Brief Definitive Report

MOLECULAR CLONING OF CD31, A PUTATIVE INTERCELLULAR ADHESION MOLECULE CLOSELY RELATED TO CARCINOEMBRYONIC ANTIGEN

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An essential property of vascular endothelial cells is the maintenance of a complete hemocompatible monolayer (1). Preserving the integrity of this monolayer requires that cell-cell contacts are established and maintained during the normal cycles of endothelial turnover. These contacts develop through adhesion molecules expressed on the cell surface, including ubiquitous integrin receptors for fibronectin, vitronectin, collagen, and laminin, and endothelial-specific molecules such as hec7 (2).

To define further endothelial cell adhesion molecules, we generated mAbs against proliferating human umbilical vein endothelial cells (HUVEC) (3) and used them to screen HUVEC cDNA libraries transiently expressed in COS cells (4). One of these mAbs, designated 9G11, recognized a 140-kD surface glycoprotein strongly expressed at cell-cell contacts. Clones encoding 9G11 epitopes were isolated, and sequence analysis revealed that this 9G11 endothelial antigen was identical to the previously identified leukocyte antigen CD31 (D. Simmons, unpublished observations). CD31 is a widely distributed, single-chain glycoprotein of mass 130–140 kD found on leukocytes (T and B cells, monocytes, granulocytes, platelets, 40% of bone marrow cells), endothelial, and smooth muscle cells (5).

Here, we report the complete sequence of CD31/9G11 and show that it is a member of the Ig superfamily (6). The extracellular domain of CD31 contains four contiguous C2-like Ig domains and is most closely related to the intercellular adhesion molecule carcinoembryonic antigen (CEA) (7, 8).

Materials and Methods

Cell Culture, Generation of mAbs, and Immunofluorescence Staining. HUVEC were established from freshly isolated full-term umbilical cords and cultured using standard methods (3). All cell lines were obtained from the ICRF cell bank and grown in DMEM/10% FCS. mAbs against HUVEC were generated using a modification of existing methods (9). Actively proliferating HUVEC were used as the immunogen. HUVEC were stained with mAb 9G11 or an anti-ICAM-1 mAb, RR-1, followed by goat anti-mouse FITC conjugate, fixed in PBS/2% formaldehyde, and photographed on an Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY) with Ilford 400 ASA film.

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Library Construction and Screening. cDNA libraries were constructed from mRNA isolated from HUVEC grown under two types of conditions; unstimulated/proliferating, and activated for 4 h with a combination of TNF at 10 ng/ml and IL-1β at 100 ng/ml. HUVEC cDNA was cloned into the expression vector CDM8 (10). An existing cDNA library (11), constructed from RNA prepared from HL60 cells (promyelocytic leukemia) induced with PMA and cloned into the πH3M vector (4), was used to isolate CD31 cDNA clones.

To isolate cDNA clones encoding surface molecules, the library was transiently expressed in COS cells (4), and cells expressing endothelial markers were panned using the anti-HUVEC mAb 9G11 as a tissue culture supernatant at 1:10 dilution. Anti-CD31 mAbs SG134 and TM3, both murine IgG1, were obtained from Dr. D. Y. Mason, John Radcliffe Hospital, Oxford, and the platelet panel of CD31 mAbs from the Fourth Leukocyte Typing Workshop (Vienna, 1989).

Blot Hybridizations and DNA Sequencing. RNA was prepared from various cell lines and 20 μg of total RNA loaded per lane, denatured in formaldehyde, subjected to electrophoresis in 1% agarose gels, transferred to Hybond N nylon, crosslinked in a Stratalinker, and hybridized with a random-primed 9G11.3 probe (4, 11). Human genomic DNA was prepared from a full-term placenta, cut with stated restriction enzymes, subjected to electrophoresis in 1% agarose, and transferred to nylon in alkaline conditions. Blots were hybridized in Church/Gilbert buffer with random-primed 9G11.3 probe (4, 11). Double-stranded sequencing was carried out by dideoxy chain termination using Sequenase (4, 11). Both strands were sequenced using a combination of sequence specific oligonucleotide primers and Pst I subclones. Certain regions were resequenced using deazaguanidine nucleotides substituted for dGTP.

