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Rapid Communication

Expression of Vascular Cell Adhesion Molecule-1 in Fibroblastlike Synoviocytes After Stimulation with Tumor Necrosis Factor

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Rapid expression of mRNA encoding vascular cell adhesion molecule-1 (VCAM-1) was induced by tumor necrosis factor (TNF) in fibroblast-like cells obtained from synovial tissue. Both alternatively spliced forms of VCAM-1 mRNA were detected by polymerase chain reaction in TNF-stimulated fibroblast-like synoviocytes. Western blotting analysis showed that two distinct proteins, reactive with an anti-VCAM-1 antisera, were expressed by 2 hours of TNF stimulation in both synoviocytes and human umbilical cord vein endothelial cells (HUVEC). The majority of HUVEC and synoviocytes displayed VCAM-1 surface expression after several hours of TNF stimulation. In contrast, dermal fibroblasts upregulated intercellular adhesion molecule-1 (ICAM-1) but not VCAM-1 expression in response to TNF. These results indicate that VCAM-1 and ICAM-1 expression can be differentially regulated and suggest tissue specific regulation of VCAM-1 expression. Furthermore, these findings may provide an explanation for the chronic retention and activation of long-lived lymphocytes and monocytes, which express VLA-4 (the receptor for VCAM-1), in the synovium in rheumatoid arthritis. (Am J Pathol 1992, 140:1055–1060)

Chronic connective tissue diseases and rheumatoid arthritis (RA) are associated with the proliferation and invasive growth of fibroblast-like cells, which contribute to tissue destruction. The pathogenesis of RA is also associated with the activation of circulating or resident lymphocytes and monocytes in the joint and the secretion of proinflammatory cytokines by interacting mononuclear cells.[1–3] To date, most studies investigating the mechanism of mononuclear cell activation in inflammatory synovitis have focused on macrophage/T cell and T cell/endothelial cell interactions.[4–6] However, lymphocyte and monocyte interactions with synovial fibroblasts clearly occur and may be intimately involved in the pathologic progression of RA.[7] Vascular cell adhesion molecule-1 (VCAM-1) is the ligand of VLA-4, which is expressed on lymphocytes and monocytes.[8] The interaction of nonvascular VCAM-1 with VLA-4 may play a major role in chronic synovitis, because nonvascular VCAM-1 expression has been detected by histologic staining on synovial lining cells.[9] However, the identity of the predominant VCAM-expressing cell type in the synovium is still being debated.[9,10]

VCAM-1 and ICAM-1 are induced by proinflammatory cytokines, interleukin-1 (IL-1), and tumor necrosis factor (TNF), on umbilical cord endothelial cells *in vitro*.[11,12] Both VCAM-1 and ICAM-1 also are expressed *in vivo* on vascular endothelium and postcapillary venules in inflammatory conditions.[9,13] They are both members of the immunoglobulin supergene family.[8] VCAM-1 is found in two alternatively spliced forms, one with six immunoglobulin-like domains (VCAM-1) and one with seven domains (VCAM-AS-I).[14,15] The receptor for VCAM-1 is the heterodimeric VLA-4 integrin (CD49d/CD29).[16] Unlike the leukocyte receptors for ICAM-1, CD11a/CD18 and CD11b/CD18, VLA-4 exhibits more restricted expression; it is found on lymphocytes and monocytes, but not neutrophils.[17]

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In this study we have examined the expression of VCAM-1 and its regulation on fibroblast-like synoviocytes. Our results suggest that the VCAM/VLA-4 specific ligand/receptor interaction may represent a mechanism by which lymphocytes and monocytes can interact with and are retained by fibroblast-like synoviocytes in inflammatory synovitis.

