Identification of a Novel High Molecular Weight Protein Preferentially Expressed by Sinusoidal Endothelial Cells in Normal Human Tissues

Sergij Goerdt, Laurence J. Walsh,* George F. Murphy,* and Jordan S. Pober
Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115; *Department of Dermatology, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6142

Abstract. Mouse mAb MS-1, raised against human spleen, detects an endothelial cell antigen abundantly expressed by the sinusoidal endothelia of spleen, lymph node, liver, and adrenal cortex, but absent from non-sinusoidal continuous endothelia in these organs. Immunoelectron microscopy of splenic tissue demonstrates that the MS-1 antigen is predominantly deposited at zones of intercellular contact between adjacent sinusoidal endothelial cells. mAb MS-1 also reacts with a variable proportion of high endothelial venules in tonsil, but not in other lymphoid tissues, and with an interstitial dendritic cell population most abundant in placenta. mAb MS-1 does not react with cultured resting or mediator-activated human umbilical vein endothelial cells, dermal fibroblasts, peripheral blood mononuclear cells, or the cell lines U937, HL-60, K562 or Mo7E; it does react with the primitive myeloid cell line KG-1. mAb MS-1 immunoprecipitates a major protein of 215 kD and minor proteins of 320 and 120 kD from splenic extracts as analyzed by SDS-PAGE with reduction. These proteins are soluble in aqueous buffers. Immunoprecipitation from KG-1 cell lysates detects four proteins of 280, 300, 205, and 120 kD; the 300-, 205-, and 120-kD species, presumably corresponding to the 320-, 215-, and 120-kD species in spleen, respectively, are secreted into the media. Under nonreducing conditions, immunoprecipitates from KG-1 cell lysates or conditioned media contain one predominant 300-kD species; upon isolation and reduction, this 300-kD species separates into the previously observed 300-, 205-, and 120-kD species. Pulse-chase experiments and limited proteolysis peptide mapping suggest that the 280-kD species is a precursor of the mature 300-kD species which may be subsequently cleaved to yield the 205- and 120-kD species. Because of its size, solubility and expression pattern, the antigen recognized by mAb MS-1 is likely to be an extracellular matrix protein utilized by endothelial cells of contorted, large caliber, or leaky microvessels that lack a well-formed basement membrane.

There is remarkable endothelial heterogeneity between the blood vessel and lymphatic systems, along the length of the vascular tree and among endothelial cells in different organs. The criteria most widely applied to categorize different endothelia are whether endothelial continuity is maintained and whether and how endothelial discontinuity is achieved. Continuity is used to describe endothelia that display both a continuous endothelial cell layer and a well-formed, continuous basement membrane as assessed by EM. Discontinuity may refer either to a discontinuous endothelial cell layer or to a discontinuous or missing basement membrane or to both. The vast majority of blood vessel endothelia belong to the continuous type. Of the blood vessel endothelia that are discontinuous, bone marrow sinusoidal endothelium forms a continuous endothelial cell layer, while hepatic and splenic sinusoidal endothelia form discontinuous endothelial layers; all three lack identifiable basement membranes by EM. Lymphatic endothelia have an uninterrupted endothelial cell layer, but are discontinuous in that they attach to substratum by anchoring fibrils rather than a well-formed basement membrane. The wall of the lymphatic sinuses of the lymph node is special among the lymphatic endothelia, consisting of two layers of Weibel-Palade body containing cells that in other respects closely resemble the lymph node parenchymal reticular cells (35).

In this spectrum of human endothelial subtypes, the splenic sinusoid endothelial cells probably deviate the most from the average endothelial cell. These cells have a unique rod-shaped appearance and contain contractile, actin-myosin basal plates (6, 33, 34, 41, 53). The sinusoids are surrounded by bands of matrix molecules called ring fibers (17, 28). The presence of sinusoids in the spleen is not a stable phylogenetic feature; even closely related species like rat (sinusoid positive) and mouse (sinusoid negative) can vary in their expression (46-49, 51). In those species that
possess sinusoids, including humans, blood exits from the arterial capillaries into the parenchyma (7, 24, 46–49) and either enters the sinusoids through their open ends (24) or squeezes into the sinusoidal lumen through interendothelial slits (32). During the latter process, the endothelial cells may contract and squeeze off intracellular inclusions (e.g., Heinz bodies) from erythrocytes. It is not known how contraction of sinusoidal endothelium is regulated or whether the ring-fibers are sufficient to anchor the endothelial cells during contractions.

Until now, no mAb probe has detected features unique to sinusoidal endothelia. In contrast, several mAbs can identify antigens that are expressed by all continuous endothelial cells. Rat mAb IF10 (23) is directed against a human endothelial cell–specific 150-kD cell surface protein, which is only weakly expressed on cultured human umbilical vein endothelial cells (HUVECs), unless they are placed on a basement membrane like substrate such as Matrigel (S. Goerd, and J. S. Pofer, unpublished observations). Mouse mAb bce7 (37) recognizes a human platelet-endothelial cell 140-kD molecule (CD31) (38) that in HUVECs cultured on collagen relocates to the endothelial junctional complexes. Mouse mAb Ec6C10 (26) inhibits Ca2+-dependent homotypic bovine endothelial cell adhesion, immunoprecipitates a 135-kD molecule, and is thus thought to recognize a bovine endothelial cell cadherin. The lack of expression of these molecules in splenic and hepatic sinusoidal endothelia prompted us to search for complementary differentiation molecules that might be selectively expressed in discontinuous endothelia.

mAb MS-1 was raised by immunizing mice with human spleen and was chosen by immunohistochemical screening for differential staining of sinusoidal and continuous endothelia in frozen sections of human spleen. mAb MS-1 detects a high molecular weight secreted protein antigen that is preferentially expressed by sinusoidal endothelia in normal human tissues, i.e., spleen, lymph node, liver, and adrenal cortex. We report here the tissue distribution and initial biochemical characterization of this novel antigen.

