

# Inhibition of Disulfide Bonding of von Willebrand Protein by Monensin Results in Small, Functionally Defective Multimers

DENISA D. WAGNER, TANYA MAYADAS, MARGARET URBAN-PICKERING, BRADLEY H. LEWIS, and VICTOR J. MARDER

*Hematology Unit, Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642*

**ABSTRACT** The biosynthesis of von Willebrand protein by human endothelial cells was impaired by the presence of the carboxylic ionophore monensin. Several processing steps that have been localized to the Golgi apparatus were affected in a dose-dependent manner, including carbohydrate processing, dimer multimerization, and precursor cleavage. Since multimerization was more susceptible to the ionophore than was precursor cleavage, it appears that these processing steps are separate events. As expected, dimer formation, which occurs in the rough endoplasmic reticulum, was unaffected by monensin. Thus, at high concentrations of monensin, only dimer molecules were produced and secreted. The observed inhibition of multimer formation and precursor cleavage were not likely the result of incomplete carbohydrate processing, since inhibition of complex carbohydrate formation by swainsonine did not interfere with the other processing steps. Monensin also affected the capacity of endothelial cells to store von Willebrand protein, as the ratio of secreted to cell-associated protein increased dramatically in the presence of monensin, and the processed forms could not be found in the treated cells. The low molecular weight multimers produced in the presence of monensin did not incorporate in the endothelial cells' extracellular matrix nor did they bind to the matrix of human foreskin fibroblasts. In summary, the presence of monensin in human endothelial cell culture produced experimental conditions that mimic Type IIA von Willebrand disease, in that the cells synthesized and secreted only low molecular weight von Willebrand protein multimers, which were functionally defective.

von Willebrand (vW)<sup>1</sup> protein is a large adhesive glycoprotein involved in the binding of platelets to exposed basement membranes during vascular injury (1, 2, 3). vW protein is present in the basement membrane (4) and in the platelet  $\alpha$ -granules (5, 6) from which it is secreted after platelet activation. vW protein is synthesized by megakaryocytes (7, 8) and by endothelial cells (9). Endothelial cells store vW protein in endothelial cell-specific organelles called Weibel-Palade bodies (10) and incorporate it into their extracellular matrix. vW protein subunit is first synthesized as a precursor polypeptide chain of 260,000-mol-wt (11, 12), which dimerizes in the endoplasmic reticulum. After transport of the dimers to the

Golgi apparatus, the carbohydrates are processed from high mannose to complex type, accompanied by an apparent increase in size of the subunits to 275,000-mol-wt. The dimers then form large multimers by disulfide bond formation and in parallel, the prosequence is cleaved to generate the final 220,000-mol-wt vW protein subunit (13). Most of the secreted vW protein consists of a 220,000-mol-wt subunit, but a small amount of the 275,000-mol-wt subunit precursor is also secreted (11).

In Type IIA vW disease, large and intermediate vW protein multimers are absent (14). The bleeding time of these patients is prolonged because the small multimers do not support efficient attachment of platelets to the vascular basement membrane (15). We were interested in whether experimental

<sup>1</sup> *Abbreviation used in this paper:* vW, von Willebrand (protein).

conditions could be found that would generate similar low molecular weight multimers in tissue culture by inhibiting the interdimer disulfide bond formation. This processing step occurs in the Golgi apparatus (13) and possibly continues in Weibel-Palade bodies, and electron microscope observations suggest that Weibel-Palade bodies originate from the Golgi apparatus (16). The carboxylic ionophore monensin, which causes equilibration of the monovalent ions  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{H}^+$  (17), has been shown to primarily affect the Golgi apparatus (18) and to inhibit the protein processing steps localized to this organelle (19). We have observed that monensin inhibits carbohydrate processing of vW protein from the high mannose to the complex type (13). We now report the effect of monensin on other steps in the biosynthesis of vW protein, in particular, multimer formation, and examine how structural defects affect the ability of vW protein to be incorporated into the extracellular matrix.

## MATERIALS AND METHODS

**Cells and Culture Conditions:** Endothelial cells were obtained from human umbilical vein by mild proteolytic digestion as described previously (10, 20). Cells were cultured in McCoy's 5A medium (Flow Laboratories, Inc., McLean, VA) containing 20% fetal bovine serum. Human foreskin fibroblasts in sixth passage, a gift from S. Schmidt (University of Rochester), were grown in the same medium. Monensin was obtained from Calbiochem-Behring Corp. (La Jolla, CA), and swainsonine was a kind gift from Drs. R. G. Arumugham and M. L. Tanzer (University of Connecticut Health Center). The drugs were dissolved in ethanol, and very small amounts of stock solutions were added to culture medium; control cells were treated with ethanol alone. Ethanol improved monensin solubility in comparison to our earlier study in which the drug was suspended in water (13). For continuous metabolic labeling, cells were grown in the presence of [ $^{35}\text{S}$ ]methionine (25  $\mu\text{Ci}/\text{ml}$ , 18.7 Ci/mmol, Amersham Corp., Arlington Heights, IL).

**Antisera:** Preparation and characterization of antisera against human vW protein were as described previously (10, 21). Some experiments used antiserum purchased from Calbiochem-Behring Corp. Monospecific antifibrinogen antiserum was a kind gift from Dr. R. O. Hynes (Massachusetts Institute of Technology) (22).

**Pulse-Chase Experiments in the Presence of Monensin:** All media contained 1  $\mu\text{M}$  monensin, and incubations were at 37°C. Cells were preincubated for 45 min in medium containing fetal bovine serum and for 15 min in serum-free medium. Medium was then removed and the cells were covered with 1 ml serum-free medium containing 1.5 mCi [ $^{35}\text{S}$ ]methionine for 20 min. Cells were then rinsed in medium containing serum and incubated in this medium for varying lengths of time. Control cells were treated identically but without monensin.