Materials and Methods

Cell Isolation and Culture

Rheumatoid synovial fibroblasts were obtained by collagenase digestion of human synovial tissue, provided by Dr. Cortland Lewis (University of Connecticut Medical Center, Newington, CT), as described.[18] Dermal fibroblasts (obtained from Dr. Steven Goldring, Massachusetts General Hospital, Boston, MA) were isolated by a trypsin/collagenase procedure[19] from human foreskin and characterized[20] as described. Fibroblast-like synoviocytes and dermal fibroblasts were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 20% fetal calf serum (FCS) (Armour Pharmaceutical Co., Kankee, IL), 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate, and 50 units/ml penicillin-50 mcg/ml streptomycin (all from Gibco). Fibroblast cultures were fed every 3–5 days and passaged every 7–14 days. Human umbilical vein endothelial cells (HUVEC) cultures were obtained from Clonetics. Fluorescent activated cell sorter (FACS) analysis for expression of various cell surface markers (CD2, CD4, CD19, CD14) demonstrated insignificant numbers (0.1–4%) of contaminating lymphocytes and monocytes in all fibroblast and HUVEC cultures. Fibroblast cultures did not contain endothelial cells as judged by the lack of uptake of fluorescent acetylated LDL, a marker for endothelial cells.[21]

Western Analysis

Fibroblast and HUVEC cultures were incubated in the absence or presence of TNF-α (200 u/ml) (Boehringer Mannheim) for the indicated times, washed three times in Mg^{2+}, Ca^{2+}-free phosphate-buffered saline (PBS), scraped off, and washed two more times before lysing. Cell pellets were lysed in sodium dodecyl (SDS) loading buffer. Cell lysates from 2×10^5 cell equivalents were separated on SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose (Schleicher and Schuell) by electroblotting and probed with a rabbit anti-peptide antisera directed to residues 1–17 of VCAM-1, prepared as described,[22] or an antisera generated against ICAM-1, purified as described.[23] Proteins binding the anti-VCAM-1 antisera or anti-ICAM-1 were detected by ^{125}I-protein A (Amersham, Arlington Heights, IL).

RNA Analysis

For Northern blotting, total RNA, isolated as described by Chomczansky and Sacchi,[24] was separated on a 1% agarose/formaldehyde gel, transferred to Nytran membrane (Schleicher and Schuell), and hybridized according to the manufacturer's protocol. VCAM-1 and VCAM-AS-I cDNAs were obtained by polymerase chain reaction (PCR) amplification of poly A^+ RNA from TNF-stimulated HUVEC using a 5′ primer consisting of nucleotide (nt). 107–130 with a XhoI site and a 3′ primer, consisting of nt. 2050–2024 with a *Pst*I site. The two cDNA fragments were isolated, digested with *Xho*I and *Pst*I, purified by gel electrophoresis, and cloned into CDM8 (Invitrogen, San Diego, CA). The VCAM-1 cDNA was excised with *Xho*I and *Pst*I, then nick-translated using a random-primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN) and used as a probe in Northern blotting. For PCR amplification of RNA, 500 ng of whole-cell RNA was subjected to reverse transcription and PCR amplification (Perkin-Elmer-Cetus GeneAMP RNA PCR kit). Reverse transcription was carried out by adding antisense VCAM-1 specific oligo (nt. 1300–1281), followed by a 35-cycle amplification with addition of sense VCAM-1 oligo (nt. 701–720) (at 94°C for 1 minute, 55°C for 2 minutes, 72°C for 4 minutes). Equal aliquots of the reaction were separated on a 1% agarose gel, blotted onto nitrocellulose, and probed with a ^{32}P-labeled VCAM-1 oligo (nt. 940–959) at 5 ng/ml at 45°C overnight before autoradiography.

FACS Analysis

Fibroblast and HUVEC cultures were stimulated with TNF as indicated, washed, and removed from tissue culture flasks by treatment with 0.02% ethylene diaminetetra acetic acid (EDTA). Cells were stained with a murine monoclonal anti-VCAM antibody (R & D Systems, Minneapolis, MN) and goat anti-mouse fluorescein isothiocyanate FITC (Jackson Immunological Research Lab, West Grove, PA) and analyzed on a FACScan flow cytometer as described.[25]

Results

Fibroblast-like synoviocyte cultures (SF) from RA patients, dermal fibroblasts (DF), and HUVEC were analyzed for protein expression by Western blotting. Unstimulated SF expressed low basal levels of VCAM-I (Figure 1A). However, two forms of VCAM-1 of about 110 kd and 100 kd were induced after 2 hours of TNF stimulation. At 8 hours, VCAM-1 expression was elevated and the higher molecular weight form was more prominently expressed. VCAM-1 expression declined at 24 hours. TNF

