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Cultured aortic endothelial cells from pigs with von Willebrand disease: *In vitro* model for studying the molecular defect(s) of the disease

(ristocetin-Willebrand factor/microfilaments/platelet-endothelial interaction)

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ABSTRACT **Aortic endothelial cells from normal pigs and pigs with von Willebrand disease have been established in long-term cultures. Both cultures appeared similar in terms of general growth characteristics, morphologic features, and ultrastructure. Immunofluorescent staining of these cultures with chicken (or rabbit) antiporcine ristocetin-Willebrand factor sera (or IgG) resulted in extensive perinuclear staining of the cells in both cultures. Additionally, staining of semiconfluent cultures of normal cells for ristocetin-Willebrand factor revealed an extensive meshwork of distinct, immunologically identifiable ristocetin-Willebrand factor-containing filaments between cells. Immunoreactive material was considerably decreased and more diffuse between cells in semiconfluent cultures from affected pigs. Through immunocytochemical staining with peroxidase-coupled antiserum, the filaments (of indeterminate length) were found to have a diameter of approximately 300 Å. Finally, washed porcine platelets interacted extensively with scrape-damaged cultures of normal endothelial cells but not with scrape-damaged cultures of affected endothelial cells. This interaction of platelets with damaged normal cultures was abolished by pretreatment of the cultures with rabbit antiporcine ristocetin-Willebrand factor IgG.**

The classic form of von Willebrand's disease (vWd) is characterized by prolonged bleeding times, decreased platelet retention in glass bead columns, decreased ristocetin-induced platelet aggregation, and decreased Factor VIII coagulant activity and Factor VIII-related antigen. Jaffe *et al.* (1, 2) have presented evidence that cultured endothelial cells synthesize and release ristocetin-Willebrand factor (RWF) and Factor VIII antigen although no Factor VIII coagulant activity (1, 2) has been demonstrated. Holmberg *et al.* (3), using an immunofluorescence staining technique, were unable to detect the presence of RWF in the endothelial cells of patients with vWd. Caen and Sultan (4) have speculated that vWd is due to an endothelial cell abnormality that either prevents them from synthesizing RWF or causes them to synthesize an abnormal RWF.

vWd that resembles the classic disease in man has also been described in dogs (5) and pigs (5, 6). The purpose of this communication is to describe the long-term culture and properties of normal and vWd porcine endothelial cells and the use of this *in vitro* culture system for studying the molecular defect(s) in vWd.

MATERIALS AND METHODS

Preparation and Culture of Cells. No detectable Willebrand factor or RWF could be demonstrated in the plasma of the vWd pigs that were used as the source of vWd aortas. Willebrand factor was determined by Laurell immunoelectrophoresis (7), and RWF activity was measured by ristocetin-induced platelet aggregation assays.

Endothelial cells were obtained from untraumatized, freshly excised normal and vWd porcine aortas (25–35 cm long) by collagenase treatment (0.5 mg/ml, CLS, Worthington Biochemical Corp., Freehold, NJ) for 15–20 min at 37°. Collagenase-treated aortas were rinsed twice with RPMI-1640 medium (GIBCO), and the endothelial cells were removed, in complete medium, by gentle agitation as described for bovine aortic endothelial cells (8). The complete medium consisted of RPMI-1640 medium, 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, penicillin (100 units/ml), streptomycin (50 μg/ml), neomycin (200 units/ml), 2 mM fresh glutamine, and 20% fetal calf serum or 20% porcine serum (the calf and porcine sera were heat-deactivated at 56° for 30 min). The final complete culture medium was adjusted to pH 7.3. Medium containing neomycin but not penicillin and streptomycin was used for subcultures.

