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CLONING OF MURINE AND RAT VASCULAR CELL ADHESION MOLECULE-1

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Vascular cell adhesion molecule-1 (VCAM1) is a member of the immunoglobulin (Ig) superfamily which interacts with the integrin very late antigen 4 (VLA4). We have cloned the cDNAs for both murine and rat VCAM1 from endotoxin-treated lung libraries. Both sequences encode proteins with seven extracellular Ig-like domains, which show 75.9% and 76.9% identity, respectively, with human VCAM1. Both murine and human cell lines show VLA4-dependent binding to COS cells transiently expressing murine and rat VCAM1. Two mAbs, M-K/1 and M-K/2, which recognize an antigen on murine bone marrow stromal cell lines, bind to murine VCAM1 expressed in COS cells and block VCAM1-dependent adhesion, confirming that these mAbs recognize murine VCAM1. © 1992 Academic Press, Inc.

VCAM1 is an adhesion molecule expressed *in vitro* on cytokine-activated endothelium (1), and *in vivo* on inflamed vascular endothelium, as well as on macrophage-like and dendritic cell types in both normal and inflamed tissue (1-3). VCAM1 interacts with the β-1 integrin VLA4 on leukocytes (4,5), and mediates both adhesion and signal transduction (6). The VCAM1/VLA4 interaction may play a pathophysiologic role both in immune responses and in leukocyte emigration to sites of inflammation (2,6). Here we report the cloning and expression of the cDNAs for murine and rat VCAM1.

MATERIALS AND METHODS

Library construction and screening. FVB female mice (Taconic Farms) were treated i.p. with lipopolysaccharide (LPS) (Difco) 4.5 mg/kg, or F344/NTAC FBR male rats (Taconic) were treated i.p. with 2.5 mg/kg LPS, for 6-8 hours, then animals sacrificed and organs frozen in

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liquid nitrogen. Lung and kidney RNA were isolated as described (7). Polyadenylated RNA was prepared by oligo(dT) cellulose chromatography (Pharmacia PL type7). Double stranded cDNA was synthesized essentially as described (8) except that MMLV reverse transcriptase (BRL) was used to obtain the first strand. Double stranded cDNA was ligated into a Not1-EcoR1 adaptor, purified on a 5%-20% potassium acetate gradient (9), ligated to EcoR1-digested λ-gt10 arms, packaged and plated on E. coli BNN102 cells (10). To obtain murine VCAM1 clones $1x10^6$ phage were plated, and replicate lifts on Genescreen Plus (NEN) filters were hybridized with a ^{32}P-labelled 30mer oligonucleotide probe (5'-AGTCTCCCCCTTCAGTAATTCAATCTCCAG-3') obtained from the sequence of a partial murine VCAM1 genomic clone (L. Burkly et al., unpublished). This sequence is highly conserved (29/30 nucleotides) between human and murine VCAM1. Filters were hybridized overnight at 65°C in plaque screen buffer (NEN) containing 10% dextran sulfate and 100 μg/ml tRNA. They were subsequently washed at 65°C in 1XSCC, 1% SDS, and exposed to film. After purification of hybridizing plaques and analysis of insert size, the two longest clones were subcloned into pNN11 for DNA sequence analysis. To screen for rat VCAM1, $2x10^6$ phage were screened with the same probe, using the above methods, except that hybridization was performed at 50°C and washing was carried out at 50°C in 4XSCC, 1% SDS.

Cell lines and antibodies. Ramos and 7OZ/3 cells were obtained from the ATCC, and grown in RPMI1640/10% fetal bovine serum. Monoclonal antibodies (mAbs) HP1/2 (IgG1) to VLA4, M-K/1 and M-K/2 (IgG2b's) to murine stromal cell antigen, PS/2 (IgG2b) to murine VLA4, and KM201 (IgG2b) to murine CD45, have been described (11-13).