**Purification of vW Protein:** vW protein was immunopurified as described previously (13), and samples were analyzed by gel electrophoresis after the radioactive count was determined.

**Endoglycosidase H Digestion:** The enzyme was a generous gift of Dr. P. W. Robbins (Massachusetts Institute of Technology) and was purified according to Tarentino et al. (23). Purified vW protein was diluted in 0.1 M Tris buffer, pH 5.8, so that the final concentration of SDS was <0.5%. Endoglycosidase H was added (3  $\mu\text{g}/\text{ml}$ ), and samples were incubated for 2 h at 37°C before analysis on gels.

**Electrophoresis Gels:** SDS polyacrylamide gels were prepared as described by Laemmli (24), and agarose horizontal slab gels were prepared using a solution of 2% agarose, 0.1% SDS in 0.05 M phosphate buffer at pH 7.0. Two-dimensional gel electrophoresis was performed as described previously (13).

**Readdition of Purified vW Protein to Monensin-treated Endothelial Cells:** Cells freshly obtained from umbilical vein were plated at high density on glass coverslips and after 2 d, the medium was exchanged for medium containing  $10^{-8}$  M monensin. Cells were grown in this medium for 4 d (with one medium exchange), during which time they started to develop extracellular matrix. At the end of the 4-d period, purified plasma vW protein was added to some coverslips (final concentration of 20  $\mu\text{g}/\text{ml}$ ) in the presence of monensin for an additional overnight incubation. The remaining coverslips were incubated in the absence of exogenous vW protein. The purified vW

protein was a side product from a preparation of factor VIII (25) and was a gift from M. Anderson (University of Rochester). Similar results were obtained when endothelial cells treated with monensin were overlaid with 3-d-old culture medium containing vW protein obtained from untreated cells. This overlay was performed in the presence of monensin. Coverslips were then washed and processed for fluorescence microscopy.

**Swainsonine Treatment of Cells for Fluorescence Microscopy:** Cells freshly obtained from umbilical veins were plated on glass coverslips and after 2 d, their medium was exchanged for medium containing 1  $\mu\text{g}/\text{ml}$  swainsonine. The cells were grown in this medium for 5 d (with one medium change) before being processed for fluorescence microscopy.

Immunofluorescence staining was performed as described previously (10).

## RESULTS

### Effect of Monensin on the Polymeric Structure of vW Protein

Human umbilical vein endothelial cells were metabolically labeled with [ $^{35}\text{S}$ ]methionine in the presence of varying concentrations of monensin. After 3 d, vW protein was purified from the culture medium and cell lysate and was analyzed nonreduced on 2% agarose gels (Fig. 1). There was a dose-dependent decrease in size of secreted (*Medium*) high molecular weight (*HMW*) multimers, with accumulation of the low molecular weight species. Low concentrations of monensin ( $1 \times 10^{-8}$  M) produced broader bands than that seen in the absence of monensin (Fig. 1). Two-dimensional gel electrophoresis of these samples with reduction in the second dimension (not shown) revealed that this heterogeneity was due to a greater proportion of precursor subunits in these small polymers. This was in contrast with untreated cultures which contained only a small amount of vW protein precursor subunit in the multimers (13).

When the concentration of monensin was increased to  $125 \times 10^{-8}$  M, only the vW protein dimer was secreted (Fig. 1). Higher monensin concentrations caused a large decrease in protein synthesis, but to the extent that vW protein was still produced, it was in dimer form and monomer secretion did not occur. Thus, interdimer disulfide bond formation was

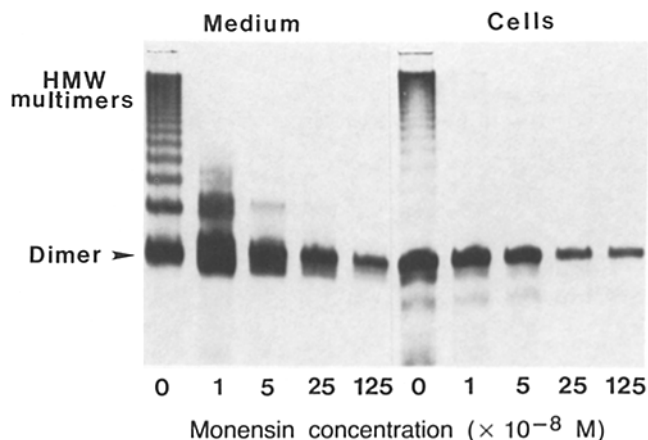


FIGURE 1 The effect of monensin on multimerization of vW protein. Human umbilical vein endothelial cells were metabolically labeled for 3 d in the presence of monensin, after which vW protein was purified from the medium and the cell lysate (*Cells*). The nonreduced vW protein was electrophoresed on 2% agarose gels, autoradiographs of which are shown in this figure. Monensin limited the size of multimers in a dose-dependent manner by inhibition of interdimer disulfide bond formation. Only dimer molecules remained in the cell lysate, while the other multimers produced in the presence of monensin were preferentially secreted.

very susceptible to the ionophore monensin, whereas dimer formation was unaffected. In four independent experiments, we have determined the total number of secreted multimer bands from overexposed autoradiographs of gels similar to that presented in Fig. 1. This showed that the formation of more than half of the multimer species was inhibited by concentrations of monensin as low as  $10^{-8}$  M, whereas a dose of monensin two orders of magnitude larger was needed to inhibit formation of tetramers (Table I). Preincubation for several hours with the same concentration of monensin as used during the metabolic labeling produced the same results.

Cellular vW protein from monensin-treated cultures (*Cells*) showed the absence of all polymers larger than dimer (Fig. 1), indicating that the smaller polymers produced in the presence of monensin were preferentially secreted rather than stored in the cells.