Typically, 25–35 cm of aorta yielded 1.5 to 4 × 10^6 cells in 20 ml of complete medium. Cells were seeded into Falcon T-25 (25 cm^2) plastic flasks or Falcon tissue culture petri dishes (35 × 10 mm) at a cell density of no more than 200 cells per cm^2. Cell patches rapidly (<1 hr) attached to the substratum and, after 1–2 hr, spread cell patches were rinsed twice with RPMI-1640 medium and fed with complete fresh medium. Medium without penicillin and streptomycin was exchanged at 72-hr intervals, and the cultures were incubated at 37°; cultures in unsealed petri dishes were incubated in a 95% air/5% CO_2 atmosphere.

After 4–6 days in culture, individual patches of tightly packed polygonal endothelial cells were selected under the phase-contrast microscope and harvested by brief (2–3 min) treatment with 0.25% trypsin/0.01% EDTA with a 4-mm-diameter cloning ring. Harvested cells were seeded into T-25 flasks or petri dishes at a density of no more than 10–40 cells per cm^2. Additional cloning of cell patches was carried out on these cultures after 4–6 days, if required. These patch-cloned cultures formed confluent monolayers in 10–14 days. Selected cultures were serially subcultured by brief trypsin treatment and routinely split 1:12 (final cell density, about 9 to 10 × 10^3 cells per cm^2).

Smooth muscle cells were obtained from porcine aortic smooth muscle segment explants and cultured as described by

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Abbreviations: vWd, von Willebrand disease; RWF, ristocetin-Willebrand factor; P_i/NaCl, 0.145 M NaCl/1 mM Na phosphate, pH 7.2; TBSS, Tyrode's balanced salt solution.

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Ross (9). These cells could be serially subcultured and were identified as smooth muscle cells by their characteristic growth, morphologic, and ultrastructural properties (9).

Electron Microscopy. Cultured cells were prepared for microscopy by fixation *in situ* for 1 hr in 2% gluteraldehyde buffered to pH 7.2 with 0.05 M Na phosphate containing 5% sucrose. Subsequently, the cells were removed from the flasks by scraping and postfixed in 1.3% OsO_4 at room temperature for 1 hr. Staining *en bloc* with uranyl acetate was followed by dehydration through a graded series of ethanol and embedding in Epon 812 (10). Ultrathin sections were double-stained with uranyl acetate and lead citrate before being examined in a Philips EM 201 electron microscope. In selected cases, sections were examined by electron microscopy without prior staining with lead citrate and uranyl acetate.

Immunochemical Staining. Chromatographically purified porcine RWF (11) was adsorbed to aluminum hydroxide gel and injected into rabbits subcutaneously. Weekly injections of 1 unit of RWF were continued for 5 weeks, after which sera collections were begun. The unadsorbed rabbit antiserum inhibited the ability of normal porcine plasma to support ristocetin-induced aggregation of human washed, gel-filtered platelets. The rabbit antiserum also exhibited a high degree of specificity with only 15% of the antiporcine activity when tested against human plasma in the same platelet aggregation assay. The rabbit antiporcine RWF serum (or IgG) formed only a single precipitin line with normal porcine plasma in both Ouchterlony and Laurell procedures. No precipitin lines were observed with plasma from vWd pigs. The rabbit antiserum also exhibited little or no inhibitory capacity toward Factor VIII coagulant activity (11). Antibodies to porcine RWF were similarly raised in chickens. The chicken antiserum gave single immunoprecipitin lines with normal porcine, human, bovine, and canine plasmas but no precipitin lines with plasma from vWd pigs.

