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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Communicated by K. M. Brinkhous, September 6, 1977

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. Immunoactive 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 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°C. 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°C 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 X 10⁶ cells in 20 ml of complete medium. Cells were seeded into Falcon T-25 (25 cm²) plastic flasks or Falcon tissue culture petri dishes (35 X 10 mm) at a cell density of no more than 200 cells per cm². 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°C; cultures in unsealed petri dishes were incubated in a 95% air/5% CO₂ 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 cm². 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 X 10⁶ cells per cm²).

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

Abbreviations: vWd, von Willebrand disease; RWF, ristocetin-Willebrand factor; P₁/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% OsO4 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.

**Immunohistochemical 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 antiserum, cover slips were immersed for 10 min in 0.145 M NaCl/1 mM Na phosphate, pH 7.2 (Pi/NaCl). When chicken antiserum was used, 4% NaCl buffered with 0.01 M Na phosphate (pH 7.2) was substituted for the Pi/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 nonimmunized 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 Pi/NaCl. The staining and washing procedures were then repeated with a 1:15 dilution of fluorescein-conjugated goat antisera 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 Pi/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 Pi/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 Pi/NaCl, fixed further with 1% gluteraldehyde buffered to pH 7.2 with 0.05 M Na phosphate, washed twice with Pi/NaCl (10 min each), and treated with 3,3′-diaminobenzidine and H2O2 to localize peroxidase activity as described (12). The cells were postfixed in 1.3% OsO4 and processed for electron microscopy as described above. Controls included (i) staining with 3,3′-diaminobenzidine plus H2O2 plus OsO4 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, H2O2, and OsO4 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 three times with modified Tyrode’s balanced salt solution (TBSS) (modified by decreasing the Ca2+ 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 platelettes (7 × 10^5 platelettes 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).

Platelettes 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. Platelettes were isolated by differential centrifugation as described for bovine platelettes (15). 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 platelettes per μl.

**RESULTS**

**Growth Behavior.** Approximately 80–85% of both normal and vWd porcine aortic endothelial cells attached to the sub-stratum 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 cm2, all resulted in established cultures of single-cell-thick confluent monolayers (1.1 to 1.2 × 10^6 cells per cm2) 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
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 19th 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.
cell cultures with fraction (Fig. 5).

Previously (13), sites exposed subendothelial microfilaments, damaged by in 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, 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
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.

The authors are indebted to Dr. D. M. Lewis, Department of Otorhinolaryngology, Ohio State University Hospitals, for providing the burro antirabbit IgG-horseradish peroxidase conjugate and G. J. Knutson for his technical assistance. This work was supported in part by grants from the National Institutes of Health (HL-17973 and HL-17430), the Chicago and Illinois Heart Associations, the Clow Foundation, and the Mayo Foundation.