 (46, 47). However, the relative importance of these transcription factors as CD40 effectors is still unclear. In this study, VEGF mRNA expression was increased by CD40 ligation, indicating that up-regulated VEGF production by CD40 ligation is attributed to the transcriptional activation of the VEGF gene. In addition, DEX completely abrogated up-regulation of VEGF mediated by CD40L. Although the exact mechanism of glucocorticoid actions remains unclear, it may be that it blocks the function of NF-κB in some way, perhaps by direct physical association of the glucocorticoid receptor with the transcription factor (33). Furthermore, DEX also stimulates the transcription of IκBα, an inhibitor of NF-κB (34). Our data, along with previous reports, suggest that CD40 ligation may stimulate NF-κB expression in FLS, and NF-κB inhibition by DEX may lead to the abrogation of VEGF production. However, since glucocorticoid receptors may also interfere with AP-1 and NF-AT (47), we cannot conclude here that a decrease in the level of VEGF, caused by DEX, can be attributed only to NF-κB inhibition. With this particular possibility in mind, we tested a single effect of the NF-κB blockade on VEGF production using a dithiocarbamate derivative, PDTC, which inhibits NF-κB translocation in transformed lymphoid cell lines, fibroblasts, and monocytes (48, 49). The result was that pretreatment with PDTC was found to abrogate the CD40-mediated up-regulation of VEGF by 55%, which showed that VEGF production by CD40L was partially mediated by NF-κB activation. However, since the production of VEGF by CD40L was still evident, notwithstanding the PDTC pretreatment, transcriptional activation of the VEGF gene by CD40 ligation could not be completely dependent upon NF-κB and is probably also induced by other transcription factors.

In summary, we observed first that CD40L stimulates VEGF secretion by synovial fibroblasts. Moreover, IL-1β, TNF-α, and TGF-β augment VEGF production by CD40 engagement of synovial cells. Transcription factor, NF-κB, appears to play a some role in the CD40-mediated activation of VEGF. The present data suggest that the CD40-CD40L interaction may be one of the major regulating pathways for VEGF production in rheumatoid FLS. In this context, the strategy to disrupt the CD40-CD40L conduit could be useful to reduce neovascularization and inflammation in RA.

Acknowledgments

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References