Materials and Methods

Cells

HUVECs were isolated and cultured by the method of Gimbrone (22) as modified by Thornton et al. (54). Human dermal fibroblast strains were established following standard procedures (16). Peripheral blood mononuclear cells were isolated by density gradient centrifugation of heparin–anti–heparinase blood using Lymphocyte Separation Media (Litton Bionetics, Kensington, MD). Human leukemia cell lines U937, HL-60, K562, Mo7E, and KG-1 were a gift from James Griffin (Dana-Farber Cancer Institute, Kensington, MD). Myeloid cell lines were propagated in Iscove's Modified Dulbecco's Medium (Gibco Laboratories, Grand Island, NY), supplemented with 20% fetal bovine serum, i.e., spleen, lymph node, liver, and adrenal cortex. We report here the tissue distribution and initial biochemical characterization of this novel antigen.

Spider

Mediators and Cell Treatments

Recombinant human tumor necrosis factor-α, used at 100 U/ml, and recombinant human interferon-γ, used at 1,000 U/ml, were obtained from Biogen (Cambridge, MA). Interleukin-1β and interleukin-4, used at 10 U/ml and 100 U/ml, respectively, were purchased from Genzyme (Boston, MA). Transforming growth factor-β1, used at 10 ng/ml, was purchased from R&D Systems (Minneapolis, MN). PMA, dexamethasone, and diethylstilbestrol, each used at a concentration of 100 nM, were purchased from Sigma Chemical Co. (St. Louis, MO). Treatments of cells were performed with cultures grown in Lab-Tek chambers (VWR Scientific, Boston, MA) for 24 h, after which the Lab-Tek slides were washed in PBS and air-dried.

Antibodies

Murine mAb MS-1 was raised by immunizing 6-wk-old female Balb/c mice with whole human spleen, stored frozen at -70°C, and sonicated before injection. The immunogen was initially applied in complete Freund's adjuvant (Sigma Chemical Co.) and two boosters were administered incomplete Freund's adjuvant (Sigma Chemical Co.) at 2-wk intervals. Splenocyte fusion partners were NS-1 myeloma cells; all procedures followed standard protocols as described (23). mAb MS-1 was detected by immunoperoxidase screening (see below) of sections of human spleen. Isotyping was performed using a mouse mAb isotyping kit purchased from Amersham Corp. (Arlington Heights, IL). Other antibodies used were the isotype-matched murine control mAb K16/16 (IgG1), kindly provided by Donna Mendrick (Brigham and Women's Hospital, Boston, MA), murine mAb EI.1/2 (IgG2b) against a mesenchymal cell surface 96-kD glycoprotein (42), murine mAb Myt1 (Becton Dickinson Monoclonal Center, Mountain View, CA) against CD34, murine mAb and rabbit polyclonal antiserum against von Willebrand factor (vWF) (DakoPatts, Glostrup, Denmark), rat mAb MA/70 against CD105 (Mac-1–cholesterol oxidase), a gift of Timothy Springer, The Center for Blood Research, Boston, MA), rat mAb against CD45 (Leucocyte Common Antigen; Zymed Laboratories, San Francisco, CA), rabbit polyclonal antiserum against human glioma–derived tenascin (Gift of Harold Erickson, Department of Cell Biology, Duke University Medical Center, Durham, NC), and irrelevant rabbit serum as control.

Immunohistochemistry

Portions of human tissues, derived from discarded tissue removed at surgery, or less commonly, necropsy, were snap frozen in liquid nitrogen and stored at -70°C. The following tissues (number of samples) were examined: spleen (9), liver (2), adrenal gland (7), lymph node (3), placenta (3), skin (3), tonsil (2), kidney (2), skeletal muscle (1), heart (1), brain (1), liver (1), small intestine (1), colon (1), and pancreas (1). Frozen sections of 6–10 µm thickness were prepared with a Histostat 855 Cryostat Microtome (Reichert Scientific Instruments, Buffalo, NY), air-dried, and fixed for 10 min in acetone. Cytospin preparations of nonadherent cells and cultured cells on Lab-Tek slides (see above) were fixed similarly. Fixed slides were subjected to quenching of endogenous peroxidase in PBS containing 10 mM sodium azide and 0.1% hydrogen peroxide for 15 min, followed by a 30-min wash. Sections were incubated in 1% BSA/1% rat serum (R-3716 Sigma, MO) for 1 h at room temperature before the application of antibody. Primary antibodies, either as hybridoma culture supernatants or as concentrated antibody fluid or ascites diluted in 1% BSA in PBS, were applied for 30 min at room temperature. For double labeling, two primary antibodies made in different species (mouse/rat, mouse/rabbit) were mixed together in 1% BSA in PBS and applied simultaneously for 30 min. After three 5-min washes in PBS, an appropriate HRP–labeled secondary antibody (Jackson Immunoresearch, West Grove, PA), diluted 1:100 in 1% BSA in PBS, was applied for 30 min. For double labeling, two appropriate double-labeled grade, species-specific secondary antibodies (Jackson Immunoresearch, West Grove, PA), the first peroxidase–, the second alkaline phosphatase–labeled, were each diluted together in 1% BSA in PBS to a final concentration of 1:50. After another washing step, peroxidase was developed with aminoethyl carbazole (30 mg/ml) in 0.1 M sodium acetate buffer for 10 min, followed for double labeling by alkaline phosphatase development with Fast Blue RR salt (240 mg/ml) in 0.01% Naphthol-AS-MX-Phosphate buffer containing 600 mg/ml levamisole for 30 min. Single-labeled slides were counterstained in Gill's Hematoxylin. All slides were mounted in glycerol-gelatin.