Results and Discussion

To identify novel adhesion molecules expressed on human endothelial cells, a set of mAbs were generated against HUVEC and screened for surface staining patterns. One of these mAbs, designated 9G11, gave a distinctive pattern in immunofluorescence assays on cultured endothelial cells. 9G11 staining was punctate and localized to cell margins and regions of cell-cell contacts. By contrast, ICAM-1 staining was diffuse and spread over the whole cell surface (Fig. 1, *a* and *b*). This suggested that the 9G11 antigen may be involved in cell-cell or cell-matrix contacts.

To isolate cDNA clones encoding the 9G11 antigen, cDNA libraries were constructed from HUVEC either actively proliferating or stimulated with TNF and IL-1β, and screened by transient expression in COS cells (4) using the mAb 9G11. After three rounds of expression and selection, 6 of 10 final round miniprep transfectants scored positive for surface staining with 9G11. One of these clones, 9G11.3 with the largest insert (2.4 kb) was pursued further.

Preliminary sequence analysis (see below) revealed that the 5′ end of the 9G11.3 clone was identical to the 5′ end of a cDNA clone encoding the CD31 antigen previously isolated from a PMA-stimulated HL60 library using the anti-CD31 mAbs SG134 and TM3 (D. Simmons, unpublished observations). In addition, COS cells transfected with the 9G11.3 cDNA clone gave positive surface immunofluorescence staining with all but one (5A2.G5) of the CD31 mAbs from the Fourth Leukocyte Typing Workshop (data not shown). Positive staining was obtained with mAbs SG134, CLB/HEC75, PvM 6G2, ES12F11, 5.6E, and CIT. Given the sequence identity and mAb reactivity profiles of the 9G11.3 and CD31.1 cDNA clones, hereafter, we will refer to the 9G11 antigen as CD31.

CD31 is expressed in proliferating HUVEC and HUVEC activated with a range of cytokines including TNF, IL-1β, and INF-γ plus TNF (Fig. 2). Two CD31 transcripts of ~3.5 and ~3.7 kb are present in all these states, and none of the cytokines appear to upregulate CD31 expression significantly. In addition, CD31 transcripts

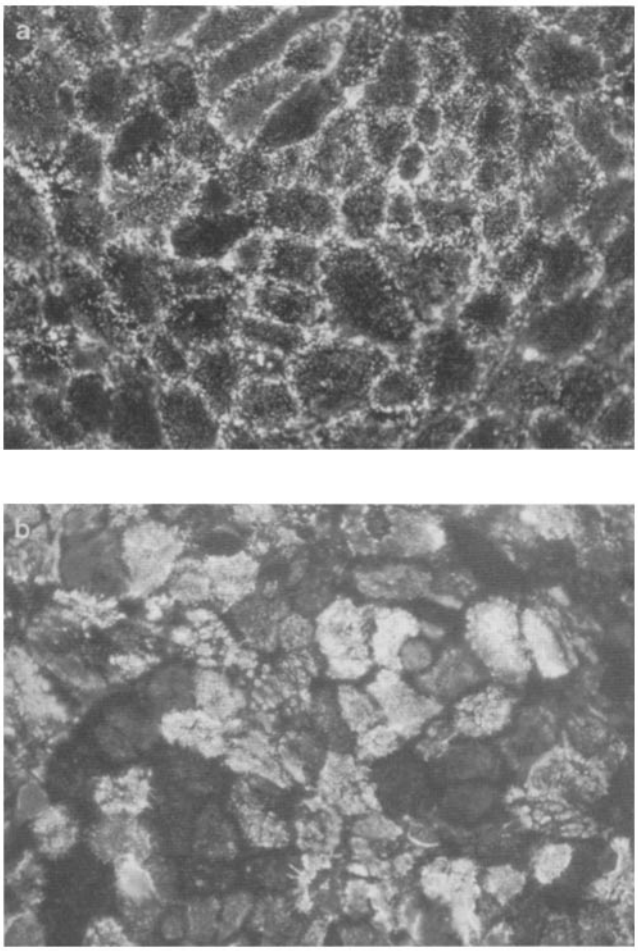

FIGURE 1. IFA analysis of CD-31. (*a*) IFA staining pattern of 9G11 mAb on confluent monolayer of HUVE cells. (*b*) IFA staining pattern of ICAM-1 mAb (RR-1) on confluent HUVEC monolayer.

were present in U937 cells (promonocytic leukemia) and U937 stimulated with INF-γ, and the myeloblastic leukemic line KG1. No transcripts were present in the melanoma lines C32 or G361, the osteosarcoma line HT1080, the erythroleukaemic line K562, the T cell lines MOLT4 or HPBALL, or the B cell lines Raji, Daudi, or JY. A sample of a metastatic colon carcinoma also contained CD31 transcripts. However, given the high level of CD31 expression in myeloid cell lines, and especially IFN-γ-activated lines, the CD31 detected there could be due to the presence of infiltrating macrophages expressing CD31.