Pulse-chase experiments confirmed the insensitivity of the dimerization step to monensin (Fig. 2). The time course of formation of dimers from the 260-kD precursor was identical in both control and monensin-treated cultures. Up to 2 h from the onset of labeling, when the protein is still mainly in the endoplasmic reticulum (13), the monensin-treated and control samples were indistinguishable by gel electrophoresis. Only later when multimerization occurred in control cells was a difference observed; this process did not take place in monensin-treated cultures (Fig. 2). The formation of fibronectin dimer was also unaffected by monensin treatment of the endothelial cells (data not shown).

In all of the pulse-chase experiments, there was an initial delay in secretion of vW protein in the monensin-treated cultures. Quantitation of secreted vW protein from two experiments showed that 2.3 times more vW protein was secreted in the control cultures than was secreted in monensin-treated cultures during the first 2 h of chase (Fig. 2). However,

this ratio decreased to 0.75 by 6 h of chase, reflecting the higher secretion of vW protein in longer monensin-treated cultures.

### Effect of Monensin on Precursor Cleavage, Carbohydrate Processing, and Intracellular Storage of vW Protein

To examine the composition and subunit size of vW protein synthesized in the presence of increasing doses of monensin, the purified protein was analyzed reduced on 5% polyacrylamide gels (Fig. 3). Secreted vW protein (*Medium*) showed a gradual increase in precursor subunit content, relative to the amount of cleaved subunit. Inhibition of precursor cleavage by monensin was quantitated by scanning several exposures of autoradiographs from three independent experiments (Table I). The precursor cleavage processing step appeared to be less susceptible to monensin than was vW protein multimerization. While  $10^{-8}$  M monensin decreased the observed number of multimer bands from more than 14 to 6, the precursor cleavage was inhibited only 18%. Also, at the highest monensin dose ( $125 \times 10^{-8}$  M), when multimerization past dimer was completely inhibited, residual cleavage of precursor subunit occurred (Table I).

Both secreted subunits (275,000-mol-wt and 220,000-mol-wt) obtained from cultures exposed to increasing concentrations of monensin migrated progressively faster on the polyacrylamide gels (Fig. 3). This effect probably was the result of increasing monensin inhibition of conversion of the high mannose carbohydrate to the complex type. This was supported by the following observations: (a) secreted vW protein synthesized in the presence of high doses of monensin (i.e.,  $1 \mu\text{M}$ ) was endoglycosidase H-sensitive (13), and (b) secreted vW protein from cells exposed to low doses of monensin did

TABLE I. Effect of Monensin on Polymeric Structure and Subunit Composition of Secreted vW Protein

	Monensin concentration ( $10^{-8}$ M)				
	0	1	5	25	125
Number of multimer bands*	>14	6	5	3	1
220,000-mol-wt subunit (% total)*	98.7 (0.3)	81.1 (4.7)	50.8 (3.3)	30.6 (1.4)	21.6 (3.3)

\* Average number of multimer bands recorded from four experiments such as that shown in Fig. 1.

\* Percentage of 220,000-mol-wt subunit measured by densitometric scan of autoradiographs of three experiments similar to that shown in Fig. 3. Values shown in parenthesis indicate standard error of mean.

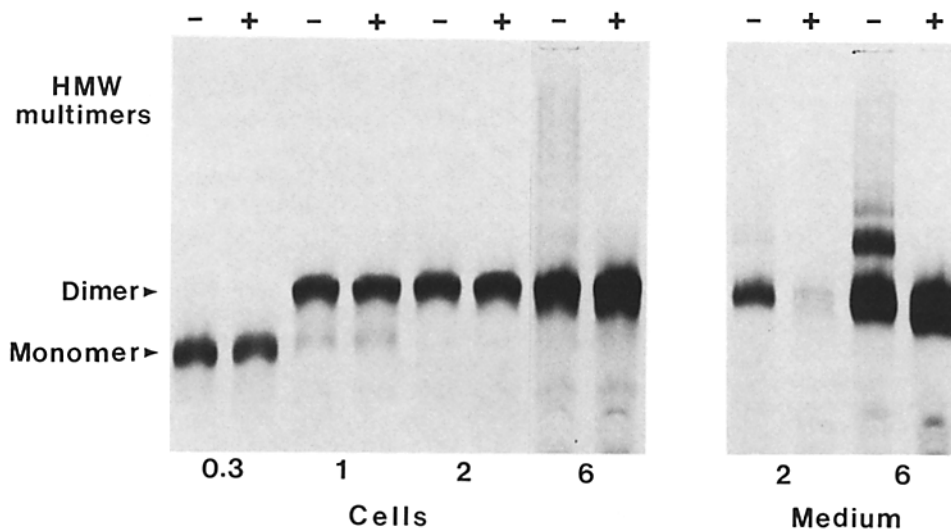


FIGURE 2 Pulse-chase experiment. Endothelial cells were labeled for 20 min with [ $^{35}\text{S}$ ]methionine and then incubated in unlabeled medium in the presence (+) or absence (-) of  $1 \mu\text{M}$  monensin. Numbers indicate the elapsed time in hours from the onset of labeling. vW protein purification and analysis was as in Fig. 1. Monensin entirely inhibited multimer formation, but dimerization was unaffected. Also note the higher amount of vW protein secreted in the control cultures in comparison with the monensin-treated culture at the 2-h time point.

not contain the largest multimers, but the protein was still endoglycosidase H-resistant (not shown).

Examination of reduced cellular vW protein (*Cells*) (Fig. 3) showed that even the lowest doses of monensin removed the majority of the 220,000-mol-wt processed subunit from the cells. The prominent subunit remaining in the cells was the 260,000-mol-wt precursor. The absence of the 220,000-mol-wt subunit resembled the absence of low molecular weight polymers from cells grown in the presence of monensin (Fig. 1). These observations are in agreement with the loss of Weibel-Palade bodies from monensin-treated endothelial cells, as shown by immunofluorescence staining with anti-vW protein antiserum and by electron microscopy (26; our unpublished observations).