Immunofluorescent staining was carried out on endothelial and smooth muscle cells that had been cultured on glass cover slips, fixed with acetone for 3 min at 4°, and air dried. Before treatment with antisera, cover slips were immersed for 10 min in 0.145 M NaCl/1 mM Na phosphate, pH 7.2 (P_i/NaCl). When chicken antiserum was used, 4% NaCl buffered with 0.01 M Na phosphate (pH 7.2) was substituted for the P_i/NaCl. The chicken antiserum was also diluted with buffered 4% NaCl. Excess saline was removed from the cover slip before addition of 2–4 drops of a 1:20 dilution of either rabbit antiporcine RWF IgG fraction or chicken antiporcine RWF serum or nonimmune rabbit IgG fraction. The cover slips then were incubated in a moist chamber for 45 min at 37°. The incubated slips were washed for 5 min in each of three changes of P_i/NaCl. The staining and washing procedures were then repeated with a 1:15 dilution of fluorescein-conjugated goat antiserum to rabbit IgG (Hyland) (in the case of the chicken antiserum, a 1:15 dilution of fluorescein-conjugated rabbit IgG fraction to chicken IgG was used, from Miles-Yeda, Ltd.). Cover slips were examined in a Leitz Orthoplan photomicroscope with an HBO 100 ultra-high-pressure mercury lamp. Photographs of immunofluorescence-stained cultures were exposed and processed under identical conditions.

Immunocytochemical staining was carried out on endothelial cells fixed *in situ* for 10 min with 5% formaldehyde in 0.05 M Na phosphate buffer, pH 7.2. Fixed cells were scraped from the culture flask, pelleted, washed twice with P_i/NaCl, and treated with a 1:20 dilution of rabbit antiporcine RWF IgG fraction for 45 min at 37°. Incubated cells were pelleted, washed twice with P_i/NaCl, and incubated with burro antirabbit IgG (Fab fragment) coupled to horseradish peroxidase (J. C. Lewis, D. A. Loegering, J. E. Maldonado, and G. J. Gleich, personal communication). Subsequently the cells were washed twice in P_i/NaCl, fixed further with 1% gluteraldehyde buffered to pH 7.2 with 0.05 M Na phosphate, washed twice with P_i/NaCl (10 min each), and treated with 3,3′-diaminobenzidine and H_2O_2 to localize peroxidase activity as described (12). The cells were postfixed in 1.3% OsO_4 and processed for electron microscopy as described above. Controls included (*i*) staining with 3,3′-diaminobenzidine plus H_2O_2 plus OsO_4 only, (*ii*) replacement of the specific antiporcine RWF IgG fraction with nonimmune rabbit IgG fraction, (*iii*) replacement of the specific antiporcine RWF IgG fraction with antiporcine RWF IgG fraction adsorbed with purified porcine RWF, and (*iv*) staining with burro antirabbit IgG (Fab fragment), 3,3′-diaminobenzidine, H_2O_2, and OsO_4 only.

Antisera and IgG fractions were all heat deactivated at 56° for 30 min. In addition, the fluoresceinated goat antirabbit and rabbit antichicken IgG sera were adsorbed with porcine liver acetone powder.

All immunofluorescence studies were performed on cultured normal and vWd porcine aortic endothelial cells and cultured normal porcine aortic smooth muscle cells.

Platelet-Endothelial Cell Interactions. Postconfluent cultures (4–6 days after reaching confluency) of normal and vWd endothelial cells grown on plastic cover slips (13) were rinsed twice with modified Tyrode's balanced salt solution (TBSS) (modified by decreasing the Ca^{2+} concentration by half), and the confluent cultures were damaged by scraping the culture surface. The scrape-damaged cultures were then incubated with either nonimmune rabbit IgG or rabbit antiporcine RWF IgG (diluted 1:30 in modified TBSS) for 30 min prior to exposure to platelets. Antibody-treated cover slips were rinsed twice for 10 min each with modified TBSS and subsequently floated on washed porcine platelets (7×10^5 platelets per μl) resuspended in modified TBSS, essentially as described (13).

Platelet–endothelial cell interactions were terminated after 4 min; the cover slips were rinsed three times with modified TBSS and then fixed and stained with methylene blue as described (13).

Platelets were obtained from freshly drawn porcine blood containing ACD (0.8% citric acid/2.2% sodium citrate/2.45% dextrose) as anticoagulant; 1 ml of ACD per 8 ml of blood. Platelets were isolated by differential centrifugation as described for bovine platelets (13). The pellet was resuspended and washed twice with 0.15 M NaCl/ACD/(30:1, vol/vol) and once with modified TBSS. The washed platelet pellet was finally resuspended in modified TBSS at a concentration of 7×10^5 platelets per μl.

