An Explanation for Minor Multimer Species in Endothelial Cell–synthesized von Willebrand Factor

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Abstract

Initial synthesis of von Willebrand factor (vWf) by cultured human endothelial cells proceeds by formation of a dimer of pro-vWf subunits. These subunits are found only within the cell and have an apparent molecular weight of 240,000–260,000, as measured by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. Posttranslational modifications, including proteolytic cleavage, glycosylation, and sulfation, result in the appearance of two additional vWf subunits. The major one migrates with the subunit of plasma vWf at an apparent molecular weight of 220,000–225,000 and the other migrates more slowly than pro-vWf at an apparent molecular weight of 260,000–275,000. These subunits oligomerize to form a set of vWf multimers, which are subsequently secreted into the culture medium. We isolated individual vWf oligomer species from the agarose gel bands and show that vWf minor, or satellite, species differ from major species in subunit composition.

Introduction

The von Willebrand factor (vWf)[1] is a large, adhesive plasma glycoprotein which is instrumental in mediating the interaction of platelets with damaged endothelial surfaces. In addition, vWf appears to function as a carrier for Factor VIIIC, the antihemophilic factor, serving to prolong the circulatory half-life of VIIIC. Inherited abnormalities in vWf function result in the bleeding disorder von Willebrand's disease (vWd), of which several different phenotypic variations are known (1, 2). vWf from normal plasma, which is thought to be synthesized in endothelial cells (3, 2), circulates as a series of self-aggregated structures called multimers, ranging from dimers of what appears to be a single subunit (apparent molecular weight ~225,000) to polymers containing more than 50 subunits (4–6). The results of various studies have established that the largest multimers are most important in the intrinsic hemostatic role of vWf (2) while multimers of all sizes can function equally as carriers for Factor VIIIC (7). When plasma from patients with vWd was examined by agarose gel electrophoresis, differing vWf patterns were observed. These patterns have aided in the classification of vWd into different types and subtypes. In type I vWd, there is a loss of multimers from all size classes, and the severity of clinical symptoms correlates well with the extent of vWf decrease. In type II vWd, there is a selective loss of the larger multimers and bleeding is quite variable and often not related to the overall vWf level. Patients with type IIA vWd have only the smallest multimers, while those with type IIB have small and intermediate multimers and lack only the largest (6). More recently, the use of higher resolution discontinuous buffer sodium dodecyl sulfate (SDS)-agarose gels has resulted in detection of satellite species to the major bands and has allowed further subclassification of vWd based on variation of satellite banding. Variants have been described which lack some of the normal bands, or have new ones present, or have alterations in the usual pattern of relative intensities (8–12). Thus, the detailed fine structure in vWf gel patterns seems to be of clinical significance. Accordingly, we have investigated the origin of the satellite bands observed in vWf produced by cultured human umbilical vein endothelial cells obtained from normal individuals.

Methods

Cell culture, labeling, and immunoisolation of vWf. Human umbilical vein endothelial cells were a gift of Dr. Michael Gimbrone (Brigham and Women's Hospital, Boston, MA). They were grown in culture as previously described (13). Cells were labeled for 16 h with [^{35}S]-methionine, lysate, and medium fractions prepared, incubated for 16 h at 4°C with a Sepharose-coupled monoclonal antibody to vWf, prepared for electrophoresis, and electrophoresed all as previously described (14, 15).

Partial proteolytic mapping. Samples were electrophoresed through a 5% polyacrylamide gel with a 3% stacking gel under reducing conditions, the gel dried onto 3MM paper (Whatman, Inc., Clifton, NJ) without prior fixation, and autoradiographed. The radioactive bands of interest were located using the radiograph and were excised from the dried gel. The excised gel pieces were then rehydrated in 50 mM ammonium bicarbonate (pH 7) containing 1 μg/ml of bovine serum albumin, 5% 2-mercaptoethanol, and 0.1% SDS. The paper was then removed, the rehydrated acrylamide minced with a scalpel, and the samples eluted by shaking at 37°C for 16 h. The eluate was lyophylized twice, redissolved in water, and the radioactive content determined. Samples of approximately equal radioactivity were adjusted to 10 μg/ml albumin, 1% SDS, 0.125 M TrisCl, pH 6.5, and digested for 1–2 h at 37°C with 15 μg/ml of *Staphylococcus aureus* V8 protease (Sigma Chemical Co., St. Louis,

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1. *Abbreviations used in this paper:* vWf, von Willebrand factor; vWd, von Willebrand's disease.