The effect of monensin on synthesis, secretion, and proportion of secreted to cell-associated vW protein was quantitated in three experiments, using metabolically labeled cultures after monensin treatment for 3 d (Table II). The lowest

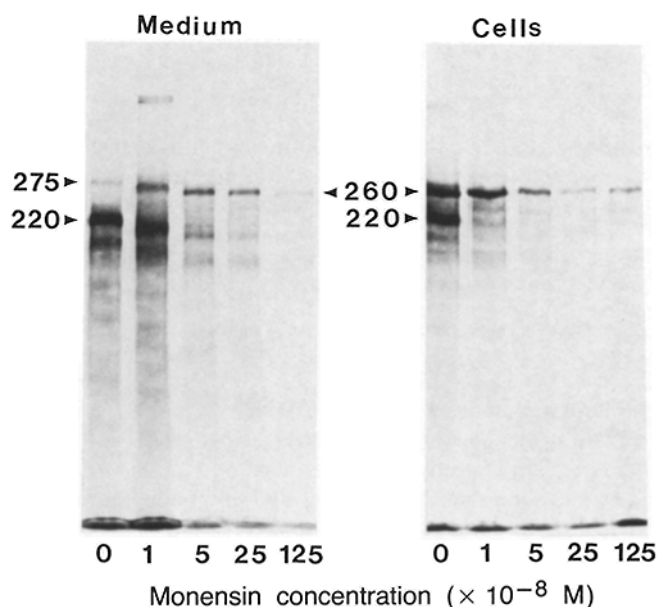


FIGURE 3 Effect of monensin on the cleavage of vW protein precursor. vW protein was purified from metabolically labeled endothelial cells and medium as described in Fig. 1 and analyzed reduced on 5% polyacrylamide gels. Monensin increased the ratio of precursor (275 or 260) to the cleaved subunit (220) in a dose-dependent manner. The migration of the secreted 275,000-mol-wt and the 220,000-mol-wt subunits increased progressively with higher concentrations of monensin, probably as the result of inhibition of carbohydrate processing. In addition, the 220,000-mol-wt subunit was preferentially secreted in the presence of monensin as it was absent from the cell lysates. The minor band migrating faster than the 220,000-mol-wt subunit (*Medium*) appears to be a fragment of vW protein which we observe often in long-term labeled cultures.

TABLE II. The Effect of Monensin on vW Protein Synthesis, Storage, and Secretion

	Monensin concentration ( $10^{-8}$ M)				
	0	1	5	25	125
A. Total vW protein*	1	1.3 (0.2)	0.8 (0.2)	0.5 (0.1)	0.4 (0.1)
B. Secreted/cell-associated vW protein	1.5 (0.3)	4.8 (0.2)	5.2 (0.6)	4.5 (0.8)	5.0 (0.5)
C. Secreted vW protein*	1	1.8 (0.1)	1.2 (0.2)	0.6 (0.1)	0.6 (0.1)

\* Values were normalized to those obtained for untreated controls; numbers in parenthesis indicate standard error of mean.

concentration of monensin stimulated vW protein synthesis slightly. Higher concentrations had an apparent inhibitory effect (Table II, A), but it is also possible that this decrease in total vW protein was due to enhanced destruction of newly made molecules. Monensin dramatically affected the secreted to cell-associated ratio of vW protein. All concentrations of monensin that were tested produced more than a threefold increase in this ratio (Table II, B), reflecting the low amount of processed vW protein remaining in the cell lysate and the absence of the vW protein storage compartment—the Weibel-Palade bodies.

#### Effect of Swainsonine on vW Protein Biosynthesis

The polymerization defect and the incomplete propeptide cleavage in monensin-treated cells could possibly be the consequence of improper carbohydrate processing. To examine the importance of complex type carbohydrate on the biosynthesis of vW protein, cells were grown in the presence of swainsonine, which inhibits the Golgi enzyme mannosidase II (27), thereby preventing the full formation of complex carbohydrate. The carbohydrates produced in the presence of swainsonine are of a hybrid type that is susceptible to digestion by the enzyme endoglycosidase H (28, 29), while complex carbohydrates are resistant to this enzyme. The results showed that vW protein synthesized in the presence of 1  $\mu$ g/ml of swainsonine was susceptible to endoglycosidase H digestion and therefore had incompletely processed carbohydrate (Fig. 4a). The ratio of precursor (260,000 or 275,000-mol-wt) to the 220,000-mol-wt cleaved subunit from the swainsonine-treated cells was unchanged (Fig. 4a), as was the polymeric structure of the nonreduced vW protein, in comparison with protein from untreated controls (Fig. 4 b). It is likely, therefore, that the presence of incompletely formed carbohydrate on vW protein molecules did not affect the ability of the protein to undergo proper biosynthetic processing that yields multimers of the 220,000-mol-wt subunit.