COS cell transfections. Transient expression of VCAM1 cDNAs was performed as described (1,14). Briefly, 20 μg VCAM1 cDNA in pCDM8, or vector alone as control, was electroporated into $1x10^7$ COS7 cells and plated in 48-well plates at 10^5 cells/well for adhesion assays, which were performed as described (15) about 65 hr post transfection. For inhibition of adhesion, either COS cells (mAbs M-K/1, M-K/2, KM201) or lymphoid cells (mAbs HP1/2, PS/2) were incubated for 30 min at 5 μg/ml prior to the assay.

Northern analyses. 5 μg polyA+ mRNA was electrophoresed through formaldehyde agarose gels and blotted as described (1). Probes were prepared by random priming (16). The murine VCAM1 probe contained nt 1-2115 of the sequence. Pyruvate kinase was used as a control housekeeping gene for mRNA quantitation.

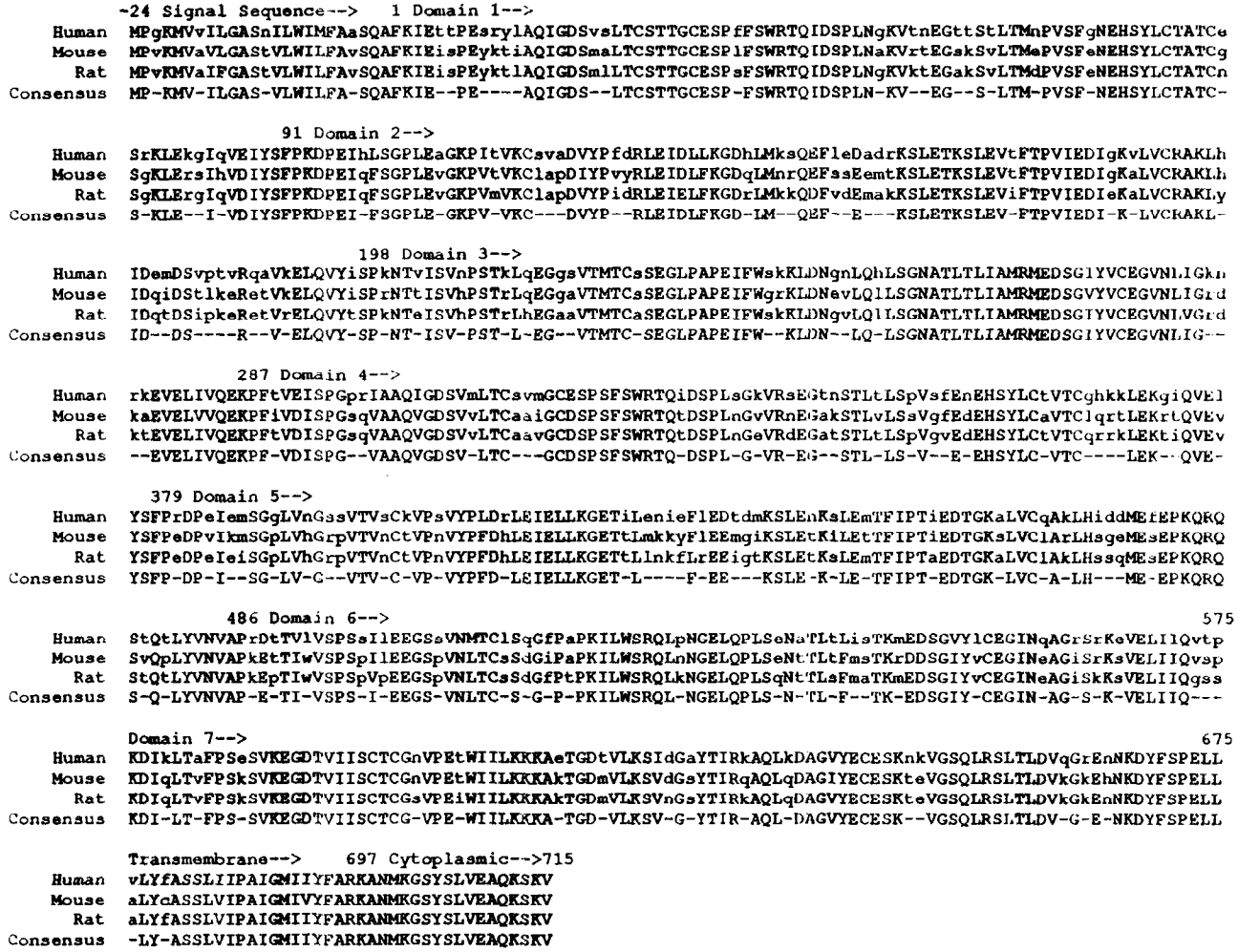
RESULTS AND DISCUSSION

Characterization of rodent VCAM1 cDNA clones. λgt10 libraries were constructed from the lungs of LPS-treated mice and rats. To obtain clones of murine VCAM1, phage were screened with a probe based on the sequence of a partial murine VCAM1 genomic clone (L. Burkly, unpublished information), highly homologous to the human cDNA sequence. 17 hybridizing plaques were purified and analysed for insert size. The two longest clones, each of 2.1 kb, were subcloned into pNN11 for DNA sequence analysis. The cDNA sequence is the

composite of the overlapping sequences of these two clones. The composite sequence is 3028 bp in length, contains 57 bp of 5' UT sequence, a 2217 bp open reading frame, and 753 bp of 3' UT sequence. To obtain clones for rat VCAM1, phage were screened with the same probe