Immunoelectron Microscopy

Normal human spleen obtained at staging laparotomy was immediately diced into 2-mm pieces and fixed in 4% paraformaldehyde in 0.01 M phosphate buffer (pH 7.2) with 7% sucrose for 3 h at 4°C. Specimens were then washed in PBS three times (20 min with agitation for each wash) and snap-frozen. Frozen sections prepared 6 µm thick were adhered to glass slides coated with 3-aminopropyltriethoxysilane, and slides subsequently

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washed in PBS and blocked for 30 min with normal horse serum diluted 1:20. Immunologic staining was achieved by the use of a sensitive avidin biotin peroxidase complex (ABC) method (Vectastain Elite, Vector Laboratories, Burlingame, CA). Briefly, sections were incubated with mAb MS-1 hybridoma supernatant or with mAb Ki/61 control hybridoma supernatant for 1 h at room temperature, washed three times in PBS; incubated with horse antimouse-biotinylated, affinity-purified, species-specific immunoglobulin (1:200) for 30 min; washed for 20 min; and finally exposed to preformed ABC (1:50) for 30 min. After additional washing in PBS for 20 min, reaction sites were developed over a 3-min interval using 3,3′-DAB (0.5 mg/ml) and 0.03% H2O2 in 0.05 M Tris HCl buffer, pH 7.6. Slides were washed in PBS for 10 min and fixed overnight in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Specimens were then dehydrated in graded ethanol and embedded in Epon using inverted bead capsules. Ultrathin sections both unstained and stained with uranyl acetate were viewed with a Hitachi 7000 transmission electron microscope to visualize electron-dense DAB reaction product.

**Metabolic Labeling**

For total labeling of KG-1 cells and KG-1 cell culture medium were isolated as described for unlabeled antigens, except that immune complexes were eluted from the Sepharose beads by shaking them two times for 5 min with 200 μl of 0.1 M glycine-HCl, pH 2.2, supplemented with 0.2% NP-40 and 0.01% BSA. The samples were then precipitated by adding 2×200 μl of a 50% TCA solution to 1 ml of glycine eluate and incubating for 1 h at 4°C. Precipitates were spun down at 12,000 g for 10 min, the supernatant was discarded, the pellet vortexed, washed in 1 ml of cold acetone, centrifuged again, and boiled in SDS-sample buffer, either containing or not containing 2-mercaptoethanol (2.5%) as indicated. Samples were run on 7.5% or 4-15% gradient polyacrylamide gels at 4.5 mA constant current overnight. Gels were fixed, equilibrated with Enlightening (NEN, Boston, MA), and exposed for autoradiography to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) at ~70°C. Radioactivity in bands was quantified either by using a Betagen gel scanner (Betagen, Cambridge, MA) or by laser densitometry (Ultra-scan, Pharmacia Fine Chemicals, Piscataway, NJ). For renaturing electrophoretically separated bands, the bands of interest were cut out of fixed and dried gels with the help of an autoradiography, put into wells of a second gel prefilled with either reducing or nonreducing sample buffer, given 15 min to equilibrate, and run at 40 mA constant current. Gels were fixed, dried, and exposed as described. In experiments designed to compare the amount of MS-1 secreted vs. that retained by KG-1 cells, one half of a 3-d-labeled cell lysate was diluted to a volume (9.5 ml) equal to one half of the conditioned medium from the same culture. Both samples were adjusted to equal Triton X-114 concentration and immunoprecipitated in parallel.

**Limited Proteolysis Peptide Mapping**

Peptide mapping was carried out by limited proteolysis during electrophoresis as pioneered by Cleveland et al. (13). Briefly, metabolically labeled KG-1 lysate samples were immunoprecipitated and subjected to SDS-PAGE as above. The gel was then equilibrated for 10 min with Enlightening without prior fixation, dried, and subjected to autoradiography. Bands of interest were cut out of the dried gel at positions indicated by the autoradiograph. These gel slices, without prior rehydration, were placed into the wells of a second gel prefilled with 40 μl of protease solution (0.5 or 0.05 mg of Staphylococcus aureus V8 protease (Cat. #P-2922: Sigma Chemical Co.), 0.125% Triton HCl, pH 6.8, 1 mM EDTA, 0.1% SDS, 10% glycerol, and trace amounts of bromphenol blue dye) and allowed to equilibrate 30 min. An additional 20 μl of protease solution were added and the samples were run into the stacking gel at 25 mA constant current. When the bromphenol blue dye front neared the interface between stacking and separating gels, current was turned off for 30 min before electrophoresis was completed at 40 mA constant current. The final gel was fixed, dried, and exposed as described.