#### Effect of Monensin and Swainsonine on vW Protein Participation in the Extracellular Matrix

In patients with Type IIA vW disease, only low molecular weight multimers of the protein circulate (30). A possible biological defect that could explain this disease state would be a decreased or absent interaction of these low molecular weight polymers with the matrix of basement membrane. Therefore, we examined the participation of the low molecular weight vW protein produced in the presence of monensin in the extracellular matrix of endothelial cells. Endothelial cells were cultured on coverslips in the presence of different concentrations of monensin for several days. In parallel, cells grown in identical concentrations of monensin were metabolically labeled to examine the extent of multimerization of secreted vW protein (Table I). While cells grown in the

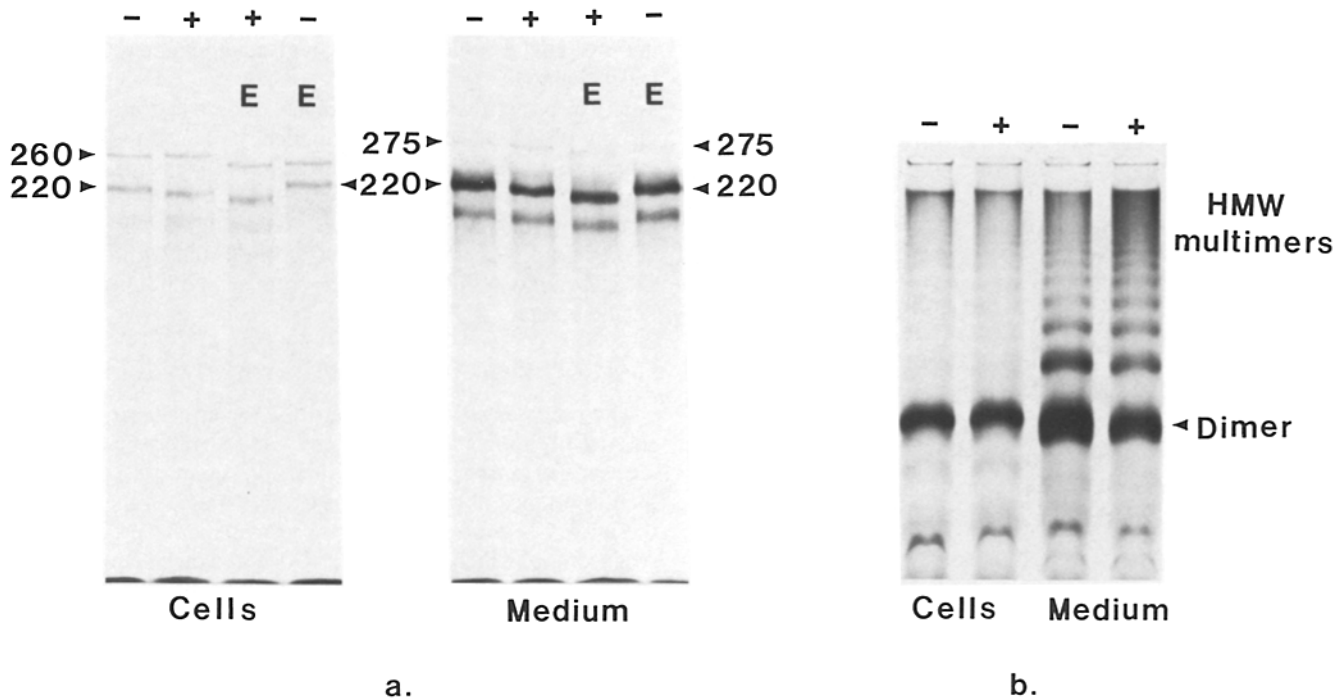


FIGURE 4 Effect of swainsonine on carbohydrate processing, precursor cleavage, and polymerization of vW protein. Endothelial cells were metabolically labeled for 2 d in the presence (+) or absence (-) of 1  $\mu\text{g}/\text{ml}$  of swainsonine. vW protein was purified and analyzed reduced on a 5% polyacrylamide gel (a) and nonreduced on a 2% agarose gel (b), autoradiographs of which are shown here. Swainsonine did not affect the ratio of precursor (275 or 260) to the cleaved subunit (220) but it did affect endoglycosidase H sensitivity (lane E) of the protein (a). While only the 260,000-mol-wt intracellular precursor of control cultures was endoglycosidase H-sensitive, the 275,000-mol-wt and 220,000-mol-wt subunits of swainsonine-treated cultures were also susceptible to endoglycosidase H. This was shown by a shift in migration of the digested protein (a). The multimeric pattern of cellular and secreted vW protein from control and swainsonine-treated cultures were very similar (b).

absence of monensin developed a normal vW protein-containing matrix as seen by immunofluorescence staining (Fig. 5a), cells treated with  $10^{-8}$  M or higher concentrations of monensin did not incorporate vW protein into their matrix (Fig. 5c). Staining with anti-fibronectin antibody showed that monensin had a concentration-dependent inhibitory effect on the production of fibronectin matrix, although it was still prominent at the  $10^{-8}$  M monensin concentration (compare Fig. 5b and 5d).

To test whether the matrix produced in the presence of monensin could bind vW protein, purified plasma vW was added (20  $\mu\text{g}/\text{ml}$  final concentration) overnight to cells grown in monensin-containing medium for 4 d. Although the matrix staining produced by the exogenous plasma vW protein was somewhat fainter than that seen in untreated cultures, the ability of the matrix to bind vW protein was clearly demonstrated (Fig. 5, e and f). The lack of binding of vW protein synthesized in the presence of monensin was not due to a decreased secretion of this protein, since more vW protein was secreted with low concentrations of monensin (Table II, C). We have also tested the ability of secreted vW protein in the monensin-treated cultures to bind to the normal extracellular matrix of human foreskin fibroblasts which do not synthesize vW protein. Culture media produced in the presence or absence of  $10^{-8}$  M monensin were incubated with human foreskin fibroblast cultures for 2 d after which the cells were fixed and stained with anti-vW protein antiserum (Fig. 6). Only vW protein from the medium of cells grown without monensin bound to the extracellular matrix of the fibroblasts. Adding monensin to the medium did not affect

the binding. It appears therefore that the vW protein lacking the high molecular weight multimers that is produced in the presence of monensin cannot participate in the endothelial cell extracellular matrix nor can it bind to foreign matrices.