but at lower stringency. Of 4 hybridizing plaques the two longest clones, of 1.7 and 2.1 kb, were subcloned and sequenced. The rat VCAM1 sequence is the composite of these two overlapping clones, and is 3016 bp in length, contains 57 bp of 5' UT sequence, a 2217 bp open reading frame, and 741 bp of 3' UT sequence. The DNA sequences have been deposited with GenBank (Accession #;s: MB4487, MB4488).

Deduced amino acid sequences of rodent VCAM1s. The deduced amino acid sequences of murine and rat VCAM1 (Fig. 1) both contain a signal peptide of 24 amino acids (aa), followed by a 674 aa extracellular sequence containing seven Ig-like domains, a 22 aa transmembrane region, and a 19 aa cytoplasmic tail, as found in the major form of human VCAM1 (1,14,17). Both sequences contain four cysteines in Ig-like domains 1 and 4, as does the human VCAM1 sequence (1,14,17), and both contain five potential N-glycosylation sites in the extracellular domains. The sequences are aligned with that of the human seven domain form of VCAM1 (14,17) in Figure 1, and show 75.9% and 76.9% identity, respectively, with the sequence of human VCAM1. By comparison, ICAM1, an adhesion molecule and Ig superfamily member which shares many similarities with VCAM1 (1,2), shows about 50% identity between murine and human sequences (18). The percent identity between individual VCAM1 domains is not only high but similar throughout the molecule, varying from 68% to 84%. Interestingly, the the cytoplasmic tail is identical in all three molecules (Fig. 1).

Northern analyses of VCAM1 expression in inflamed lung. Northern analyses of both untreated and LPS-treated murine lung tissues are