RESULTS

Growth Behavior. Approximately 80–85% of both normal and vWd porcine aortic endothelial cells attached to the substratum within 30 min. No differences were observed in the rate of cell attachment, spreading of cell clumps, or growth morphology in normal and vWd cultures. With cells from both sources, primary and subculture inocula, varying from 10 to 1.2×10^4 cells per cm^2, all resulted in established cultures of single-cell-thick confluent monolayers (1.1 to 1.2×10^5 cells per cm^2) of tightly packed polygonal cells when cultured in RPMI-1640 medium supplemented with 20% fetal calf or porcine serum. Population doubling times of about 22–24 hr (normal and vWd) were observed during the logarithmic phase of growth.

Selected cultures of aortic endothelial cells from six separate

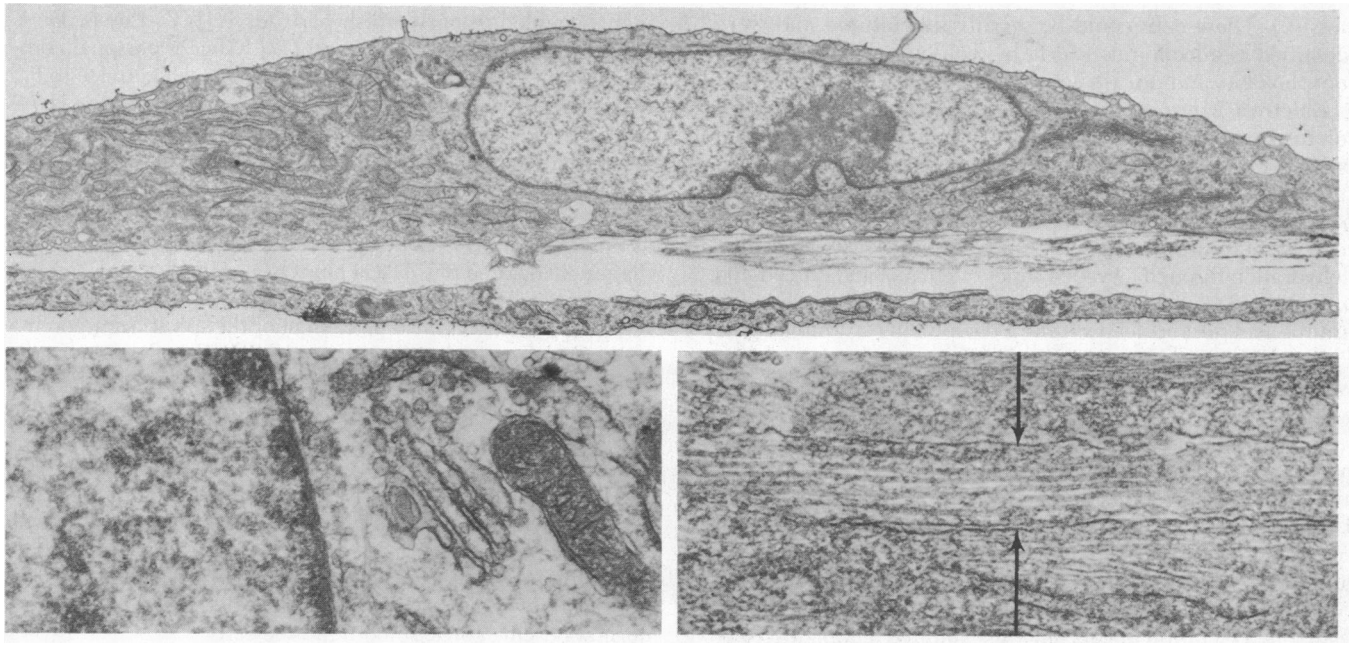

FIG. 1. (*Upper*) Subcultured vWd porcine aortic endothelial cell in ninth passage. (×9000.) (*Lower Left*) High magnification of a Golgi region in a subcultured (12th passage) vWd endothelial cell, showing vesicles and flattened saccules. (×41,200.) (*Lower Right*) Perinuclear cytoplasm from a subcultured (12th passage) vWd porcine endothelial cell, showing structure resembling Weibel–Palade body (between arrows). (×52,500.)