**Dot Blot Assay**

15 μl of Mono-Q–purified human glioma–derived tenasin (150 μg/ml; gift of Harold Erickson, Department of Cell Biology, Duke University Medical Center, Durham, NC) and 15 μl of purified plasma-derived fibronectin (2 mg/ml; New York Blood Center, New York, NY), serving as a negative control, were applied to a 0.45 nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was dried, preincubated with 5% nonfat dried milk and 0.2% FCS containing RPMI 1640 for 1 h, incubated with ascitic fluid or rabbit antisera for 1 h at a 1:1,000 dilution in 20% FCS containing RPMI 1640, washed with 10 mM Tris-HCl, pH 8.5, 150 mM NaCl with 0.005% Tween 20, incubated for another hour with a 1:3,000 dilution of the appropriate alkaline phosphatase–labeled secondary antibody (Jackson Im-

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mAb M1/70 of capillaries and larger vessels strongly stained the sinusoidal endothelium, but did not stain other endothelia or any other structure in the spleen, was selected and subsequently cloned. This hybridoma, named MS-1, yielded stable subclones that produced high titer antibody of IgG1 isotype in either culture supernatant or ascites.

Results

To identify molecular markers of discontinuous endothelium, mAbs against whole human spleen were produced in mice and screened for their ability to bind selectively to splenic sinusoidal endothelial cells. From 473 hybridomas in one fusion, one hybridoma, producing antibody that stained the sinusoidal endothelium, but did not stain other endothelia or any other structure in the spleen, was selected and subsequently cloned. This hybridoma, named MS-1, yielded stable subclones that produced high titer antibody of IgG1 isotype in either culture supernatant or ascites.

Tissue Distribution

In the spleen, mAb MS-1 strongly stained the sinusoidal endothelium in the red pulp outlining the sinusoidal spaces (Fig. 1, A and B). No staining was observed in the follicular areas nor in the capsular or trabecular systems including the larger arteries and veins (Fig. 1 A). Double labeling with mAb IF10 (23), specific for all continuous endothelial cells, whether arterial, arteriolar, capillary, or venular, revealed a staining pattern almost complementary for the two antibodies (Fig. 1 C). Only the endothelium of the transitional venules, into which the sinuses open, exhibited limited double labeling (Fig. 1 D). However, MS-1 staining of these transitional venules was relatively weak compared to that of the sinusoidal endothelium. As expected, mAb MS-1 and antivWF antibodies double labeled the sinusoidal endothelium, while anti-vWF antibodies stained all other continuous endothelium and platelets as well. No double labeling between MS-1 and anti-CD11b antibodies was observed, the latter staining myeloid cells mainly in the red pulp and in the outer edge of the follicles.

Ultrastructural examination (Fig. 2) confirmed that the antigen recognized by mAb MS-1 is, in spleen, present exclusively on sinusoidal endothelial cells. The antigen was observed in these cells both as small clusters of cytoplasmic reactivity, as well as on the entire perimeter of the plasma membrane (Fig. 2, A and B), including the abluminal surface (Fig. 2 E) where the cell interfaces with underlying interstitium. Staining was most intense at points of sinusoidal endothelial cell apposition (Fig. 2 F). Immuno-electron microscopy corroborated complete absence of MS-1 reactivity on nonsinusoidal continuous endothelium in the spleen (Fig. 2, B and D).

In the liver, MS-1–positive cells were identified in the sinuses (Fig. 1 E). MS-1 staining did not completely outline the sinusoidal channels, but rather appeared to stain the perinuclear region of single cells. As a result, it could not be decided from the single antibody staining pattern whether these cells were the hepatic sinusoidal endothelial cells or some other hepatic sinusoidal cell population, e.g., the Kupffer cells. To address this question, we performed double-labeling experiments using a combination of MS-1 and anti-CD11b antibodies (Fig. 1 F) or MS-1 and anti-CD45 antibodies. Such double-labeled sections of liver showed two distinct populations of cells adjacent to one another in the hepatic sinuses: the Kupffer cells expressing CD11b and CD45, and the hepatic sinusoidal endothelial cells expressing the MS-1 antigen. Since vWF and the IF10-antigen are expressed at very low levels in the hepatic sinusoidal endothelial cells and are not useful for double labeling in this organ, we cannot formally exclude that the MS-1–positive cell population might comprise some other sinusoidal element, e.g., the Ito- or fat-storing cell, but in the light of the overall staining pattern of mAb MS-1, we regard this possibility as highly unlikely.

Staining of MS-1 in the adrenal gland was complex. This organ has a blood vascular system in its cortex whose vessels are of large caliber and irregular outline and are considered, for this reason, to be sinuses. In contrast to the sinusoidal endothelial cells of spleen and liver, however, adrenal cortical sinusoidal endothelial cells sit on a well-formed basement membrane and form a continuous layer of endothelial cells, which express the continuous endothelial antigens CD31 and IF10. The MS-1 staining pattern in the cortical sinuses was focal (Fig. 1 G). Double labeling with MS-1 and IF10 mAbs demonstrated that most MS-1–positive cells were also positive for IF10 (Fig. 1 H). In contrast, mAb IF10 recognized much more of the total sinusoidal area than MS-1.