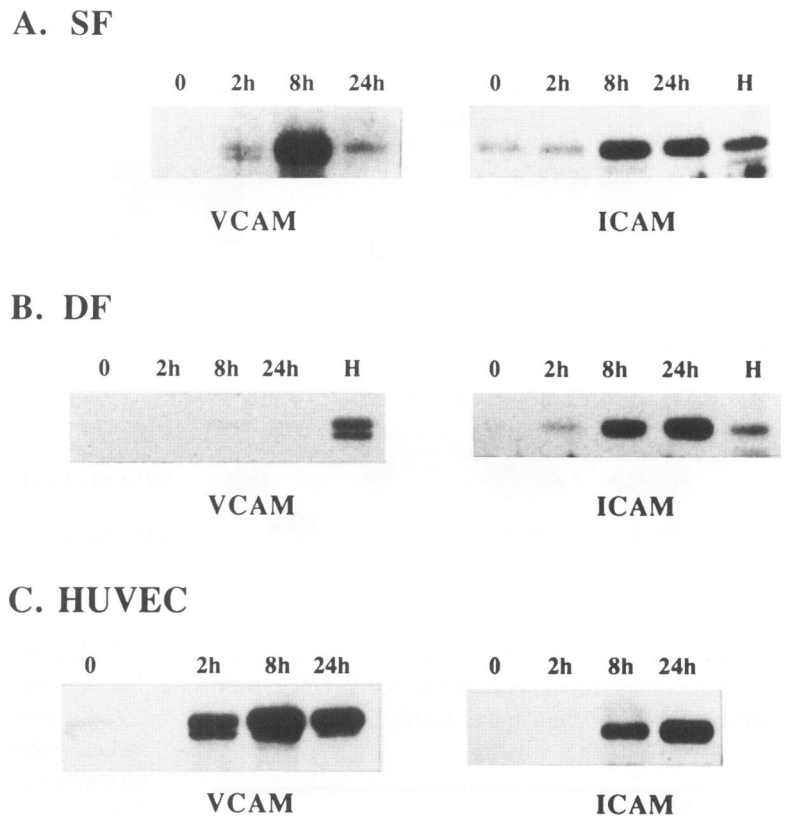

Figure 1. *Induction of VCAM-1 and ICAM-1 expression. VCAM-1 and ICAM-1 protein expression was analyzed by Western blotting in synovial fibroblasts (SF)* **(A)**, *dermal fibroblasts (DF)* **(B)**, *and HUVEC* **(C)** *stimulated for 0, 2, 8, and 24 hours with TNF as described in Materials and Methods. HUVEC control (H) represents 2-hour TNF-stimulated cells. The ICAM blot in* **(C)** *was subjected to a shorter length of autoradiographic exposure time than the ICAM blots in* **(A)** *and* **(B)**.

induced VCAM-1 expression in SF cultures at all passages tested (up to 40). Interestingly, even synovial fibroblasts from normal tissue (one sample only) could express substantial amounts of VCAM-1 (not shown). In contrast, dermal fibroblasts produced little VCAM-1 under identical conditions (Figure 1B). HUVEC cultures exhibited kinetics of VCAM-1 protein expression similar to SF cultures (Figure 1C). However, induction of ICAM-1 expression by TNF, in contrast to VCAM-1 expression, was similar in SF (Figure 1A), dermal fibroblasts (Figure 1B), and HUVEC (Figure 1C), suggesting differential regulation of VCAM-1 and ICAM-1 expression.

To determine whether VCAM-1 expression was regulated at the transcriptional level, VCAM-1 mRNA levels were analyzed by Northern blotting and PCR amplification. Total cellular RNA was extracted from HUVEC and SF, which had been treated with TNF for various length of time. The RNA was probed for the expression of VCAM-1 RNA by Northern blotting (Figure 2A, B). VCAM-1 mRNA was not detectable in uninduced HUVEC and SF (Figure 2A, B, time = 0). However, significant amounts of VCAM-1 mRNA was expressed in HUVEC (A) and SF cultures (B) by 2 hours. VCAM-1 mRNA was not detectable in RNA from dermal fibroblasts under similar conditions. Because Northern blotting does not allow distinction between the two known alternatively spliced forms of VCAM-1,[14] PCR was used to determine the presence of alternatively spliced VCAM-1 messages. Both alternatively spliced VCAM-1 mRNA species were present in HUVEC and SF (lanes 1, 2, and lanes 4, 5, respectively) and both were detectable in reduced quantities in RNA from dermal fibroblast cultures (Figure 2C, lane 8). Although our PCR amplification procedure was not quantitative, mRNA for VCAM-AS-I was more abundant as has been shown previously.[14,15]

