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Fibronectin Biosynthesis and Cell-Surface Expression by Cardiac and Non-Cardiac Endothelial Cells

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We examined the biosynthesis and surface expression of fibronectin, an adhesive glycoprotein, in several types of cultured porcine endothelial cells: pulmonary artery, thoracic aorta, coronary artery, aortic valve, and mitral valve. We used immunocytochemical staining to compare the levels of fibronectin present in these same tissues* in vivo. *Using endogenous radiolabeling, we found that all cell types except aortic valve endothelial cells synthesized and released into the culture media substantial quantities of fibronectin. Using radioiodination of intact cells, we found that, whereas both thoracic aorta and pulmonary artery cells had measurable fibronectin on the surface, aortic valve, mitral valve, and coronary artery cells had little cell-surface fibronectin present. Immunocytochemical staining showed that all endothelial regions except aortic valve had substantial quantities of immunoreactive fibronectin* in vivo. *These data suggest that the aortic valve endothelium may be distinct from other endothelia. Such differences could be important for the pathogenesis of valvular disease. (Am J Pathol 1993, 142:1401–1408)

Fibronectin (FN) is a 440–450,000 molecular weight dimeric glycoprotein found in the plasma and on cell surfaces. FN has been shown to have important functions in cellular adhesion, both to other cells and to components of the extracellular matrix (reviewed in refs. 1–3). FN is a major biosynthetic product of many cultured cells,[4] particularly endothelial cells,[5] and is an important modulator of normal tissue growth,[6] tissue repair after injury,[7] and platelet interactions with the vessel wall.[8] We have previously shown that cultured porcine aortic valve endothelial cells, compared with porcine thoracic aorta endothelial cells, exhibited a relative deficiency of FN synthesis.[9] This difference in FN synthesis was not observed with other glycoproteins.[10] It is possible that such a selective difference in the production of this important adhesive protein could contribute to the pathogenesis of disorders of the cardiac valve. In the present studies we wished to answer several questions: 1) Do such differences in FN synthesis extend to endothelial cells derived from other cardiac sites? 2) Are there differences in surface expression of FN on cultured endothelial cells? and 3) Are there differences in FN expression between cardiac and non-cardiac endothelial surfaces *in vivo*?

Materials and Methods

Cell Isolation and Culture

Porcine endothelial cells derived from the thoracic aorta, aortic valve, mitral valve, pulmonary artery, and coronary artery were isolated from normal pigs as we have previously described.[9,11] These cells were plated at limiting dilution after primary isolation from the tissues, thereby producing pure cell lines derived from single growth foci.[9] All cells were grown in medium 199 supplemented with 10% fetal bovine serum (both from Hyclone, Logan, UT), penicillin G (100 U/ml), and gentamicin (both from GIBCO-BRL, Gaithersburg, MD). All tissue culture supplies were from Falcon (Oxnard, CA). When confluent, cultures were passaged by treatment with trypsin/EDTA (GIBCO). All cultures used in these experiments were derived from several ani-

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mals and were studied when just confluent at from fourth to seventh passage.

Radiolabeling of Cell-Surface Proteins

Endothelial cell cultures were surface-labeled as we have previously described.[12] This technique uses lactoperoxidase/glucose oxidase immobilized on micro-carrier beads (Bio-Rad, Richmond, CA) to catalyze radioiodination of cellular proteins under conditions that confine the labeling reaction to the fluid phase above the cellular monolayer. This permits selective radiolabeling of proteins on the apical surface of the cells with accessible tyrosine residues.[13] After termination of the radioiodination reaction with sodium azide, the monolayers were extracted with 1% 3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonate (CHAPS, Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS) and gel-filtered through Bio-Gel P-10DG (Bio-Rad) in extraction buffer to remove unincorporated ^{125}I. Samples were then stored frozen at −20 C for later analysis.