The lymph node has a delicate system of lymphatic sinuses: the marginal sinus just below the capsule collecting the afferent lymph; the intermediate sinuses surrounding the follicles; and the medullary sinuses recollecting the lymph for outbound transport. In this organ, mAb MS-1 detected a network of lymphatic endothelial cells in all of the lymphatic sinuses (Fig. 3 A). In transverse sections of lymphatic sinuses the MS-1–positive cells could be seen to line the ves-
Figure 2. Ultrastructural localization of MS-1 antigen in normal human spleen. Specimens were labeled using immunoperoxidase. Sites of reactivity display electron dense DAB reaction product. Nuclei are stained with uranyl acetate. The cytoplasm is unstained. (A) Splenic sinusoidal endothelial cell surrounding luminal space (L) shows DAB reaction product, indicating sites of MS-1 antigen reactivity, circumferentially distributed along the plasma membrane and in small aggregates within the cytoplasm. (B) Small splenic arteriole lined by endothelium devoid of MS-1 reactivity; granular material within lumen (L) represents serum protein. (C) Higher magnification of luminal plasma membrane of sinusoidal endothelial cell depicted in A; note fine, electron dense DAB reaction product (arrowhead). (D) Higher magnification of arteriolar endothelial cell depicted in B; there is no DAB reaction product decorating the endothelial cell which rests upon a well-developed basement membrane (arrows). (E) These sinusoidal endothelial cells exhibited little luminal (L) reactivity, although multiple foci (arrowheads) of reactivity are observed at interfaces between abluminal plasma membranes and interstitial tissue. A patchy cytoplasmic pattern is apparent above these regions. (F) Plasma membrane activity for MS-1 antigen was often strong and continuous in zones of apposition between adjacent sinusoidal endothelial cells.

Figure 3. Tissue distribution of MS-1 antigen by immunohistochemistry. Lymph node (A–D), placenta (E), tonsil (F), and KG-1 cells (G) were either labeled with mAb MS-1 using immunoperoxidase (red reaction product) and hematoxylin as a counterstain (blue) (A, B, E, F, and G), or double labeled with mAb MS-1 (red) and either anticontinuous endothelial cell mAb IF10 (D) or an anti-vWF polyclonal antibody (C) using alkaline-phosphatase (blue reaction product). (A) In the lymph node, the marginal sinuses below the capsule (arrowheads) and the intermediate sinuses surrounding the follicles stain strongly positive with mAb MS-1. (B) At higher magnification, a transverse section of a lymphatic sinus shows MS-1-positive cells lining the vessel wall. (C) Most of the MS-1-positive cells in the lymphatic sinuses (red) are double labeled with anti-vWF (blue), while continuous endothelia are exclusively vWF positive. (D) Staining by mAb IF10 of the continuous endothelium of capillaries, high endothelial venules and larger vessels (blue), and by mAb MS-1 of the endothelial cells of the lymphatic sinuses is complementary. (E) In placenta, an interstitial cell population, predominantly found in the villi, is strongly stained by mAb MS-1. Endothelial cells of placental blood vessels are MS-1 negative (arrowhead). (F) A tonsillar high endothelial venule is reactive with mAb MS-1 (center). (G) KG-1 cells exhibit focal and granular MS-1 staining in ~40% of cells.
Table I. Reaction of mAb MS-1 with Human Cells and Cell Lines, Stimulated with Various Cytokines and Drugs

| Cell type                        | MS-1 staining |
|----------------------------------|---------------|
| HUVEC                            | -             |
| control                          | -             |
| + interleukin-1β                 | -             |
| + interleukin-4                  | -             |
| + tumor necrosis factor-α        | -             |
| + interferon-γ                   | -             |
| + transforming growth factor-β1  | -             |
| + PMA                            | -             |
| + dexamethasone                  | -             |
| + diethylstilbestrol             | -             |
| Fibroblasts                      | -             |
| Peripheral blood mononuclear cells | -         |
| U937                             | -             |
| HL-60                            | -             |
| K562                             | -             |
| Mo7E                             | -             |
| KG-1                             | +             |
| KG-1a                            | +             |

Most of these MS-1-positive cells were found to coexpress vWF (Fig. 3 C), consistent with the ultrastructural finding that lymphatic sinus endothelial cells contain Weibel-Palade bodies (35) and, thus, underlining their endothelial nature. These MS-1-positive endothelial cells did not coexpress the 1F10 antigen, which instead marked all continuous endothelia in the lymph node, including the high endothelial venules (Fig. 3 D). Cells expressing CD11b or CD45 were only occasionally double labeled with MS-1 and were not very common in the lymphatic sinuses. This is in accord with the suggestion proposing that the so-called sinus histiocytes, traversing the sinus lumen in cellular cords, may be a subset of the sinus lining cells rather than true tissue macrophages (35, 57).

In the tonsil, the only secondary human lymphatic organ without a blood vascular or lymphatic sinus system, a subset of postcapillary venules, which appear to be the high endothelial venules of this organ, were stained with mAb MS-1 (Fig. 3 F). It cannot be excluded, however, that MS-1 antigen expression on this subset of tonsillar endothelial cells is due to inflammatory induction, since both specimens of tonsil examined showed, at least focally, reactive changes, including neutrophil infiltration.

The vasculature in all other organs examined was MS-1 negative. However, in several organs, mAb MS-1 was found to stain varying numbers of interstitial cells. MS-1-positive interstitial cells were most prominent in placenta (Fig. 3 E), but were also seen in skin, gut, pancreas, and cardiac and skeletal muscle. In contrast, no MS-1-positive cells were found in brain. In double-labeling experiments, MS-1-positive interstitial cells were demonstrated to be 1F10 negative. Thus, these MS-1-positive cells could be lymphatic endothelial cells and/or subtypes of tissue macrophages and interstitial dendritic cells (52), such as placent macrophages (36) and dermal dendritic cells (39).