Analysis of VCAM-1 surface expression by FACS analysis showed that the majority of both SF cells (Figure 3a) and HUVEC (Figure 3c), but not dermal fibroblasts (Figure 3b) express VCAM-1 on the cell surface after treatment with TNF. Little surface VCAM-1 was expressed in the absence of TNF treatment (not shown).

Discussion

We have demonstrated by mRNA analysis, protein analysis, and cell-surface labeling that SF express VCAM-1 in response to TNF stimulation. PCR analysis indicated that both the seven- and six-domain form of VCAM-1 are expressed in synoviocytes. Fibroblast-like synoviocytes expressed both VCAM-1 and ICAM-1 in response to TNF. However, dermal fibroblasts upregulated ICAM-1 but not VCAM-1 after TNF stimulation. This suggests that VCAM-1 and ICAM-1 expression are differentially regu-

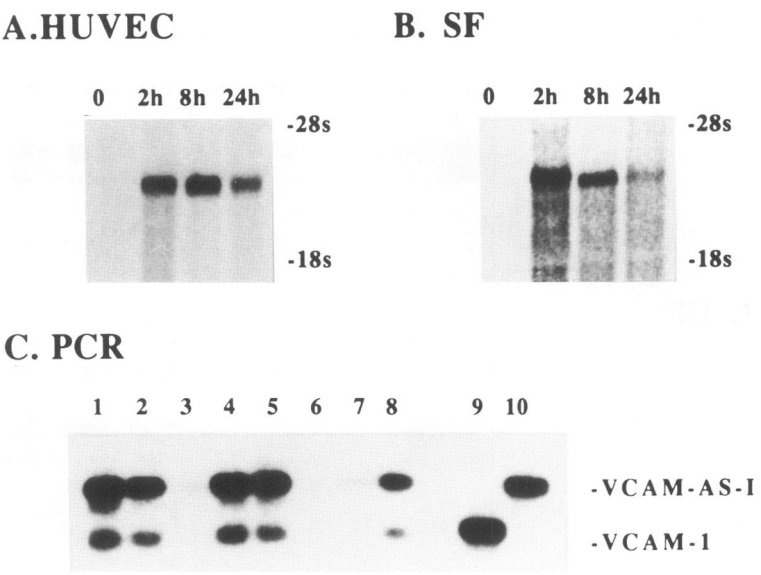

Figure 2. *Analysis of VCAM mRNA. Northern blot analysis of total RNA from HUVEC* (**A**) *and SF (synovial fibroblasts)* (**B**), *stimulated for 0, 2, 8, or 24 hours with TNF. PCR amplification of VCAM-1 and VCAM-AS-I* (**C**) *was carried out on RNA extracted from HUVEC stimulated for 2 hours (lane 1) or 24 hours (lane 2) with TNF. RNA from unstimulated rheumatoid synovial fibroblasts is represented in lane 3, cells stimulated for 2 hours are shown in lane 4, and cells stimulated for 8 hours are represented in lane 5. RNA from untreated dermal fibroblasts is shown in lane 6, 2-hour stimulated cells are shown in lane 7, and 8-hour stimulated cells are shown in lane 8. Lane 9 represents VCAM-1 and lane 10 VCAM-AS-I cDNA.*

lated in fibroblast-like synoviocytes and dermal fibroblasts and indicates that induction of VCAM-1 expression can be uncoupled from that of ICAM-1.