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MO). Samples were then made 5% in SDS and 5% in 2-mercaptoethanol, boiled for 5 min, and electrophoresed through a 12% polyacrylamide gel with a 3% stacking gel.

Analysis of subunit content of individual vWf oligomer bands. Before gel fixation, a 0.8/2% discontinuous buffer SDS agarose gel was cut longitudinally through the lanes in which samples from endothelial cell lysate and culture medium had been run. One-half of each lane was processed to produce an autoradiogram while the other was wrapped in plastic and stored at 4°C (for 4 d). The resultant autoradiogram was then used to locate the regions of the gel containing the desired vWf species. The agarose containing them was removed and melted at 90°C in the presence of 5% 2-mercaptoethanol and 5% SDS. The molten agarose was then applied to a 5% polyacrylamide gel with a 3% stacking gel and electrophoresed at 80 V for 16 h.

Results and Discussion

The electrophoretic relationship among the three subunit species of vWf found in [^{35}S]-methionine–labeled endothelial cells is shown in Fig. 1, which displays an autoradiogram of a polyacrylamide gel through which reduced samples of immunoisolated vWf were electrophoresed. The primary species found in the culture medium migrates at the same rate as the plasma vWf subunit (apparent molecular weight 220,000–225,000) and is referred to as mature vWf. The cell lysate contains some mature vWf as well as the previously characterized (15, 16) primary translation product, the pro-vWf subunit (apparent molecular weight 240,000–260,000). The culture medium has, in addition to the mature vWf subunit, a second species (apparent molecular weight 260,000–275,000) which migrates more slowly than pro-vWf (16). Smaller amounts of this material are also present in cell lysate samples. Although this more slowly migrating species can be coimmunoisolated with mature vWf using a variety of polyclonal and monoclonal antibodies, this is not evidence that it is an authentic vWf gene product. To clarify this point, partial proteolytic mapping was performed on this protein. In Fig. 2, it may be seen that this species has a peptide pattern very similar to that of mature vWf, and thus, would appear to be derived

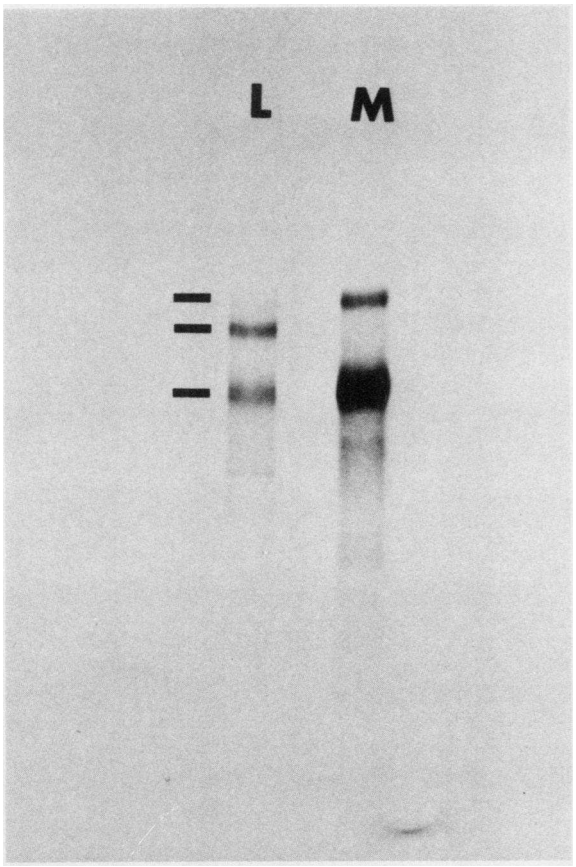

Figure 1. Subunit species of vWf. Autoradiogram of a 5% SDS polyacrylamide gel through which [^{35}S]-methionine–labeled vWf immunoisolates of endothelial cell lysate (L) and culture medium (M) were electrophoresed after treatment with 2-mercaptoethanol. The horizontal bars on the left denote the migration positions of the vWf species observed. From the top down, they are referred to as: large vWf subunit, pro-vWf subunit, and mature vWf subunit.