normal and three separate vWd porcine aortas were serially subcultured and maintained for 8–14 months (10–24 passages) and 10–14 months (18–22 passages), respectively. All these cells retained their closely opposed tight packing and population doubling times (approximately 22–28 hr) and formed confluent monolayers as readily as did primary cultures.

Electron Microscopy. Normal and vWd cultured cells (primary and subcultures in 2nd, 5th, 9th, and 12th passages) were ultrastructurally similar to *in vivo* porcine aortic endothelial cells.

The cells in all cultures were oval to fusiform in shape and had centrally located electron-lucent nuclei with prominent nucleoli. An abundance of rough endoplasmic reticulum and many mitochondria with transverse cristae and electron-opaque matrices were observed randomly positioned throughout the peripheral cytoplasm (Fig. 1). The perinuclear cytoplasm was characterized by the presence of extensive Golgi regions comprised of vesicles and flattened saccules. Membrane-enclosed tubular structures often resembling Weibel–Palade bodies were found in some of the cells. This observation was infrequent: these organelles were noted in only two or three out of several hundred cell sections studied. Cultured porcine aortic endothelial cells (normal and vWd) were ultrastructurally distinct from cultured smooth muscle cells from the same source.

Immunofluorescence Studies. Low-intensity immunofluorescence staining for RWF was associated with entire cells in normal primary and subcultured (3rd, 7th, 11th, and 13th passages) porcine aortic endothelial cell cultures. The staining was most prominent in the perinuclear region of these cells (Fig. 2 *left*). Similar results were obtained when either monospecific chicken antiporcine RWF serum or rabbit antiporcine RWF IgG fraction was used. Cellular and perinuclear staining were completely abolished by adsorption of the chicken antiserum or rabbit IgG fraction with purified porcine RWF.

Immunofluorescent staining for RWF was also observed in entire cells, most prominently in the perinuclear region, of vWd primary cultures and subcultured (2nd, 5th, 9th, and 17th passages) cells (Fig. 2 *right*). Although the apparent extent of perinuclear staining for RWF was similar in normal and vWd endothelial cells, the nature of the staining was different. In general, perinuclear staining in normal cells appeared somewhat more diffuse as compared to the more granular staining observed in the vWd cells.

In addition to the cellular and perinuclear staining for RWF observed in these cells, treatment of semiconfluent cultures (normal and vWd) with monospecific chicken (or rabbit) antiserum to purified porcine RWF showed the presence of an extensive meshwork of distinct immunofluorescent filaments in the spaces between normal porcine cells (Fig. 3 *upper*). The immunofluorescence in the spaces between vWd porcine cells was more diffuse and considerably decreased (Fig. 3 *lower*). Distinct immunofluorescently stained filaments were not as apparent in the vWd cultures as in the normal cultures. Staining (perinuclear and filaments) of semiconfluent cultures was completely abolished by adsorption of the chicken antiserum or rabbit IgG fraction with purified porcine RWF.

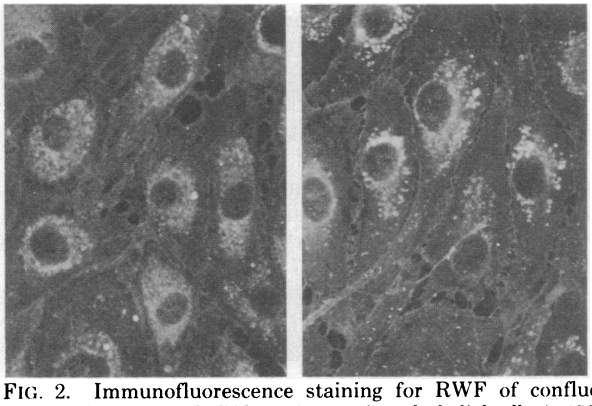

FIG. 2. Immunofluorescence staining for RWF of confluent subcultured normal and vWd porcine aortic endothelial cells. (×1500.) (*Left*) Normal culture, seventh passage. (*Right*) vWd culture, fifth passage. Note perinuclear staining.