Cells and Cell Lines

HUVECs in culture, either unstimulated or stimulated by various cytokines and drugs, and three different strains of dermal fibroblasts did not express the MS-1 antigen (Table I). In light of the presence of MS-1-positive interstitial cells in various organs, some of which may be bone marrow derived, peripheral blood mononuclear cells, myeloid leukemia cell lines U937, HL-60, KG-1, and KG-1a, erythroleukemia cell line K562, and megakaryoblastic cell line Mo7E were examined (Table I). Of these cell types, only the early hematopoietic progenitor cell lines KG-1 and KG-1a expressed the MS-1 antigen in a somewhat focal and granular pattern (Fig. 3 G). No cell surface expression of the MS-1 an-
Figure 5. Solubility and distribution of MS-1 antigen from metabolically labeled KG-1 cells, analyzed by SDS-PAGE (7.5% gel) and autoradiography. MS-1 immunoprecipitates are prepared from Triton X-114 cell extracts before partition (PRE; lane 1) and after phase separation into aqueous (AQ, lane 2) and detergent (DET, lane 3) phases. As quantified by gel scanning, \( \approx 80\% \) of the antigen partitions into the aqueous phase. In a separate experiment, samples of Triton X-114 cell lysate before partition (KG-1L, lanes 4 and 5) and of conditioned medium (KG-1M, lanes 6 and 7) from the same culture are immunoprecipitated in parallel with mAb MS-1 (lanes 5 and 6) or with isotype-matched irrelevant mAb K 16/16 (lanes 4 and 7). As quantified by gel scanning, \( \approx 40\% \) of the total MS-1 antigen was recovered from the medium after a 72-h labeling period.

**Identification of the Protein Recognized by mAb MS-1**

MAb MS-1 immunoprecipitated a 215-kD protein (reduced) from spleen extracts (Fig. 4), which was strongly stained by silver. The molecular weight of this band was estimated from an electrophoretic migration slightly lower than laminin B1 chain (210 kD) and slightly ahead of the mature form of vWF (220 kD) (not shown). Fainter staining bands at \( \sim 320 \) and \( \sim 120 \) kD (reduced) were also consistently identified in the immunoprecipitates from the spleen. No other silver-stained bands could be identified as specific in MS-1 precipitates. Most of the MS-1 antigen was found in the aqueous phase of Triton X-114 extracts, and antigen could also be extracted from spleen using aqueous buffers. Aqueously extracted antigen remained soluble despite ultracentrifugation (1 h at 100,000 g).

Immunoprecipitation from KG-1 cell lysates yielded four bands: a faint, thin band at \( \sim 280 \) kD, stronger bands at \( \sim 300 \) and \( \sim 205 \) kD, and a broad band of variable intensity at \( \sim 120 \) kD (all reduced) (Figs. 4 and 5). Approximately 80% of the MS-1 antigen recovered after phase separation with Triton X-114, was found in the aqueous phase (Fig. 5). Analysis of KG-1 cell culture medium revealed that the 300-, 205-, and 120-kD species were secreted (Figs. 4 and 5); secreted MS-1 antigen accounted for \( \approx 40\% \) of the total (cellular and secreted) MS-1 antigen synthesized during a 72-h labeling period (Fig. 5). MS-1 precipitates from the KG-1 supernatants electrophoresed without prior reduction, appeared as one predominant 300-kD band (Fig. 6 A). When cut out from the gel and reelectrophoresed under reducing conditions, this unreduced 300-kD band separates into three major bands corresponding to the previously observed 300-, 205-, and 120-kD species (Fig. 6 B).

To further analyze the relationship among the immunopre-
Figure 7. Pulse-chase analysis of MS-1 antigen in KG-1 cells. Cells were labeled with $^{35}$S-methionine and $^{35}$S-cysteine for 50 min (lanes 1–9) and subsequently chased in cold medium for 0 min (lanes 1 and 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), 8 h (lane 7), 24 h (lane 8), and 48 h (lane 9). Cells were lysed and immunoprecipitation was carried out with mAb MS-1 (lanes 2–9) or isotype-matched irrelevant mAb K16/16 as control (lane 1). Samples were analyzed by SDS-PAGE (7.5% gel) and autoradiography.

Figure 8. Limited proteolysis of MS-1 antigen species from metabolically labeled KG-1 cell lysates by Staphylococcus aureus V8 protease. The 300-kD (lanes 2 and 5), 205-kD (lanes 3 and 4) and 120-kD (lanes 1 and 6) species were isolated by SDS-PAGE, subjected to Staphylococcus aureus V8 protease at high (lanes 1–3) or low (lanes 4–6) concentrations, reelectrophoresed on a 7.5–15% linear gradient gel, and analyzed by autoradiography. Note the shared fragments of the 300- and 205-kD species (arrowheads) and of the 300- and 120-kD species (arrows).