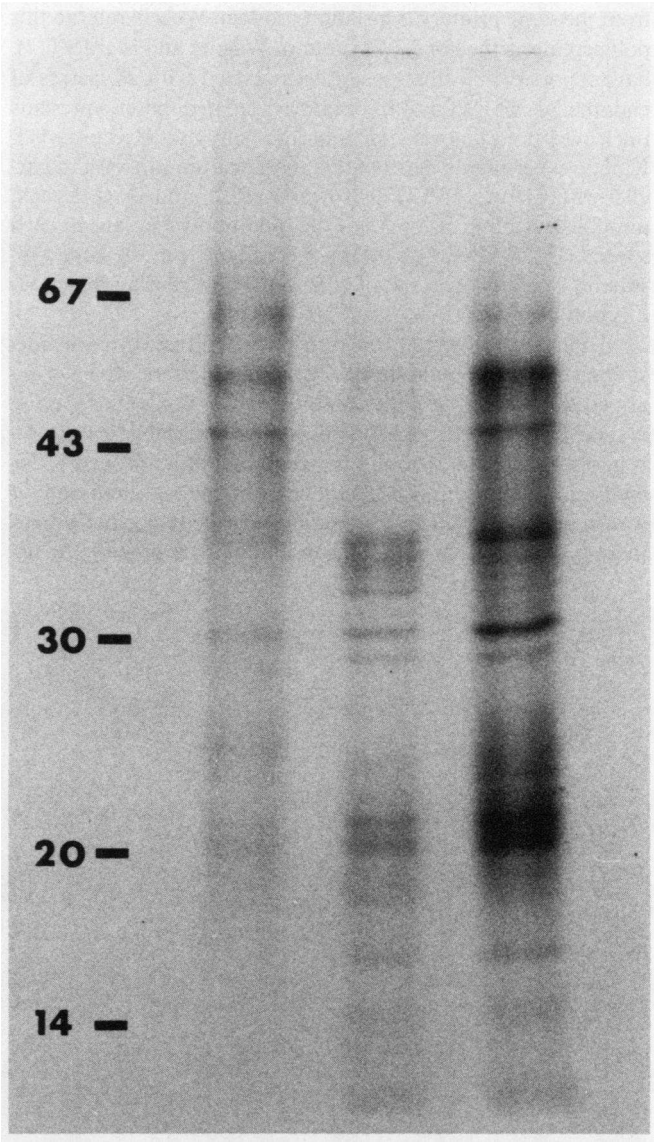

Figure 2. Partial proteolytic digestion of vWf isolated from endothelial cell culture medium. Autoradiogram of a 12% SDS-polyacrylamide gel through which *S. aureus* V8 protease digests of large and mature vWf subunits were electrophoresed. *Left* lane contains large vWf subunit digested for 1 h as described in Methods. *Center* and *right* lanes contain mature vWf subunit digested for 2 and 1 h, respectively. The horizontal bars on the left indicate the migration position of protein standards whose molecular weight in thousands is indicated by the numerals.

from the same primary translation product. We will refer to this polypeptide as the large vWf subunit. Wagner and Marder (17), have reported that this species is associated with all classes of endothelial cell vWf multimers and speculated that it represents uncleaved but otherwise posttranslationally modified pro-vWf. Kinetic experiments indicate that it arises from pro-vWf in parallel with mature vWf (17, Browning, P. J., and D. C. Lynch, unpublished observations) and the addition of sulfate to each species (18). While its exact origin is still unclear, the large vWf subunit is clearly the result of differential posttranslational modification of pro-vWf.