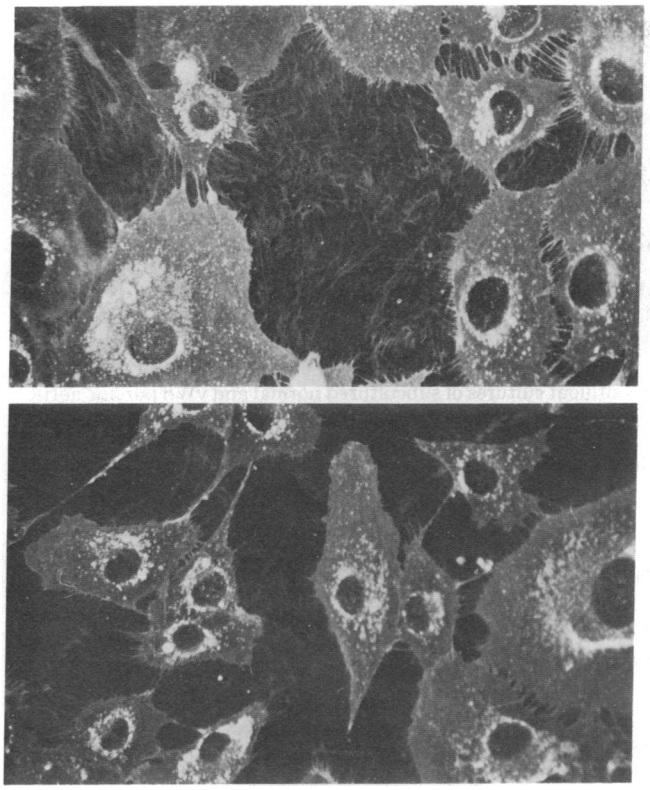

FIG. 3. Immunofluorescent staining for RWF of semiconfluent subcultured normal and vWd porcine aortic endothelial cells. (×1400.) (*Upper*) Normal (13th passage) culture showing extensive meshwork of distinct immunofluorescent filaments in spaces between cells. (*Lower*) vWd (11th passage) culture showing decreased and diffuse immunofluorescent material in spaces between cells.

Similar immunofluorescent staining was observed with six different established normal and three established vWd porcine aortic endothelial cell cultures.

Immunofluorescent staining for RWF was not observed with cultured porcine aortic smooth muscle cells.

Immunochemical Studies. Staining of formaldehyde-fixed normal primary and subcultured (seventh passage) porcine endothelial cells, by using monospecific rabbit antiporcine RWF IgG fraction, consistently showed the presence of a meshwork of horseradish peroxidase-decorated extracellular filaments (Fig. 4 *left*). When observed at high magnification, the electron opaque 3,3′-diaminobenzidine oxidation product appeared to enshroud a filament having a diameter in the range of 300–400 Å (Fig. 4 *right*). No peroxidase reaction was associated with smaller filaments.

Similar treatment of vWd subcultured (7th and 12th passages) endothelial cells did not show any extracellular horseradish peroxidase-decorated material. None of the reaction controls with either the normal or the vWd cultured cells showed specific intracellular or extracellular staining.