The complete nucleotide sequence of the insert in the 9G11.3 clone consists of 2,442 bp with a single long open reading frame of 736 amino acids (Fig. 3 *a*). This sequence is identical to partial sequence obtained for a CD31.1 cDNA clone previously isolated from a PMA-activated HL60 library using anti-CD31 mAbs from the Third Leukocyte Typing Workshop (5). This clone was submitted to the Fourth Workshop and used as a COS transfectant to score CD31 mAbs. The predicted polypeptide sequence consists of an NH_2-terminal signal peptide of 23 residues (potentially

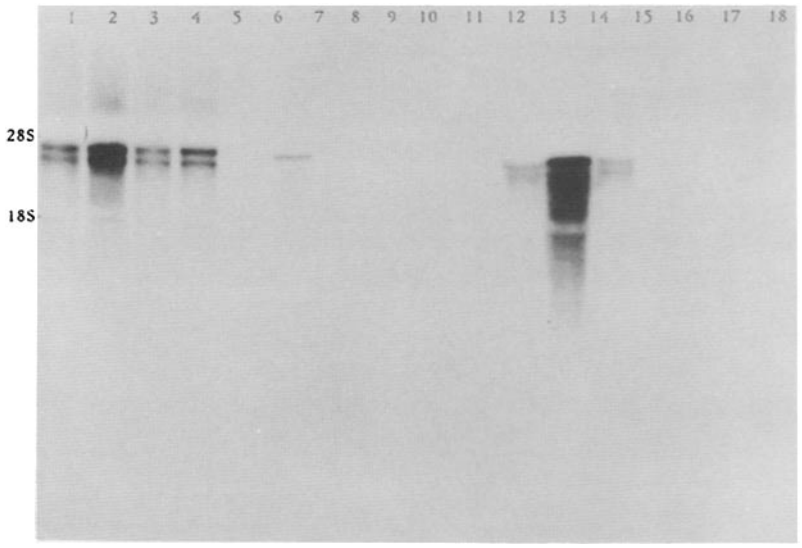

FIGURE 2. CD31 RNA expression. RNA blot analysis of 20 μg of total RNA isolated from various cell lines and tissues: lane *1*, proliferating HUVEC; lane *2*, HUVEC activated with TNF; lane *3*, HUVEC activated with IL-1β; lane *4*, HUVEC activated with TNF plus IFN-γ; lane *5*, normal colon; lane *6*, colon carcinoma; lane *7*, melanoma line C32; lane *8*, melanoma line G361; lane *9*, fibrosarcoma line HT1080; lane *10*, T cell line MOLT4; lane *11*, T cell line HPBALL; lane *12*, promonocytic leukemic line U937; lane *13*, U937 activated with IFN-γ (100 U/ml, 12 h); lane *14*, myeloblastic leukemic line KG1; lane *15*, erythroleukemic line K562; lane *16*, B cell line Raji; lane *17*, B cell line Daudi; and lane *18*, B cell line JY.

cleaved between leucine 23 and glutamic acid 24 [12]), an extracellular domain of 577 residues, a single membrane-spanning region of 19 hydrophobic residues, and a long cytoplasmic domain of 110 residues. Mature CD31 must be extensively glycosylated, as the predicted polypeptide mass is only 82 kD, compared with the observed mass of 130–140 kD. The extracellular domain contains nine potential sites (Asn-X-Ser/Thr) for the addition of N-linked carbohydrate and numerous serine/threonine rich regions for O-linked sugar attachment.