The defective matrix-binding of vW protein produced in the presence of monensin could possibly be due to the absence of complex type carbohydrate on the molecules rather than to the lack of high molecular weight multimers. Endothelial cells grown for 5 d in the presence of swainsonine before staining with anti-vW protein antiserum had normal Weibel-Palade bodies and a normal vW protein extracellular matrix (Fig. 7). Therefore, the absence of complex type carbohydrate did not affect the storage of vW protein in the Weibel-Palade bodies or the participation of secreted vW protein in the extracellular matrix.

## DISCUSSION

### *Effect of Monensin on vW Protein Production, Storage, and Secretion*

The impact monensin had on vW protein synthesis was dependent upon the monensin concentration (Table II, A). At all concentrations used in our study ( $1-125 \times 10^{-8}$  M), monensin favored secretion of vW protein, in the sense that a greater proportion of synthesized vW protein was released into the culture medium (Table II, B). This increase in exocytosis of vW protein was perhaps due to an increase in the availability of intracellular  $\text{Ca}^{2+}$ , caused indirectly by monensin, as was observed for catecholamine secretion from secretory granules (31). On the other hand, a very high dose

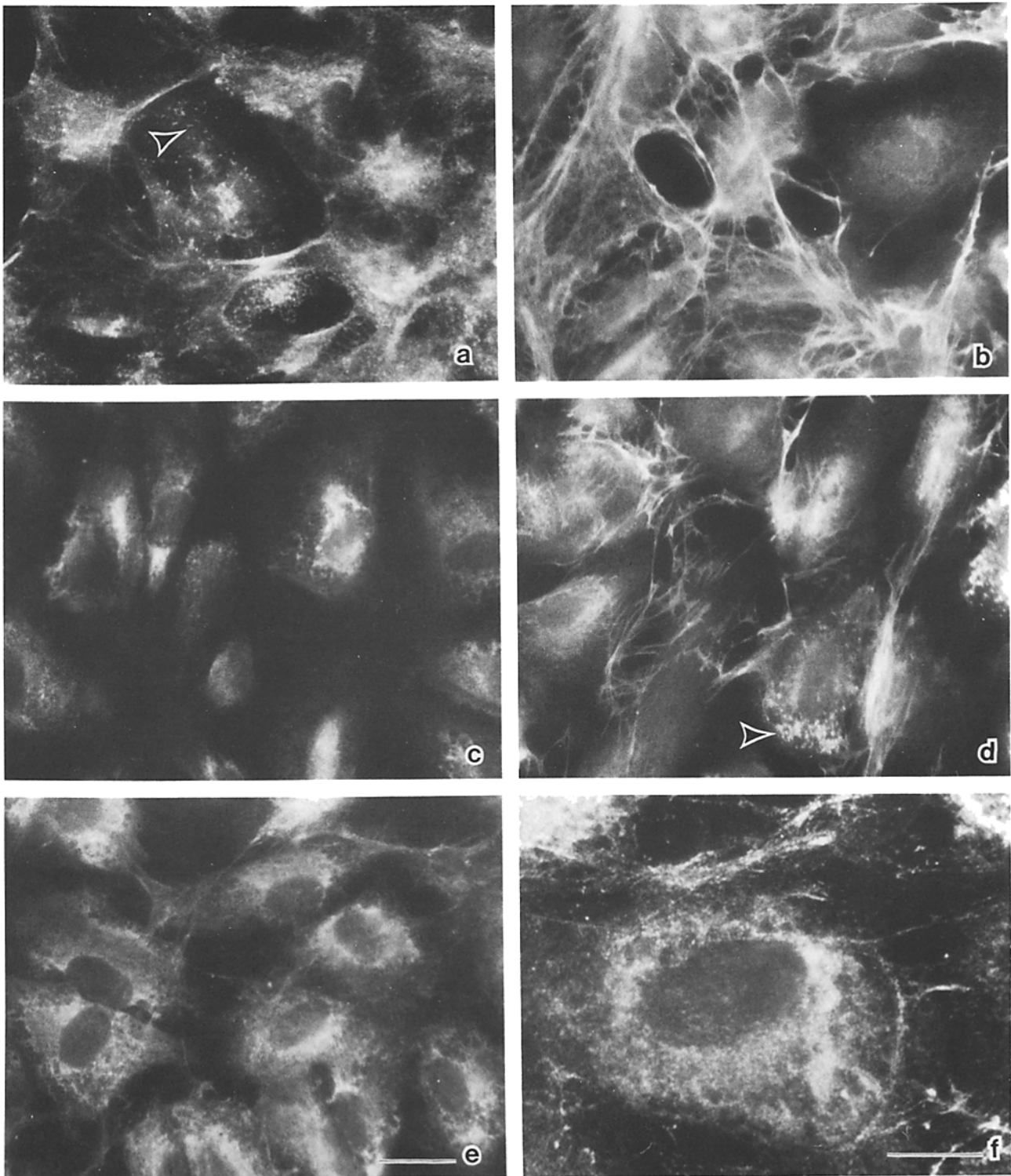


FIGURE 5 Effect of monensin on vW protein incorporation into endothelial cell extracellular matrix. Immunofluorescent staining with anti-vW protein antiserum (a, c, e, and f) or antifibronectin antiserum (b and d) of permeabilized 1-wk-old human umbilical vein endothelial cell cultures. Cells grown in the absence of monensin developed a prominent matrix that was positive for vW protein (a) and fibronectin (b). Weibel-Palade bodies containing vW protein are indicated by the arrowhead in a. While the presence of  $10^{-8}$  M monensin reduced the amount of fibronectin-containing matrix filaments present in the culture (d), the participation of vW protein in the matrix was completely abolished (c). Intracellular protein distribution was also different, as no structures resembling Weibel-Palade bodies were present (c and f). As observed by others (references 26 and 41), intracellular accumulation of fibronectin occurred in the presence of monensin (arrowhead, d). The addition of 20  $\mu\text{g}/\text{ml}$  of purified plasma vW protein to the monensin-treated culture, in the presence of added monensin, resulted in staining of the matrix with anti-vW protein antiserum (e). Panel f shows a higher magnification of this matrix decoration of monensin-treated cells with exogenous vW protein. Bars: a-e, 20  $\mu\text{m}$ ; f, 10  $\mu\text{m}$ .