Endogenous Radiolabeling of Cellular Proteins

Endothelial cell cultures were endogenously radiolabeled as we have previously described.[9,10] Monolayers were incubated for 24 hours in RPMI 1640 medium depleted of methionine (GIBCO) containing 10% fetal bovine serum, 14 μCi/ml of [^{35}S]methionine (Amersham), and 50 kallikrein U/ml of aprotinin A (Sigma). At the end of the incubation the media were removed, the dishes washed with PBS, and the monolayers extracted with 1% CHAPS in PBS. The dishes were then washed again with PBS and residual matrix material extracted with 2% sodium dodecyl sulfate (SDS) in the form of electrophoresis sample preparation buffer.[14] Media samples were precipitated overnight at 4 C with 50% saturated ammonium sulfate, redissolved in 0.05 mol/L Tris-hydroxymethyl aminomethane/0.1 mol/L NaCl, pH 7.40 (TBS) made 1 mmol/L phenylmethyl sulfonyl fluoride and then dialyzed exhaustively against TBS. Media and cellular matrix extract samples were then stored frozen at −20 C for later analysis. Total cellular protein in the CHAPS extracts was determined using the BCA assay (Pierce Chemical, Rockford, IL).

Immunoprecipitation, Electrophoresis, and Immunoblotting

Samples of surface-labeled proteins and endogenously radiolabeled media proteins were analyzed by immunoprecipitation with our previously described rabbit anti-porcine FN antibody.[9] Samples derived from all of the various endothelial cell types were first pre-cleared by addition of normal rabbit IgG, followed by incubation overnight at 4 C with protein A-Sepharose (Sigma). The samples were then spun in a microcentrifuge and the supernatants incubated with the anti-FN antibody, followed by protein A-Sepharose. The resin pellets were then washed three times with TBS and bound radioactivity released by treatment with 2% SDS in the form of electrophoresis sample preparation buffer. These samples were then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels under reducing conditions according to Laemmli.[14] Gels containing ^{125}I-labeled samples were dried and processed as autoradiograms. Gels containing [^{35}S]methionine-labeled samples were processed as fluoro-autoradiograms by treatment with Enhance (NEN Products, Boston, MA). Cell matrix samples were subjected to SDS-PAGE followed by transfer to nitrocellulose membranes in 20% methanol according to Towbin.[15] The sample volumes were normalized to total cellular protein in the sample. We have found that, for these endothelial cells, total cellular protein correlates well with cell number (CM Johnson, unpublished observations). These membranes were then processed as immunoblots using the above anti-FN antibody,[10] after which the dried membranes were exposed as autoradiograms.

Immunocytochemical Staining

Hearts derived from normal pigs similar in age and breed to those used to provide the endothelial cell cultures were fixed in formalin. Tissues were embedded in paraffin and cut to include portions of the ascending aorta and aortic valve in the same tissue section. Samples of coronary and proximal pulmonary artery were also prepared in similar fashion. All of these tissue samples derived from the same areas that provided the endothelial cells used in the above *in vitro* studies. The samples were cleared of paraffin and processed for immunocytochemistry in a single batch using the peroxidase-antiperoxidase technique[16]: the primary antibody was the above

anti-FN (20 μg/ml), with normal rabbit IgG in equal concentration serving as the control. All slides were counterstained with hematoxylin.

Enzyme-Linked Immunosorbent Assay

An enzyme-linked immunosorbent assay (ELISA) for cellular FN receptor was set up as follows. Endothelial cells derived from the thoracic aorta and aortic valve were grown to confluence in 96-well plates and then fixed with 70% methanol. Some wells were digested with 0.1 mol/L NaOH for later protein assay instead of being fixed. The methanol-treated wells were washed with TBS and replicate wells incubated with 10 μg/ml of either control mouse IgG (Sigma) or a mouse monoclonal antibody directed against the a α_5 subunit of human integrin (clone Ab-1, Oncogene Science, Uniondale, NY). Both antibodies were diluted in 1% bovine serum albumin/0.1% normal goat IgG (Sigma)/TBS and added at 0.05 ml/well. After a 2-hour incubation at room temperature, the wells were washed three times with TBS and alkaline phosphatase-linked goat anti-rabbit IgG (Sigma), diluted 1:200 in 1% bovine serum albumin/TBS, added at 0.05 ml/well. After a 2-hour incubation at room temperature, the wells were washed three times with TBS and developed with *p*-nitrophenyl phosphate (Sigma) for 30 minutes. The plates were then read in an ELISA reader at 405 nm. The NaOH-digested wells were assayed for total protein using the BCA assay. The ELISA results were then expressed as the mean absorbance value for anti-integrin wells minus the mean absorbance value for normal mouse IgG wells, normalized to total cellular protein.