Comparison with Other High Molecular Weight Secreted Proteins

Distribution among endothelial cells in various tissues, extracellular secretion, intercellular deposition, and high molecular weight all suggest that the MS-1 antigen is likely to be an extracellular adhesion/matrix molecule. Of the various characterized extracellular adhesion/matrix molecules, only vWF and tenascin (reviewed in 19) share two key features with the MS-1 antigen: both are expressed by splenic sinusoidal endothelium (8, 10) and both are of similar size to the bands precipitated by mAb MS-1 (260- and 275-kD precursors and a 220-kD mature form for vWF; 320-, 230-, and 220-kD isoforms for tenascin) (4, 58). Therefore, we investigated whether the MS-1 antigen could be a form of these molecules. As already described (see above), vWF is strongly expressed in sinusoidal endothelial cells of the spleen, lymph node, and adrenal cortex, but not of liver. Furthermore, vWF is expressed in all continuous endothelial cells, including HUVECs in culture, while it is not expressed in KG-1 cells (Table II). In addition, mature vWF is of slightly higher molecular weight than the MS-1 215-kD band in the spleen, as demonstrated by direct comparison on SDS-page gels. Most convincingly, preabsorption of splenic extracts to remove all immunoprecipitable vWF does not remove the MS-1 antigen (not shown). Thus, vWF and the MS-1 antigen are not identical.
**Table II. Comparison of vWF, Tenascin, and MS-1 Antigen Expression in Selected Cell Types**

| Cell type | MS-1 | α-vWF | α-Tenascin |
|-----------|------|-------|-----------|
| HUVEC     | -    | +     | -         |
| Fibroblasts| -    | -     | +         |
| KG-1      | +    | -     | -         |

Tenasin is a family of related molecules, some of which may as yet be uncharacterized. Using a polyclonal antitenasin antibody, which is reported to mimic staining of all known antitenasin mAbs, including an antitenasin mAb specific for the 320-kD isoform (see ref. 30a), we found that all MS-1-positive structures in human tissues can be stained. However, minor differences in staining were noticed as the splenic sinusoids were not as crisp and well-outlined as with MS-1, and the liver sinusoids appeared more clearly visible as vascular channels with antitenasin than with MS-1. In addition to the structures that apparently coexpressed the proteins, tenasin was expressed in many other structures, e.g., in elongated elements in the follicles of lymph node and spleen, in cells at the outer edge of the follicles in tonsil, in the outer wall of arteries and arterioles, and at the epithelial-mesenchymal border in skin, tonsil and gut (as described in 3, 31). More critically, the MS-1-negative fibroblasts were, as expected (10), brightly tenasin positive, while the MS-1-positive KG-1 cells were tenasin negative (Table II). Moreover, also expected from the literature (31), splenic tenasin was not solubilized using the Triton X-114 procedure applied for MS-1 immunoprecipitation (data not shown). Finally, Mono-Q-purified human glioma-derived tenasin, reactive with various antitenasin mAbs and the polyclonal antitenasin antiserum (4), did not react with mAb MS-1 in a dot blot assay (not shown). Thus, the known tenasin isoforms and the MS-1 antigen are not identical.

**Discussion**

We report here on the generation of a mAb, designated MS-1, raised in the mouse against human spleen, that detects a previously undescribed antigen, expressed by the sinusoidal endothelia of spleen, lymph node, liver, and adrenal cortex, but not by nonsinusoidal continuous endothelia and renal glomerular endothelium. Additional expression is seen in endothelial cells of tonsillar high endothelial venules and in an interstitial cell population in several organs such as placenta, skin, and gut, but not brain. Of several myeloid cell lines examined, only the early myeloid cell lines KG-1 and KG-la express and secrete the MS-1 antigen; no MS-1 antigen expression is found on the cell surface of these cells. Preliminary studies in skin suggest that the MS-1-positive interstitial cells consist of two distinct populations: vWF-positive lymphatic endothelial cells and factor XIIIa-positive dermal dendritic cells (L. J. Walsh, S. Goerdt, J. S. Pober and G. F. Murphy, manuscript in preparation). By ultrastructure, the MS-1 antigen in spleen appears to be deposited at zones of contact between sinusoidal endothelial cells.

The antigen immunoprecipitated from human spleen by mAb MS-1 is a high molecular weight protein consisting of a prominent 215-kD and minor 320- and 120-kD proteins. mAb MS-1 precipitates a minor 280-kD and prominent 300-, 205-, and 120-kD proteins from KG-1 cells, of which the 300-, 205-, and 120-kD species are secreted in substantial amounts. The differences in molecular weight between the similarly sized species in spleen and in KG-1 probably arise from slight differences in the posttranslational processing or from differential splicing of mRNA in the two cell types, and it seems highly unlikely that they are unrelated, cross-reacting proteins. The 280-kD protein of KG-1 cells is not identified in immunoprecipitates from spleen. The amount of the 280-kD KG-1 protein is low, and thus may escape detection in cold splenic extracts. Alternatively, splenic MS-1 proteins may predominantly be solubilized from extracellular matrix deposits, which would not be expected to contain the 280-kD species, since it seems not to be secreted.