To investigate directly the distribution of these three distinct subunit species in endothelial cell vWf multimers, we have analyzed the subunit content of individual vWf oligomer species. A typical pattern seen when [^{35}S]-methionine–labeled vWf immunoisolates were analyzed by electrophoresis through a discontinuous agarose gel run under nonreducing conditions is shown on the left in Fig. 3. In addition to the major multimers, there is a prominent satellite banding pattern present. As described in Methods, the location of individual oligomer species in the gel was determined, the agarose containing them removed, melted in the presence of reducing agent, and then applied to an SDS-polyacrylamide gel. The resultant autoradiogram, which reveals the subunit content of the numbered major and satellite bands, may be seen in the correspondingly numbered lanes on the right in Fig. 3. As we had previously demonstrated by another technique (14), the pro-vWf dimer band from the cell lysate (lane *1* in Fig. 3) is composed exclusively of pro-vWf subunits. In the culture medium, there is a major dimer band (lane *2*) and two trailing satellites (lanes *3* and *4*). The major band is composed exclusively of mature vWf subunits while the slowest satellite contains only the large vWf subunit (lane *4*). The intermediate satellite species contains approximately equal amounts of large and mature vWf subunits (lane *3*). An analogous pattern may be seen for the first trailing satellite of the tetramer (lane *6*) which has a much higher ratio of large to mature subunits than does the major tetramer (lane *5*). This is also the case for the hexamer species (lanes *7* and *8*), but the data are