Results

The results of radiolabeling of cell-surface proteins are displayed in Figure 1, which shows an autoradiogram of samples derived from all the endothelial cells studied: thoracic aorta, pulmonary artery, coronary artery, aortic valve, and mitral valve. It can be seen that, in general, the complement of cell-surface proteins available to the labeling reaction varied little between the cell types. However, a difference was observed between cardiac and non-cardiac cells in the relative density of a band of 220,000 in apparent molecular weight. Figure 2 shows an immunoprecipitation of the surface-labeled samples using anti-FN, demonstrating the virtual absence of surface-labeled FN on cells derived from either of the cardiac valves or the coronary artery. In contrast, cells derived from both thoracic aorta and pulmonary artery showed substantial amounts of surface-labeled FN. These data suggest that the high molecular weight band that distinguished cardiac from non-cardiac endothelial cells in Figure 1 was FN.

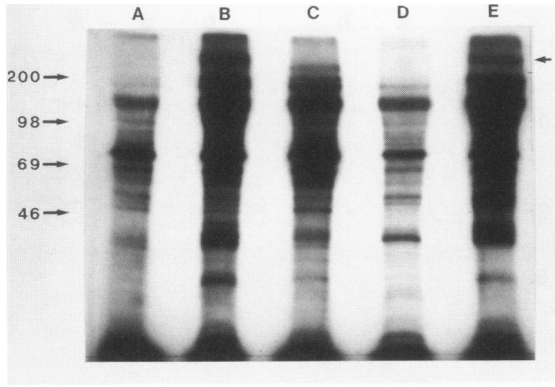

Figure 1. *Radiolabeled endothelial cell-surface proteins. Intact cells were radiolabeled as described in Materials and Methods, and the extracts electrophoresed on a 10% polyacrylamide gel under reducing conditions. Sample loads were normalized to total cellular protein. Migration distances of standard proteins are indicated at left by arrows. The sources of the cell extracts are as follows:* **lane A,** *aortic valve;* **lane B,** *pulmonary artery;* **lane C,** *mitral valve;* **lane D,** *coronary artery;* **lane E,** *thoracic aorta. It appears that the pulmonary artery* **(B)** *and the thoracic aorta* **(E)** *lanes contained a band corresponding to a protein of 220 kd that was much less apparent in the other lanes (right arrow).*

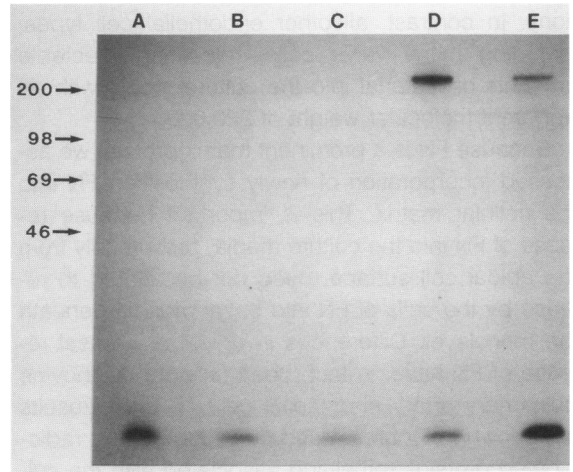

Figure 2. *Immunoprecipitation of radiolabeled endothelial cell-surface proteins with anti-FN. The surface-labeled extracts shown in Figure 1 were immunoprecipitated with anti-FN and processed for SDS-PAGE as described in the text. The contents of the lanes are as follows:* **(A)** *aortic valve,* **(B)** *mitral valve,* **(C)** *coronary artery,* **(D)** *pulmonary artery, and* **(E)** *thoracic aorta. Migration distances of standard proteins are indicated at left by arrows ($\times 10^{-3}$ kd). Note that virtually no surface-labeled FN was detected by this technique in any of the cardiac endothelial cell extracts: aortic valve, mitral valve, or coronary artery. In contrast, a band corresponding to an apparent molecular weight of 220 kd, the predicted molecular weight of reduced FN, was present in both thoracic aorta and pulmonary artery samples.*