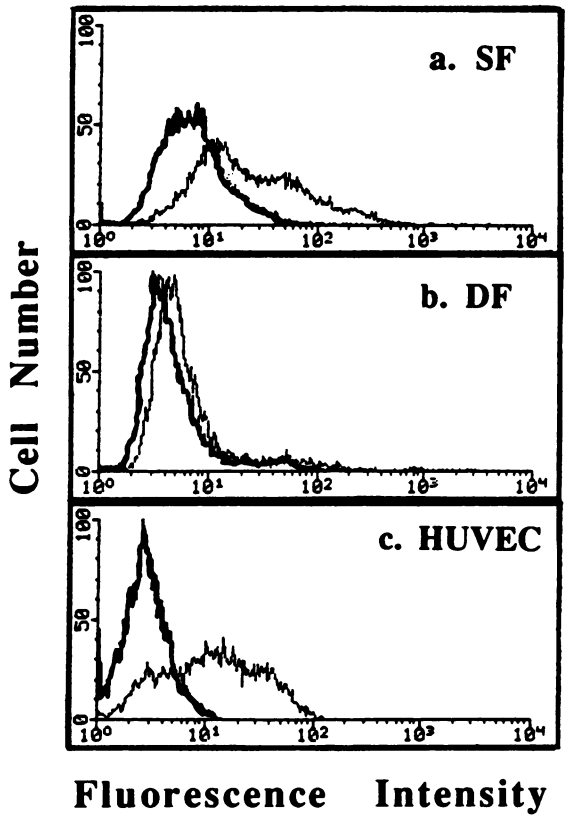

Figure 3. *VCAM-1 surface expression. Synovial fibroblasts* (**a**), *dermal fibroblasts* (**b**), *and HUVEC* (**c**) *were treated for 8 hours with TNF and stained with monoclonal anti-VCAM, followed by goat anti-mouse antibody conjugated to FITC. Heavy lines represent control samples and thin lines anti-VCAM-treated samples.*

Induction of mRNA encoding VCAM-1 in response to TNF was rapid, with the seven-domain form being expressed more prominently. We also demonstrated the induction of two protein species of about 110 kd and 100 kd reactive with an anti-VCAM-1 antisera in synoviocytes. The greatest expression of the 100 kd form was at 2 hours and its expression was more transient than that of the 110 kd species. The sizes of these proteins and kinetics of induction (Figure 1) suggest that they represent the seven- and six-domain forms of VCAM-1, VCAM-AS-I, and VCAM-1. Protein expression was consistent with the kinetics of induction of the two alternatively spliced forms of VCAM-1 mRNA (Figure 2). The differential kinetics of 110 kd and 100 kd suggest a functional difference, although both forms reportedly bind to VLA-4.[14] Although we could not detect the lower molecular weight protein in TNF-treated dermal fibroblasts (Figure 1B), PCR amplification (Figure 2C, lane 8) showed that both forms, although clearly at much lower levels, were inducible.

Basal levels of VCAM-1 and ICAM-1 protein expression is low or undetectable in synoviocytes, dermal fibroblasts, and HUVEC (Figure 1). Both VCAM-1 and ICAM-1 are upregulated by TNF in HUVEC and fibroblast-like synoviocytes (Figure 1A and C), but only ICAM-1 expression is induced in dermal fibroblasts (Figure 1B). This suggests that there is a tissue-specific regulation of VCAM-1 and not a defect in the ability of dermal fibroblasts to respond to TNF. However, we cannot distinguish between a fundamental difference in gene regulation and regulatory extrinsic factors such as the local cytokine millieu. Whether synovial fibroblasts and dermal fibroblasts used here (obtained from foreskin) represent different stages of differentiation or possess tissue specific characteristics, is presently unclear.

Surface expression of VCAM-1 on fibroblast-like synoviocytes suggests a potential mechanism by which lymphocytes or monocytes can adhere and become activated via the VLA-4/VCAM-1 interaction in the synovium. TNF and other proinflammatory cytokines are clearly elevated in the chronically inflamed joint[26] and therefore synoviocytes may display VCAM-1 *in vivo*. Furthermore, VCAM-1 expression on synoviocytes may not only function in the transient homing of lymphocytes and monocytes to the inflamed joint, but may also serve to retain these long-lived cells and prevent them from recirculating. The retention of monocytes and lymphocytes by VCAM-1–expressing synoviocytes could contribute to the activation of interacting cells by stimulation of further cytokine secretion and release of degradative enzymes by fibroblasts and activation of lymphocytes via VCAM-1/VLA-4,[27,28] leading to chronic tissue injury and destruction.

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