What are the relationships among the differently sized MS-1 protein species? From the analysis of KG-1 cells, the most likely relationship of the proteins precipitated by mAb MS-1 is that of precursors and products. Specifically, we propose that a 280-kD precursor is converted to a 300-kD species which, in turn, is cleaved to 205- and 120-kD species. This interpretation is supported by three lines of evidence: pulse-chase experiments, comparison of reduced and non-reduced precipitates, and limited proteolytic fragmentation. First, in pulse-chase experiments, label appears to be chased from the 280-kD species into the 300-kD species and there is extensive, though not complete, chase of radioactivity from the 300-kD species into the 205-kD species. Momen-sin, a drug known to affect transportation of proteins into the Golgi apparatus and to inhibit complex carbohydrate formation in vWF and fibronectin biosynthesis (30, 58), inhibits the appearance of the 300-kD protein species and causes accumulation of radioactivity in the 280-kD band, further supporting the interpretation that the 280-kD protein is the precursor of the 300-kD protein. The 120-kD protein species was not clearly seen in the late time points of the pulse-chase experiments; this may be explained by the fact that there is less net radioactivity in these samples, that the 120-kD species labels less intensely than the other species, and that the radioactivity of the 120-kD species is spread over a range of molecular weight rather than being concentrated in a concise band. Second, studies comparing reduced and non-reduced precipitated forms of MS-1 antigen and conversion of unreduced 300-kD to 205- and 120-kD species upon reduction also suggest a common origin of the 300-kD species and the smaller bands. These data further suggest that the 205- and 120-kD fragments must be held together by disulfide bonds. Finally, the common origin of the 205- and 120-kD species from the 300-kD precursor is directly supported by limited proteolysis peptide mapping. Endogenous proteolytic conversion may be more efficient in splenic sinusoidal endothelial cells than in KG-1 cells, accounting for the fact that the 215-kD protein is relatively more abundant (compared to the other protein species) in splenic extracts than the 205-kD species is in KG-1 cell lysate or culture medium.

The antigen recognized by mAb MS-1 differs significantly from a number of other large extracellular proteins described to date. In particular, we have shown that the MS-1 protein is not identical with vWF or the three known isoforms of tenasin, though it does share several features with them.
Laminin and fibronectin, including the fibronectin splice variant ED1 (40, 56), are clearly not identical to MS-1 because of differences in molecular weight and/or tissue distribution. Other high molecular weight secreted proteins that were considered, but ruled out because of the lack of any substantial endothelial cell association, are: coagulation factor VIII, thought to be synthesized preferentially in hepatocytes (59); coagulation factor V, synthesized in hepatocytes and megakaryocytes and found in spleen only in platelets (21); and the members of the high molecular weight tumor-associated glycoprotein family that are highly restricted to certain epithelia (1, 14).

We have also considered whether MS-1 might be related to a previously described endothelial cell molecule. As noted earlier, mAbs 1F10 (23, and results), hec7 (37), and Ec6Cl0 (26) recognize a subset of endothelia almost mutually exclusive with that detected by MS-1. mAbs aHCl (43) and B721 (50) also share a lack of reactivity in splenic and hepatic sinusoids, and exhibit far more cross-reactions, e.g., with hairy cell leukemia cells and epidermal basal cells and with activated B- and T-cells, smooth muscle cells, and syncytiotrophoblast, respectively. mAb EN11/4/25 (25) is directed against a subset of endothelia in inflammatory diseases and tumor tissues, but does not stain any normal endothelial cells, including splenic or hepatic sinusoidal endothelium. Liver sinusoidal endothelial cells and endothelial cells of high endothelial venules in secondary lymphatic organs express the monocyte-endothelial cell antigens CD14 (55) and HECA-452 (18), but these molecules are not found in splenic sinusoidal endothelium. Of this antienhanced mAbs that do react with splenic sinusoidal endothelium, BW200 (2), E431 (50), and EN3/EN4 (15) are pan-endothelial markers. CD36 (29), a monocyte-platelet-endothelial cell-surface glycoprotein of 90 kD functioning as a thrombospondin receptor, and the antigen recognized by mAb PAL-E (44), are expressed on all microvascular endothelia, including the splenic sinusoidal endothelium, and to a lesser extent in the endothelium of larger vessels, especially larger arteries and arterioles. Expression of CD71, the transferrin receptor, shared among splenic sinusoidal (11) and brain capillary (27) endothelia in situ, may bear some functional significance in the splenic sinusoids in light of their involvement in erythrocyte selection, pitting, and degradation. Expression of CD8 by the splenic sinusoidal endothelial cells, as detected by immunoenzymatic staining with mAbs Leu2a OKT8 (11, 55), is unprecedented not only among endothelial cells, but among all non-T-cells, and, if confirmed, could possibly be of great importance for sinusoidal endothelial cell biology. CD33 and CD34 (5, 12), which, like MS-1, are somewhat selectively expressed in hemopoietic progenitor cells, differ from MS-1 in molecular mass (70 and 110 kD, respectively) and tissue distribution. Interestingly, CD34 is an endothelial cell protein as well as that, unlike MS-1, preferentially expressed in situ in continuous type endothelial cell microvessels (20) and on abuminal endothelial cell microprocesses in tumor stroma (45), but is absent from splenic (S. Goerd and J. S. Pober, unpublished observations) and hepatic sinusoids (20).

The function of the molecule recognized by mAb MS-1 is not yet known. A possible function may be inferred from the unique in situ distribution of this molecule and its biochemical characteristics. The expression of MS-1 antigen groups together the sinusoidal endothelium that had not previously been thought to have much in common other than the fact that they are microvessels of large caliber and irregular outline and (with the exception of the adrenal cortex) lack a well-formed basement membrane. In preliminary studies, we have found that MS-1 antigen is also expressed by the endothelium of the bone marrow sinusoids, which also lack a basement membrane, and by the endothelium of certain tumor vessels, characterized by tortuosity and leakiness. The large size of MS-1 antigen, the secretion of the molecule by K5-1 cells, and the ultrastructural accumulation of the protein at zones of intercellular contact between splenic sinusoidal endothelial cells all point to a role as an extracellular adhesion/matrix protein. We offer the hypothesis that MS-1 protein serves as an adhesive function for endothelial cells in those vessels which because of their irregular shape, large caliber, leakiness, or lack of proper basement membrane, must use macromolecules different from those utilized by continuous endothelia to achieve and maintain their integrity.

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