Comparison (13) of the CD31 polypeptide sequence with the NBRF database (14) revealed extensive homology to the carcinoembryonic antigen (7), the CEA-related protein NCA-1 (15), and members of the Ig superfamily (6). The extracellular domain of CD31 (residues 160–600) contains four contiguous Ig domains of the short C2 type (~40–50-residue spacing between cysteines) (Fig. 3 *b*). Monte Carlo calculations of the best alignment of CD31 with CEA gives a score of 14.73 SD above the mean score of the two sequences randomly permutated (16).

CEA has recently been shown to function as a homotypic adhesion molecule when cDNA clones encoding CEA were expressed in both hamster and human cell lines (8). Given the high degree of sequence and domain similarity between CEA and CD31, and the distinctive staining pattern of the CD3/9G1 mAbs on HUVEC, we speculate that CD31 also functions as an ICAM. CD31 may therefore play a role in maintaining the integrity of the endothelial barrier. In addition, its presence on leukocyte and tumor cell lines suggests that it may also be involved in interactions between the vascular endothelium and some circulating cells.

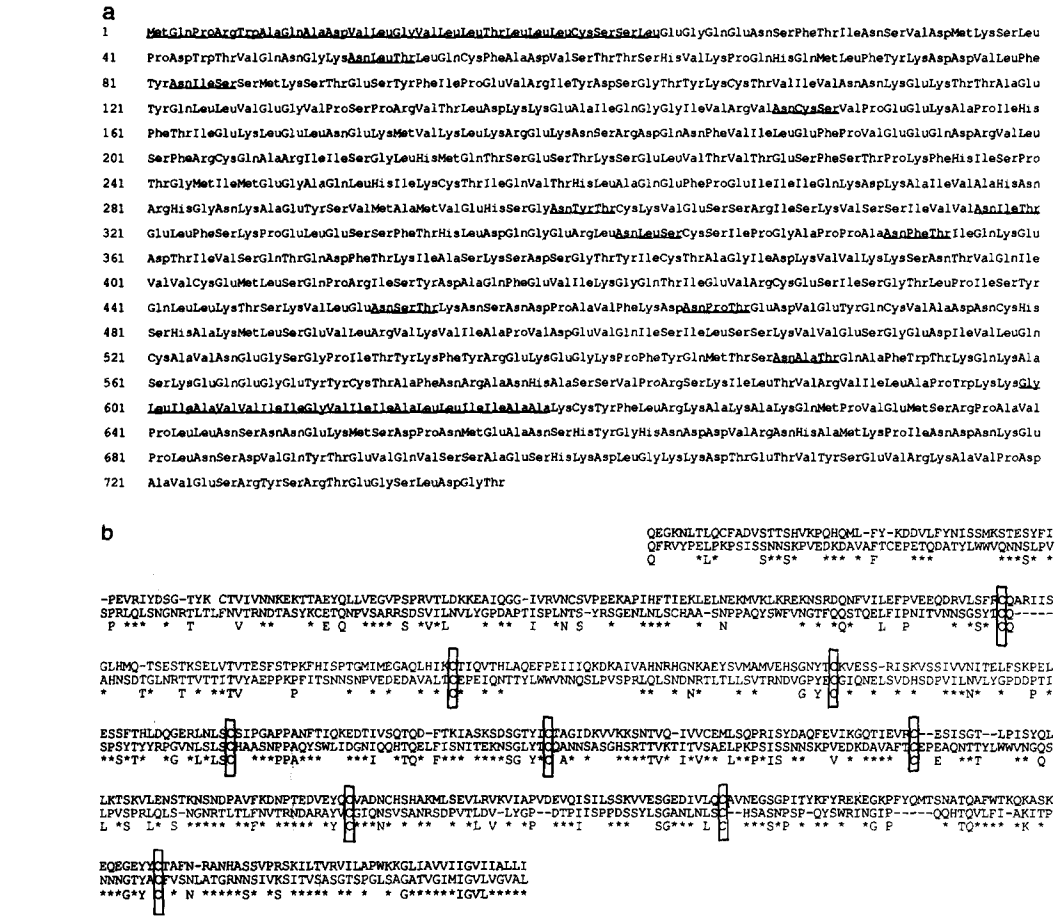

FIGURE 3. CD31 sequence analysis and alignment with carcinoembryonic antigen. (*a*) Predicted polypeptide sequence of CD31 NH_2-terminal signal peptide, potential N-linked glycosylation sites (Asn-X-Ser/Thr), and COOH-terminal transmembrane domain are underlined (We thank Dr. Peter Newman for unpublished sequence to aid in our analysis). (*b*) Alignment of CD31 peptide sequence with carcinoembryonic antigen (CEA, 7) using the ALIGN program (13, 14). Identities are represented by the residue, similarities by an asterisk. Cysteines involved in potential Ig domains are boxed.