Platelet–Endothelial Cell Interaction. Washed normal porcine platelets resuspended in modified TBSS did not interact with undamaged confluent normal porcine aortic endothelial cells in culture. However, confluent normal cultures that were damaged by scraping reacted extensively with platelets at the exposed sites of damage. This *in vitro* interaction of platelets with exposed subendothelial microfilaments, described previously (13), was not affected in damaged normal control endothelial cell cultures pretreated with nonimmune rabbit IgG fraction (Fig. 5). Pretreatment of damaged normal endothelial cell cultures with monospecific rabbit antiporcine RWF IgG

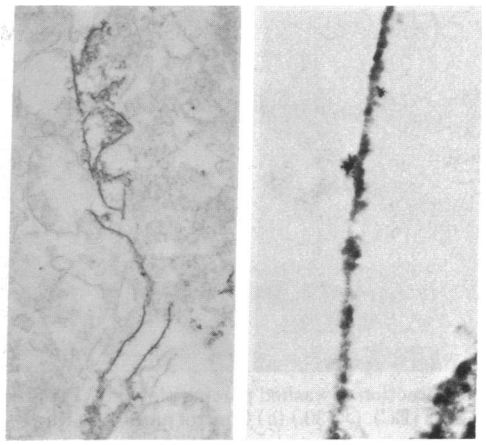

FIG. 4. Electron micrographs of immunocytochemical staining for RWF in subcultured (seventh passage) normal porcine aortic endothelial cells using burro antirabbit IgG (Fab fragment) coupled to horseradish peroxidase. (*Left*) Unstained section, showing peroxidase-positive extracellular filaments. (×22,300.) (*Right*) Higher magnification of filament enshrouded with diaminobenzidine oxidation product. Section stained before viewing. (×60,000.)

fraction, however, abolished all platelet interaction. In the case of vWd endothelial cell cultures, no platelet interaction was observed with either the damaged vWd control cultures pretreated with nonimmune rabbit IgG fraction or with the damaged vWd cultures pretreated with monospecific rabbit antiporcine RWF IgG fraction (Fig. 5).

DISCUSSION

Normal and vWd porcine aortic endothelial cells have been established in long-term culture. These cells exhibit the same characteristic ultrastructural properties as those described for bovine and porcine aortic (8, 14) and human umbilical cord endothelial cells (15, 16).

Jaffe *et al.* (1, 2) have previously shown that cultured human umbilical cord endothelial cells synthesize and release RWF. By using monospecific rabbit antiporcine RWF IgG fraction (11) and an immunofluorescent staining technique, we have confirmed the presence of RWF in endothelial cells in long-term cultures of both normal and vWd porcine aortic endothelial cells. Immunofluorescent staining with monospecific antibody to RWF showed perinuclear staining in normal and vWd porcine cells. In addition, immunofluorescent staining for RWF of semiconfluent cultures (normal and vWd) showed the presence of an extensive meshwork of filaments between cells in normal cultures only. Immunocytochemical staining of normal cultured porcine endothelial cells, with a horseradish peroxidase-linked antibody, confirmed the presence of extracellular microfilaments that consist, at least in part, of immunologically detectable RWF in normal cultures only.

From these immunological data on the nature and presence of RWF in cultured vWd porcine aortic endothelial cells, we have concluded that (*i*) RWF is produced in a normal molecular form but is present in decreased amounts due to decreased synthesis, (*ii*) RWF is produced in normal molecular form in normal amounts but cannot be transported and/or assembled into a biologically and immunologically reactive extracellular molecular form (RWF-containing filaments), (*iii*) RWF is produced in an abnormal molecular form and is transported and/or assembled into an abnormal extracellular molecular form that is biologically and immunologically less reactive, or (*iv*) RWF is not produced by these cells but is accumulated by