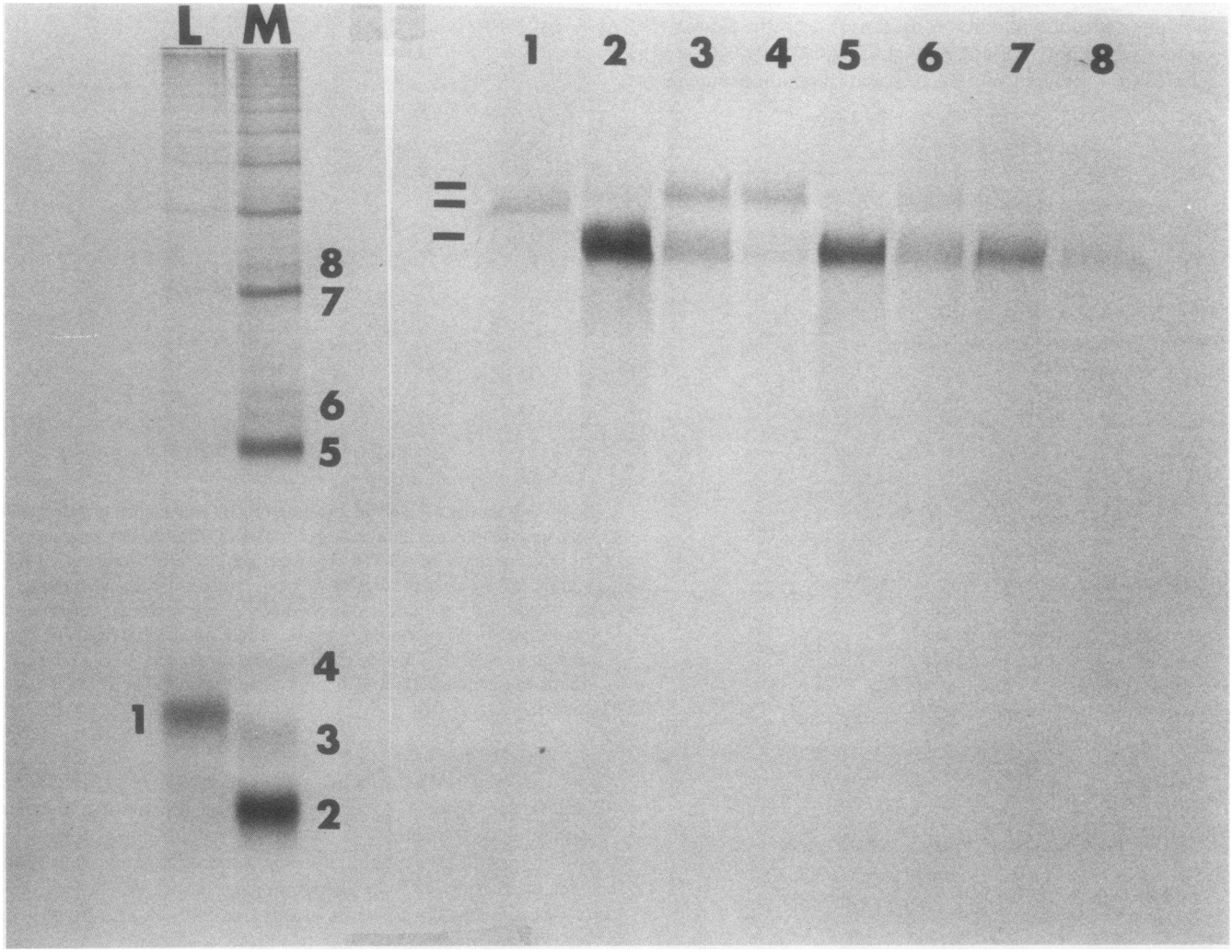

Figure 3. Subunit composition of discrete vWf oligomer species. *Left.* Autoradiogram of a 2% SDS agarose gel through which [^{35}S]-methionine–labeled vWf immunoisolates of endothelial cell lysate (L) and culture medium (M) were electrophoresed under nonreducing conditions. *Right.* Autoradiogram of a 5% SDS polyacrylamide gel through which material eluted from agarose gel bands was electrophoresed after reduction with 2-mercaptoethanol as described in Methods. The entire gel is presented, including the sample wells and dye front. Agarose derived from the numbered bands on the left was loaded into the correspondingly numbered lanes on the right. The horizontal bars denote, from the top down, the migration positions of the large, pro, and mature vWf subunits, respectively.

not well reproduced on the photograph. Thus, it appears that variation in apparent molecular weight of the constituent vWf subunits is sufficient to explain the appearance of two satellites to the major dimer band and is at least a partial explanation for the appearance of satellite bands in higher order multimers.

vWf in the cell lysate has a much lower overall ratio of large to mature subunit and much less intense satellite banding, but all of the satellite species observed in the culture medium are present in the lysate. We have observed no changes in the number and migration rate of satellite bands associated with each major vWf multimer produced by cells from different normal individuals or as the cells are passaged in culture. The relative intensity of satellites to major species in both medium and lysate does vary in either direction by severalfold between successive passages, and we do not yet know why this occurs. The number of discrete satellite species associated with each major band increases with size (best seen on the original autoradiograms), but limits of gel resolution prevent ready quantitation beyond the hexamer level. In contrast to the increasing number of satellite species, the ratio of large to mature vWf subunits decreases with multimer size to the extent that it is undetectable in the largest multimers (see Fig. 5 from reference 14). While there is no obvious overall structural model that may be inferred from these observations, it does appear that the large subunit is preferrentially represented in the smaller multimers and, thus, may be associated with termination of vWf polymerization. Whether the large subunit results from failure to cleave pro-vWf or aberrant modification of mature vWf (e.g., increased sulfation) is unclear at present.

We believe that the importance of these observations is twofold. First, they give a rational explanation for what initially may have appeared to be an electrophoretic artifact. Secondly, they suggest that differential posttranslational modification of vWf is responsible for at least some vWf satellite species produced by cultured endothelial cells. Since vWf satellite variability is a major abnormality observed in some variants of von Willebrand's disease (8–12), the origin of such variability is of interest. Zimmerman et al. (12) have recently described vWf proteolytic fragments associated with multimeric vWf purified from normal and patient plasmas. This finding suggests that association of proteolyzed fragments with normal subunits may be responsible for some satellite species formation. The results presented here suggest the possibility that differential posttranslational modification may also contribute to the abnormal satellite species observed in some von Willebrand's disease phenotypes.

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