-24 Signal Sequence--> 1 Domain 1-->
Human MPgKMVvILGASnILWIMFAaSQAFKIEttPEsrylAQIGDSvsLTCSTTGCESPfFSWRTQIDSPLNgKVtnEGttStLTMnPVSFgNEHSYLCTATCe
Mouse MPvKMVaVLGAStVLWILFAvSQAFKIEisPEyktiAQIGDSmaLTCSTTGCESPlFSWRTQIDSPLNaKVrtEGskSvLTMaPVSFeNEHSYLCTATCg
Rat MPvKMVaIFGAStVLWILFAvSQAFKIEisPEyktlAQIGDSmlLTCSTTGCESPsFSWRTQIDSPLNgKVktEGakSvLTMdPVSFeNEHSYLCTATCn
Consensus MP-KMV-ILGAS-VLWILFA-SQAFKIE--PE----AQIGDS--LTCSTTGCESP-FSWRTQIDSPLN-KV--EG--S-LTM-PVSF-NEHSYLCTATC-
91 Domain 2-->
Human SrKLEkgIqVEIYSFPKDPEIhLSGPLEaGKPItVKCsvaDVYPfdRLEIDLLKGDhLMksQEFleDadrKSLETKSLEVtFTPVIEDIgKvLVCRAKLh
Mouse SgKLErsIhVDIYSFPKDPEIqFSGPLEvGKPVtVKClapDIYPvyRLEIDLFKGDqLMnrQEFssEemtKSLETKSLEVtFTPVIEDIgKaLVCRAKLh
Rat SgKLErgIqVDIYSFPKDPEIqFSGPLEvGKPVmVKClapDVYPidRLEIELFKGDrLMkkQDFvdEmakKSLETKSLEViFTPVIEDIeKaLVCRAKLy
Consensus S-KLE--I-VDIYSFPKDPEI-FSGPLE-GKPV-VKC---DVYP--RLEIDLFKGD-LM--QEF--E---KSLETKSLEV-FTPVIEDI-K-LVCRAKL-
198 Domain 3-->
Human IDemDSvptvRqaVkELQVYiSPkNTvISVnPSTkLqEGgsVTMTCsSEGLPAPEIFWskKLDNgnLQhLSGNATLTLIAMRMEDSGlYVCEGVNLIGkn
Mouse IDqiDStlkaRetVkELQVYiSPrNTtISVhPSTrLqEGgaVTMTCsSEGLPAPEIFWgrKLDNevLQlLSGNATLTLIAMRMEDSGVYVCEGVNLIGrd
Rat IDqtDSipkeRetVrELQVYtSPkNTaISVhPSTrLhEGaaVTMTCaSEGLPAPEIFWskKLDNgvLQlLSGNATLTLIAMRMEDSGIYVCEGVNLVGrd
Consensus ID--DS----R--V-ELQVY-SP-NT-ISV-PST-L-EG--VTMTC-SEGLPAPEIFW--KLDN--LQ-LSGNATLTLIAMRMEDSGIYVCEGVNLIG--
287 Domain 4-->
Human rkEVELIVQEKPFtVEISPGprIAAQIGDSVmLTCsvmGCESPSFSWRTQiDSPLsGkVRsEGtnSTLtLSpVsfEnEHSYLCtVTCghkkLEKgiQVEl
Mouse kaEVELVVQEKPFiVDISPGsqVAAQVGDSVvLTCaaiGCDSPSFSWRTQtDSPLnGvVRnEGakSTLvLSsVgfEdEHSYLCaVTClqrtLEKrtQVEv
Rat ktEVELIVQEKPFtVDISPGsqVAAQVGDSVvLTCaavGCDSPSFSWRTQtDSPLnGeVRdEGatSTLtLSpVgvEdEHSYLCtVTCqrrkLEKtiQVEv