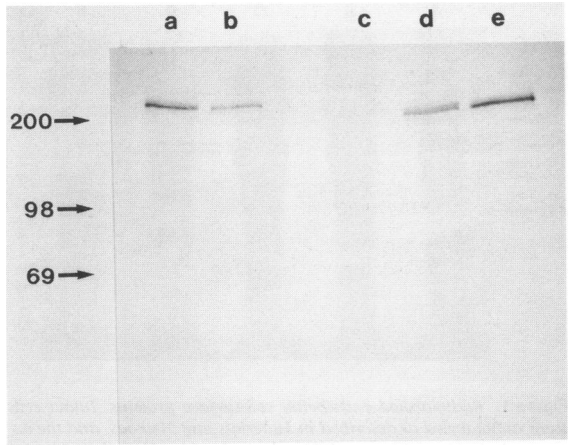

Figure 3. *Immunoprecipitation of radiolabeled endothelial cell secreted proteins with anti-FN. As described in the text, endogenously radiolabeled proteins released by the various endothelial cells into the culture media were immunoprecipitated with the anti-FN antibody and analyzed on a 10% gel under reducing conditions. Migration distances of standard proteins are indicated at left (× 10^{-3} kd). Sample lanes are as follows:* (a) *coronary artery,* (b) *mitral valve,* (c) *aortic valve,* (d) *thoracic aorta, and* (e) *pulmonary artery. Note that the anti-FN antibody precipitated a protein with an apparent molecular weight of 220 kd in all of the samples except that of aortic valve.*

Figure 3 shows results of immunoprecipitations of endogenously radiolabeled cellular proteins that were secreted into the culture media. It can be seen that the endothelial cells differed in their release of newly synthesized FN. In agreement with our previous work,[9] the aortic valve endothelial cells secreted virtually no detectable FN under these conditions. In contrast, all other endothelial cell types, including mitral valve cells, released detectable amounts of material into the culture media with an apparent molecular weight of 220,000.

Because FN is a prominent matrix protein, we assessed incorporation of newly synthesized FN into the cellular matrix. This is important because release of FN into the culture media, presumably from the apical cell surface, need not be related to release by the cells of FN into basal regions beneath the monolayer. Differences in apical and basal release of FN have, in fact, been reported for bovine pulmonary artery endothelial cells.[17] These results are shown in Figures 4 and 5. Endogenously radiolabeled proteins remaining associated with the culture dishes after CHAPS extraction were electrophoresed and transferred to nitrocellulose as described in Materials and Methods. This membrane was then processed both as an immunoblot using anti-FN and as an autoradiogram. The relative protein concentrations of the various samples were equivalent. Specific radioactivity (cpm/μg protein) was also similar between the samples. Figure 4 displays the membrane developed as an autoradiogram; Figure 5 shows a photograph of the immunoblot. Overall, endothelial cells derived from the various sites had similar repertoires of matrix proteins. However, Figure 4 does suggest that there was some difference between the cells in biosynthesis of a protein migrating at an apparent molecular weight of 220,000, with the aortic valve cells showing the weakest signal. Figure 5 shows differences among the cells in FN detected using the polyclonal antibody. The majority of this signal corresponded to the 220-kd band identified in Figure 4, the predicted size of FN, with the aortic valve cells again exhibiting the weakest signal. Figure 5 also shows differences between the cells in the relative quantities of a smaller protein migrating at a position corresponding to an apparent mass of 206 kd. This smaller species was weakly detected by the anti-FN antibody, and it is possible that it corresponded to an isoform of FN present only in the matrix. Production of such different FN species are known to result from alternative splicing of the primary FN gene transcript.[18] If the 206-kd protein was an FN isoform, this suggests that the antibody, originally raised against porcine plasma FN, discriminated among the various FN molecules. However, such an explanation for the detection by the

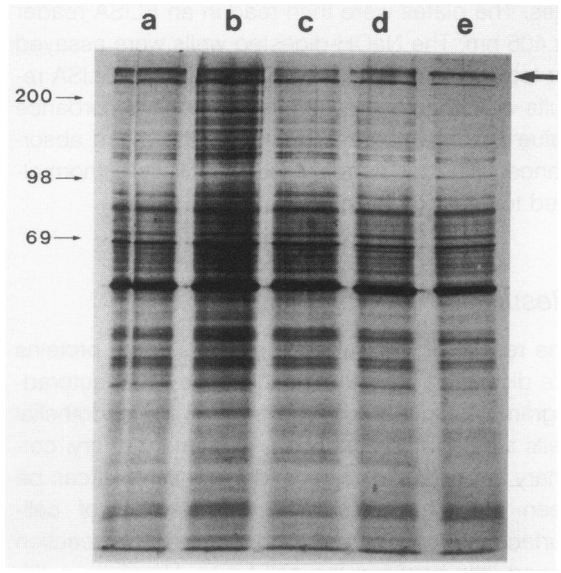

Figure 4. *Radiolabeled endothelial cell matrix proteins. The endogenously labeled samples were electrophoresed on a 10% gel under reducing conditions and then transferred to nitrocellulose as described in the text and exposed as an autoradiogram. Migration distances of standard proteins are indicated at left (× 10^{-3} kd). Sample lanes are as follows:* (a) *coronary artery,* (b) *mitral valve,* (c) *aortic valve,* (d) *thoracic aorta, and* (e) *pulmonary artery. As described in the text, although the complement of CHAPS-resistant proteins was similar between the cells, there were some differences. In particular, the aortic valvular sample was relatively deficient in the density of a band corresponding to a protein with a mass of 220 kd (right arrow).*