Summary

cDNA clones encoding CD31 have been isolated by transient expression. The sequence of CD31 expressed on human umbilical vein endothelial cells (HUVEC) is identical to that expressed on the monocyte-like cell line HL60. In HUVEC, CD31 is concentrated in regions of cell-cell contacts. CD31 is a member of the Ig superfamily and is most closely related to the carcinoembryonic antigen CEA, consisting of four contiguous C2 domains. The localization of CD31 to regions of cell-cell contacts, and the sequence similarity to CEA, a known intercellular adhesion molecule (ICAM), strongly suggest that CD31 may function as an ICAM, possibly mediating

endothelial cell-cell contacts and also promoting interactions between leukocytes and endothelial cells.

We thank Dr. Peter Newman for providing unpublished sequence information to aid in our analysis. Dr. Newman's sequence data have been submitted to the EMBL/GenBank/DDBJ Data Libraries and have been given the accession number M28526.

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References

1. Gordon, J. L., and J. D. Pearson. 1987. Biology of the vascular endothelium. *In* Haemostasis and Thrombosis. A. L. Bloom and D. P. Thomas, editors. Churchill Livingstone, Edinburgh. 303–321.
2. Muller, W. A., C. M. Ratti, S. L. McDonnell, and Z. C. Chon. 1989. A human endothelial cell-restricted, externally disposed plasmalemmal protein enriched in intercellular junctions. *J. Exp. Med.* 170:399.
3. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* 52:2745.
4. Seed, B., and S. Aruffo. 1987. Molecular cloning of a CD28 cDNA by a high efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA.* 84:8573.
5. McMichael, A. J., P. C. L. Beverley, S., Cobbold, M. J. Crumpton, W. Gills, F. M. Gotch, N. Hogg, M. Horton, N. Ling, I. C. M. MacLennan, D. Y. Mason, C. Milstein, D. Spiegelhalter, and S. H. Waldmann. 1987. Leukocyte Typing III. White Cell Differentiation Antigens. Oxford University Press, Oxford. 589–590.
6. Williams, A. and A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily: domains for cell surface recognition. *Annu. Rev. Immunol.* 6:381.
7. Beuchemin, N., S. Benchimol, D. Cournoyer, A. Fuks, and C. P. Stanners. 1987. Isolation and characterization of full-length functional cDNA clones for human carcinoembryonic antigen. *Mol. Cell. Biol.* 7:3221.
8. Benchimol, S., A. Fuks, S. Jothy, N. Beauchemin, K. Shirota, and C. P. Stanners. 1989. Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. *Cell.* 57:327.
9. Oi, V. T., and L. A. Herzenberg. 1980. Immunoglobulin-producing hybrid cell lines. *In* Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi, editors. W. H. Freeman and Company. New York. 351–578.
10. Seed, B. 1987. LFA-3 is a phospholipid linked membrane protein homologous to its ligand CD2. *Nature (Lond.).* 329:840.
11. Simmons, D. L., M. W. Makgoba, and B. Seed. 1988. ICAM-1, an adhesion ligand for LFA-1, is homologous to neural cell adhesion molecule NCAM. *Nature (Lond.).* 331:624.
12. von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14:4683.
13. Wilbur, W. J., and D. J. Lipman. 1983. Rapid similarity searches of nucleic acid and protein data banks. *Proc. Natl. Acad. Sci. USA.* 80:726.
14. Dayhoff, M. O., W. C. Barker, and L. T. Hunt. 1983. Establishing homologies in protein sequences. *Methods Enzymol.* 91:524.
15. Paxton, R. J., G. Mooser, H. Pande, T. D. Lee, and J. E. Shively. 1987. Sequence analysis of carcinoembryonic antigen: identification of glycosylation sites and homology with immunoglobulin supergene family. *Proc. Natl. Acad. Sci. USA.* 84:920.
16. Doolittle, R. F. 1981. Similar amino acid sequences: chance or common ancestry. *Science (Wash. DC).* 214:149.