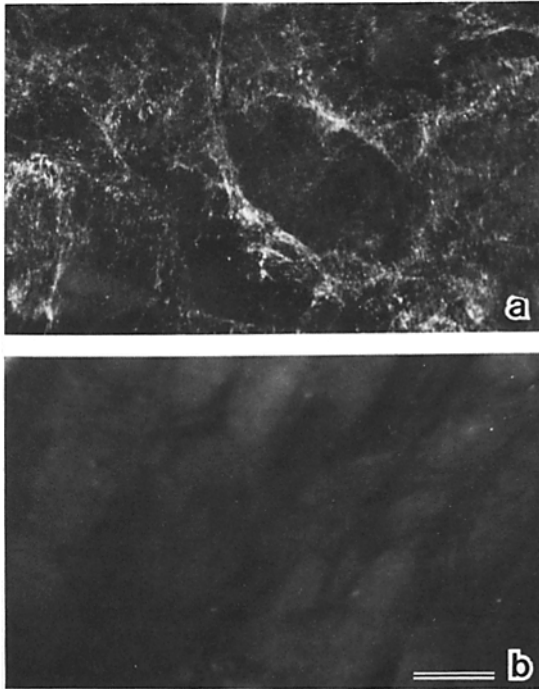


FIGURE 6 Binding of vW protein to the matrix of human foreskin fibroblasts. Fibroblasts grown on glass coverslips were overlaid with 3-d-old culture medium from endothelial cells grown in the absence (a) or presence (b) of  $10^{-8}$  M monensin. After 2 d, the fibroblasts were fixed, permeabilized, and stained by immunofluorescence with anti-vW protein antiserum. vW protein produced in the presence of monensin did not bind to the fibroblast extracellular matrix. Bar, 20  $\mu$ m.

of monensin ( $10^{-5}$  M) inhibited vW protein secretion entirely (26). The secretion of human chorionic gonadotropin is also affected differently at different concentrations of monensin. Peters et al. reported that monensin at  $5 \times 10^{-7}$  M inhibited carbohydrate processing of the hormone without significantly affecting secretion, while 10-fold more monensin inhibited secretion drastically (32). Considering these observations and our results, it appears to be advantageous to study the effect of monensin on protein processing and secretion at several different concentrations.

Pulse-chase experiments have shown that 1  $\mu$ M monensin slightly increased the time required from synthesis to secretion of vW protein (Fig. 2). Similarly, monensin has been observed to decrease the rate of intracellular movement of procollagen and fibronectin (33) and other proteins (17). It is unlikely that the longer time required for transport of vW protein in the presence of monensin is due to its accumulation in a post-Golgi compartment because there was no cellular accumulation of low molecular weight multimers in the presence of monensin. These multimers appeared to be preferentially secreted and were not easily detected in the cells (Fig. 1).

One of the major consequences of monensin treatment, even at concentrations as low as  $10^{-8}$  M, was the disappearance of Weibel-Palade bodies (26; Fig. 5). The data do not allow us to determine whether new Weibel-Palade bodies could not be produced by the Golgi apparatus or if their possible recycling was inhibited by monensin. In either case, vW protein could not be stored in the endothelial cells and the ratio of secreted to cell-associated vW protein increased dramatically (Table II, B). Furthermore, the more processed

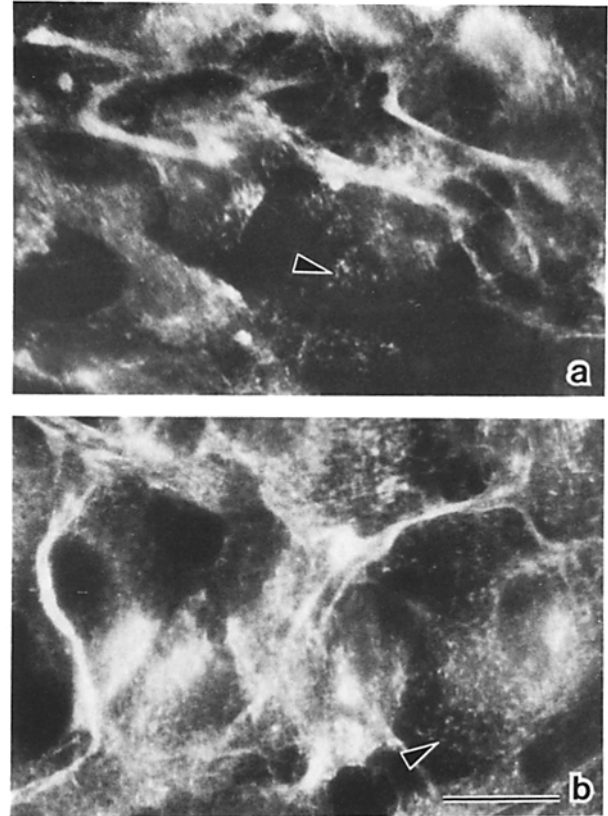


FIGURE 7 Effect of swainsonine on vW protein distribution in endothelial cells. Human umbilical vein endothelial cells cultured in the absence (a) or the presence (b) of 1  $\mu$ g/ml swainsonine were permeabilized and stained with anti-vW protein antiserum. The distribution of vW protein in the treated and untreated cultures was very similar. Note the presence of Weibel-Palade bodies (arrowheads) and matrix filaments in both a and b. Bar, 20  $\mu$ m.

forms of vW protein produced in the presence of a low concentration of monensin, i.e., multimers larger than a dimer which contain the 220,000-mol-wt cleaved subunit, were found only in the secreted fraction (Figs. 1 and 3). The depletion of all vW protein forms except the 260,000-mol-wt dimers in monensin-treated cells again indicates that this dimer was present in a monensin-insensitive compartment and was not stored in the Weibel-Palade bodies.