Consensus --EVELIVQEKPF-VDISPG--VAAQVGDSV-LTC---GCDSPSFSWRTQ-DSPL-G-VR-EG--STL-LS-V--E-EHSYLC-VTC----LEK--QVE-
379 Domain 5-->
Human YSFPrDPeIemSGgLVnGssVTVsCkVPsVYPLDrLEIELLKGETiLenieFlEDtdmKSLEnKsLEmTFIPTiEDTGKaLVCqAkLHiddMEfEPKQRQ
Mouse YSFPeDPvIkmSGpLVhGrpVTVnCtVPnVYPFDhLEIELLKGETtLmkkyFlEEmgiKSLEtKiLEtTFIPTiEDTGKsLVClArLHsgeMEsEPKQRQ
Rat YSFPeDPeIeiSGpLVhGrpVTVnCtVPnVYPFDhLEIELLKGETtLlnkfLrEEigtKSLEtKsLEmTFIPTaEDTGKaLVClAkLHssqMEsEPKQRQ
Consensus YSFP-DP-I--SG-LV-G--VTV-C-VP-VYPFD-LEIELLKGET-L----F-EE---KSLE-K-LE-TFIPT-EDTGK-LVC-A-LH---ME-EPKQRQ
486 Domain 6--> 575
Human StQtLYVNVAPrDtTVlVSPSsIlEEGSsVNMTClSqGfPaPKILWSRQLpNGELQPLSeNaTLtLisTKmEDSGVYlCEGINqAGrSrKeVELIlQvtp
Mouse SvQpLYVNVAPkEtTIwVSPSpIlEEGSpVNLTCsSdGiPaPKILWSRQLnNGELQPLSeNtTLtFmsTKrDDSGIYvCEGINeAGiSrKsVELIIQvsp
Rat StQtLYVNVAPkEpTIwVSPSpVpEEGSpVNLTCsSdGfPtPKILWSRQLkNGELQPLSqNtTLsFmaTKmEDSGIYvCEGINeAGiSkKsVELIIQgss
Consensus S-Q-LYVNVAP-E-TI-VSPS-I-EEGS-VNLTC-S-G-P-PKILWSRQL-NGELQPLS-N-TL-F--TK-EDSGIY-CEGIN-AG-S-K-VELIIQ---
Domain 7--> 675
Human KDIkLTaFPSeSVKEGDTVIISCTCGnVPEtWIILKKKAeTGDtVLKSIdGaYTIRkAQLkDAGVYECESKnkVGSQLRSLTLDVqGrEnNKDYFSPELL
Mouse KDIqLTvFPSkSVKEGDTVIISCTCGnVPEtWIILKKKAkTGDmVLKSVdGsYTIRqAQLqDAGIYECESKteVGSQLRSLTLDVkGkEhNKDYFSPELL
Rat KDIqLTvFPSkSVKEGDTVIISCTCGsVPEiWIILKKKAkTGDmVLKSVnGsYTIRkAQLqDAGVYECESKteVGSQLRSLTLDVkGkEnNKDYFSPELL
Consensus KDI-LT-FPS-SVKEGDTVIISCTCG-VPE-WIILKKKA-TGD-VLKSV-G-YTIR-AQL-DAGVYECESK--VGSQLRSLTLDV-G-E-NKDYFSPELL
Transmembrane--> 697 Cytoplasmic-->715
Human vLYfASSLIIPAIGMIIYFARKANMKGSYSLVEAQKSKV
Mouse aLYcASSLVIPAIGMIVYFARKANMKGSYSLVEAQKSKV
Rat aLYfASSLVIPAIGMIIYFARKANMKGSYSLVEAQKSKV
Consensus -LY-ASSLVIPAIGMIIYFARKANMKGSYSLVEAQKSKV