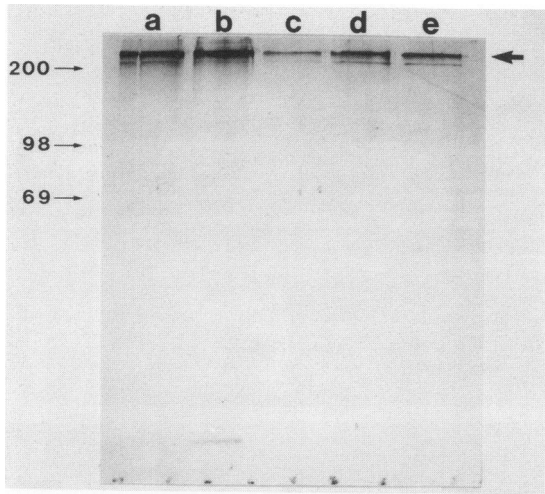

Figure 5. *Immunoblot of endothelial cell matrix proteins using anti-FN. This figure shows the same nitrocellulose membrane displayed in Figure 4 probed as an immunoblot using anti-FN. Migration distances for molecular weight standards are indicated at left ($\times 10^{-3}$ kd). It can be seen that the antibody identified the high molecular weight protein that discriminated between cell types in Figure 4 as FN (right arrow). As discussed in the text, the figure also shows relative differences in a band detected by the antibody at a position corresponding to a mass of 206 kd. It is possible that this lower band may be a smaller isoform of FN.*

antibody of both a strong signal at 220 kd and a weak signal at 206 kd in the matrix samples is not totally convincing. For example, the aortic valve and thoracic aorta samples appear to show equal amounts of the 206-kd protein in Figure 4, the autoradiogram, yet are quite disparate in Figure 5, the immunoblot. Furthermore, the mitral valve sample shows a stronger signal in Figure 4 than that of pulmonary artery cells for the 206-kd band, yet the reverse is true for the immunoblot.

Figure 6 shows the results of immunocytochemical staining of porcine tissues for FN using the same antibody that demonstrated the differences observed on the cultured cells described above. All sections were processed at the same time and showed no positive staining with nonimmune rabbit IgG (data not shown). It can be seen in Figure 6 that there were differences between the various sites in the presence of immunoreactive FN. In general, coronary artery, thoracic aorta, and pulmonary artery all showed positive staining for FN in the endothelial regions. In contrast, aortic valve tissues stained only weakly for FN, and little of this positive staining was associated with the endothelial layer. It is important to note that the aortic valve specimen was part of the same tissue section that included the thoracic aorta, and thus received the identical antibody solution, incubation time, washing steps, and substrate solution.

As described in Materials and Methods, an ELISA was set up for an endothelial cell integrin, as measured by reactivity with a monoclonal antibody to the α_5 subunit of the human heterodimer. When normalized to total cellular protein, 50% more integrin was detected in the thoracic aorta cells than in the aortic valve endothelial cells: thoracic aorta wells had a specific absorbance of 0.033 ± 0.002 absorbance units/μg cellular protein, whereas aortic valve wells had a specific absorbance of 0.021 ± 0.001 absorbance units/μg cellular protein. This difference was significant ($P < 0.05$) by a two-tailed *t*-test. Potential differences in integrin expression were further assessed by immunoblot studies. Cell extracts, equalized for total protein, derived from all of the cell types (aortic valve, mitral valve, pulmonary artery, coronary artery, and thoracic aorta) were electrophoresed and transferred to nitrocellulose. This membrane was then probed as an immunoblot using the anti-integrin antibody. No differences were observed between the cell types in the presence of an 140-kd band corresponding to the α_5 subunit molecule (data not shown).