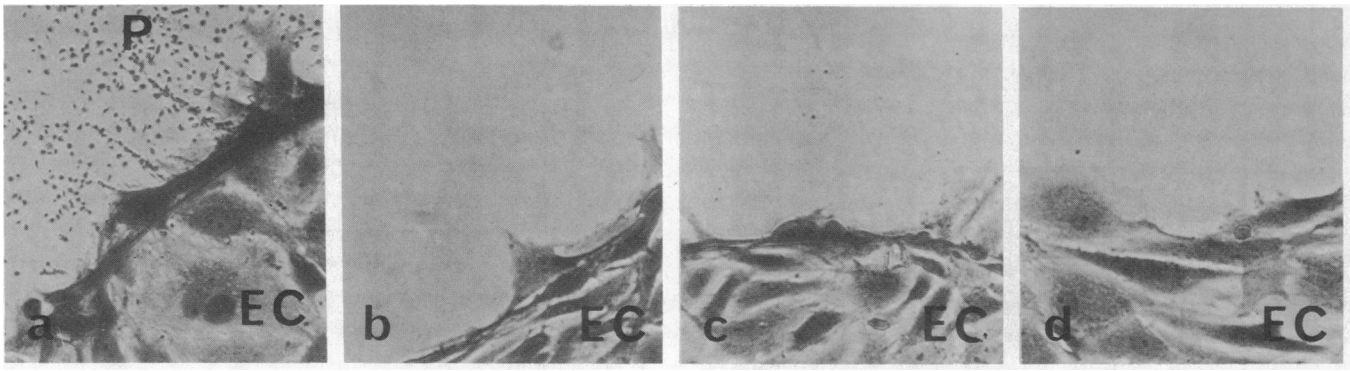

FIG. 5. Interaction of washed porcine platelets (P) with scrape-damaged confluent cultures of subcultured normal and vWd porcine aortic endothelial cells (EC). (×430.) (*a*) Control normal culture (eighth passage) treated with nonimmune rabbit IgG (diluted 1:30) prior to exposure to platelets. (*b*) Normal culture (eighth passage) treated with rabbit antiporcine RWF IgG (diluted 1:30) prior to exposure to platelets. (*c*) Control vWd culture (eighth passage) treated as described in *a*. (*d*) vWd culture (eighth passage) treated as described in *b*. No platelet interaction was observed with the vWd cultures *c* and *d*.

the concentration or adsorption of RWF from the serum in the medium.

Of the three cultured vWD porcine endothelial cell lines studied thus far, only one has provided cells that showed an apparent decrease in the extent of perinuclear immunofluorescent staining for RWF. If this apparent perinuclear staining were due only to a decreased synthesis of RWF, we would also expect to see decreased amounts of an extracellular molecular form of normal RWF (RWF-containing filaments) in cultured vWd endothelial cells. We have been unable to demonstrate the presence of any distinct RWF-containing filaments in semiconfluent cultured vWd endothelial cells by using immunofluorescent staining techniques.

Porcine aortic endothelial cells (normal and vWd) have been maintained and serially subcultured (three to four passages) in normal and vWd porcine sera without any apparent differences in the extent and nature of perinuclear immunofluorescent staining for RWF. In addition, we have been unable to detect any RWF in fetal calf serum or in vWd porcine serum. Finally, following a standard immunization protocol, we have been unable to develop a demonstrable antibody titer against RWF in a homozygous vWd pig. These data suggest that RWF is, in fact, produced in vWd endothelial cells and is not accumulated from the serum in the culture medium.

The presence of immunologically reactive RWF in vWd porcine aortic endothelial cells, reported here, differs from the observations by Holmberg *et al.* (3). These investigators reported the apparent absence of RWF from endothelial cells in biopsy material from human patients with vWd, on the basis of an immunofluorescent staining technique. The differences in the results are unexplained.

The *in vitro* interaction of porcine platelets with damaged normal porcine endothelial cells and the subsequent inhibition of this interaction by rabbit antiporcine RWF IgG fraction, in conjunction with the absence of platelet interaction and an immunologically detectable extracellular RWF form (RWF-containing filament) in vWd endothelial cells, suggest a possible role for RWF in platelet–endothelial cell (vessel wall) interaction. The exact nature of this relationship remains to be demonstrated.

Data presented here show that established cultures of vWd porcine aortic endothelial cells exhibit differences in the distribution and nature of immunologically detectable RWF as well as decreased platelet interaction. This culture system, therefore, provides a unique model system for studying the molecular defect(s) of this disease *in vitro*.

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