#### *Inhibition by Monensin of the Processing Steps Localized in the Golgi Apparatus and the Weibel-Palade Bodies*

The presence of the carboxylic ionophore monensin had an inhibitory effect on the biosynthetic processing steps of vW protein that have been assigned to the Golgi apparatus and the Weibel-Palade bodies. Earlier processing steps that occur in the rough endoplasmic reticulum (13) were not inhibited by the presence of the ionophore. Monensin inhibited both multimerization and precursor cleavage in a dose-dependent manner (Table I). Although we did not examine directly the extent of carbohydrate processing as a function of monensin concentration, it is likely that it also varied with the monensin dose, inasmuch as the size of the secreted subunits decreased gradually with increasing concentrations of monensin (Fig. 3).

Pulse-chase experiments have shown that multimerization and precursor cleavage occur at about the same time (13), but the susceptibility of these two processing steps to monensin was very different (Table I). This disparity argues in favor of the two processing steps being separate enzymatic events. The interdimer disulfide bond formation in vW protein is the first example described of disulfide bond formation occurring in the Golgi apparatus where it can be inhibited by monensin. It is likely that vW is not unique in this respect and that other proteins form interchain disulfide bonds in the Golgi apparatus. It appears that this Golgi-localized multimerization step requires special conditions, for example, low pH (34), and likely a specific enzyme, and therefore it would not be expected to occur spontaneously after the protein was secreted (Fig. 1). It is also unlikely that an equilibrium between the different polymeric sizes could exist outside the cell.

The inhibition of polymerization and precursor cleavage did not appear to be consequences of the absence of carbohydrate processing in the presence of monensin. To begin, vW protein synthesized by cells cultured in the presence of a very low concentration of monensin lacked the highest molecular weight multimers but was still endoglycosidase H-resistant (not shown). Further, swainsonine, a drug which inhibits the Golgi enzyme mannosidase II (27) and therefore inhibits biosynthesis of complex type carbohydrate, did not affect either precursor cleavage or high molecular weight multimer formation of vW protein (Fig. 4). The carbohydrate processing was indeed inhibited in the presence of swainsonine as could be demonstrated by the susceptibility of the protein to the enzyme endoglycosidase H (Fig. 4a).

At high concentrations of monensin, the major vW protein species secreted was the dimer of the 260,000-mol-wt subunits (Figs. 1 and 3). Since this species is normally present in the endoplasmic reticulum and is not secreted (13), monensin produced a functional or possibly physical bypass of the Golgi apparatus. This should be elucidated by a cell fractionation study.

#### *Low Molecular Weight Multimers Produced in the Presence of Monensin Do Not Participate in the Extracellular Matrix*

vW protein mediates the binding of platelets to subendothelium, an in vitro model of which is the endothelial cell extracellular matrix. vW protein was demonstrated in the matrix (Fig. 5a) where it co-distributed with other components such as fibronectin and collagens (35, 36). Cells grown in  $10^{-8}$  M monensin produced a decreased quantity of fibronectin-containing matrix, from which vW protein was completely lacking, as determined by fluorescence staining (Fig. 5c). The matrix produced in the presence of monensin was still able to bind exogenous normal vW protein, from control cell culture medium (not shown) or from human plasma (Fig. 5, e and f). vW protein synthesized and secreted in the presence of monensin also could not bind to the extracellular matrix of human foreskin fibroblasts, whereas vW protein from untreated cultures did bind (Fig. 6). The data strongly support the conclusion that the vW protein produced in the presence of monensin was functionally defective. It does not appear likely that the presence of <20% precursor subunit in the dimer and multimers was blocking the binding of the protein to the matrix, but we cannot rule out this possibility.

We were more concerned about the possible role of complex type carbohydrate in the binding of vW protein to the extracellular matrix. This concern was suggested by observations of several patients with a variant of vW disease who had carbohydrate deficiency, in addition to other defects in vW protein (37, 38). However, endothelial cells grown in the presence of swainsonine produced vW protein with incompletely processed carbohydrates that remained endoglycosidase H-sensitive (Fig. 4a), but these cells had an apparently normal extracellular matrix containing vW protein (Fig. 7). Therefore, the defect in matrix participation of vW protein in the presence of monensin was not due to the absence of complex type carbohydrate.

The major difference between vW protein produced in control and  $10^{-8}$  M monensin-treated cultures that we have observed was the absence of large and medium-sized multimers in the monensin-treated cultures (Fig. 1; Table I). Type IIA vW disease has been attributed to the absence of these multimers. The importance of the large multimers may be explained if polyvalency was required for proper binding of the protein to the extracellular matrix or in vivo to the basement membrane. For instance, sequences related to the known cell binding sequence of fibronectin (39) are present several times within each fibronectin subunit (40).

The addition of monensin to endothelial cell cultures elicited changes in synthesized vW protein that mimic the situation existing in Type IIA vW disease. Thus, the high molecular weight multimers which are lacking in the human disease state were also missing from the endothelial cell cultures. Furthermore, the monensin-treated cells synthesized only low molecular weight multimers, similar in size to those found in Type IIA patients, and these were functionally defective, as is the case in the disease state. As monensin inhibits posttranslational modifications localized to the Golgi apparatus, the data further indicate that a defect in the Golgi-controlled interdimer disulfide bond formation might be the pathogenetic mechanism underlying Type IIA vW disease.

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