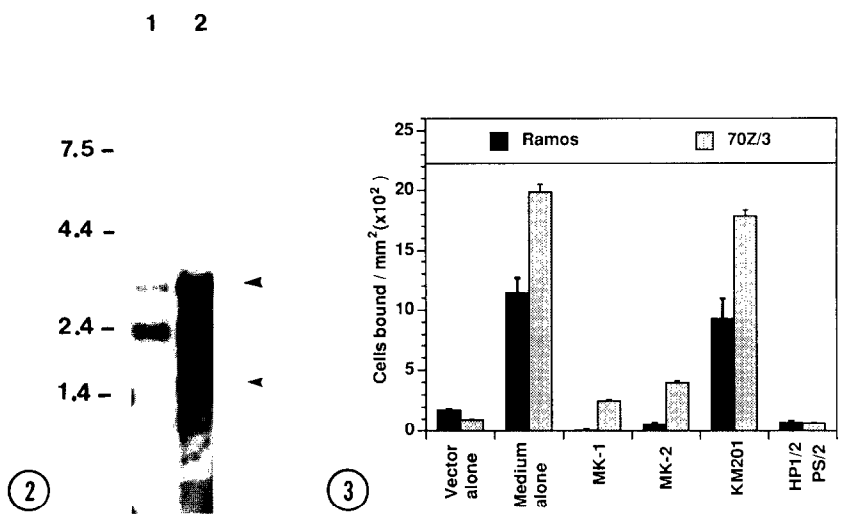


Figure 2. Induction of murine VCAM1 mRNAs in LPS-treated lung. Five micrograms of polyA+ mRNA from control (lane 1) and LPS-treated (lane 2) murine lung tissue were electrophoresed through an agarose-formaldehyde gel, transferred, and probed with a radiolabelled murine VCAM1 insert. Hybridizing mRNAs in the induced lung tissue are indicated with arrows. Molecular weight markers are indicated at right.

Figure 3. Adhesion of cell lines to COS cells transiently transfected with murine VCAM1 cDNA. The adhesion to murine VCAM1 of human Ramos cells or murine 7OZ/3 cells was examined, in the presence or absence of anti-human (HP1/2) or anti-murine (PS/2) VLA4 mAbs, or of anti-murine VCAM1 (MK-1, MK-2) or isotype-matched control (KM201) mAbs.

shown in Figure 2. In normal lung a 3.2 kb VCAM1 mRNA is present at low levels, and is induced approximately ten-fold in LPS-treated lung tissue. In the induced lung tissue another mRNA can be seen at about 1.6 kb. The nature of this RNA is currently under study. Such smaller cross-hybridizing mRNAs have also been seen in inflammed murine kidney (19), in cytokine-treated murine tubular epithelial cells (19), and in murine endothelial cells (13). Similar results have been obtained with normal and inflammed rat lung (not shown).

Transient expression of the murine VCAM1 cDNA. COS cells were transiently transfected with the cDNA for murine VCAM1, and cell adhesion assessed (Fig. 3). Both human Ramos cells and murine 7OZ/3

Figure 1. Homology between human, murine, and rat VCAM1 protein sequences. The deduced amino acid sequences are aligned, along with a consensus sequence indicating amino acid residues identical in both sequences. The beginning of each of the seven Ig-like domains is indicated (17), as are the transmembrane region and the cytoplasmic tail.

cells adhere well to transfected but not control COS cells, and this adhesion is totally inhibited by blocking anti-VLA4 mAbs. We have obtained similar results with the rat cell line RBL-1 binding to transiently expressed rat VCAM1 (not shown). We have also examined two putative mAbs, M-K/1 and M-K/2, to murine VCAM1 (12,13). Both mAbs inhibit the binding of either Ramos or 7OZ/3 cells (Fig. 3) to murine VCAM1-expressing transfectants, while the isotype-matched control mAb KM201 is without effect. In contrast, neither mAb blocks VLA4-dependent adhesion to human VCAM1 (not shown).

Adhesion molecules are important for retention of maturing lymphocyte precursors in the bone marrow, where they closely interact with stromal cells. Two mAbs, M-K/1 and M-K/2 prepared by immunizing with adherent fibroblast-like cells define a counter-receptor for VLA4 on murine stromal cell lines (12,13). The mAbs selectively interfere with B lymphocyte formation when included in long-term bone marrow cultures (13). The antigen recognized by these mAbs is constitutively expressed on cultured murine endothelial cells, correlates with the presence of mRNA hybridizing to a human VCAM1 cDNA probe, and has an N-terminal sequence homologous to that of human VCAM1 (1,13), strongly suggesting that it is, or is closely related to, murine VCAM1. The predicted N-terminal sequence of murine VCAM1 (Fig. 1) matches that obtained from the N-terminal sequence of the murine antigen. In addition, both M-K mAbs bind to COS cells transiently transfected with the murine VCAM1 cDNA but not to control COS cells (not shown), and block VLA4-dependent cell adhesion to murine VCAM1-expressing COS cells (Fig. 3). Taken jointly, the results confirm that the murine stromal cell antigen recognized by mAbs M-K/1 and M-K/2 is indeed murine VCAM1, and confirm that the VCAM1/VLA4 pathway is important in lymphopoeisis (12,13).

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