Discussion

In the present studies we have substantially extended our previous experiments on FN synthesis by porcine vascular endothelial cells. We had previously shown that aortic valve endothelial cells synthesized relatively little FN in comparison with endothelial cells derived from the thoracic aorta,[9] and that this difference in FN synthesis was not paralleled by differences in synthesis of other glycoproteins.[10] These previous results raised several important questions.

First, it was not clear whether this difference in FN synthesis was shared by vascular endothelial cells derived from other sites. Of particular interest in this regard would be potential differences between cardiac and non-cardiac endothelial cells. Any phenotypic variation between endothelial cells with regard to FN gene regulation could be important in the pathogenesis of tissue-specific vascular disease. Second, it was unknown whether these differences in FN synthesis were reflected in differences in the amount of FN actually found on the cell surface. It has been shown by immunofluorescence studies that endothelial cells, compared with other cultured cells, express relatively little FN on their apical surface,[19] in spite of the observation by us[9] and others[5] that FN comprises a major proportion of newly synthesized proteins secreted by cultured

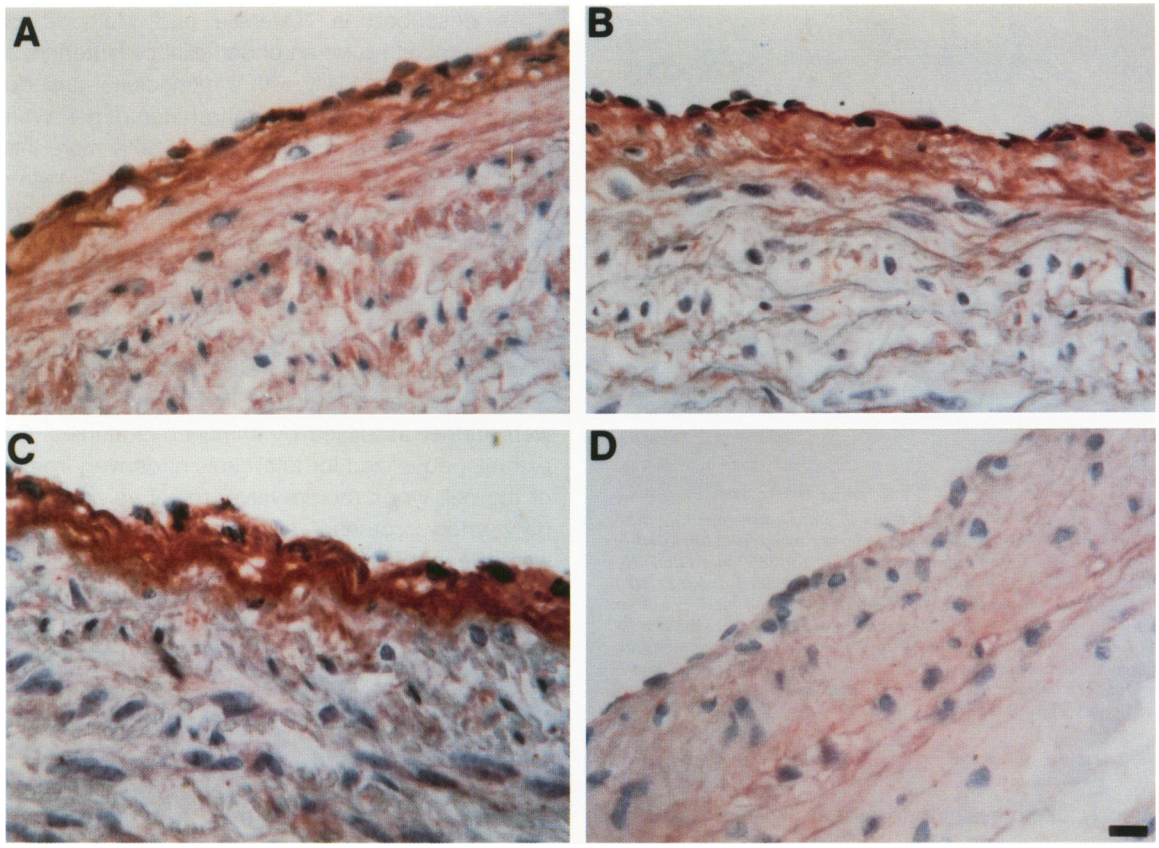

Figure 6. *Immunocytochemical staining of porcine cardiac and vascular tissues. Normal porcine tissues were processed for immunocytochemical staining with anti-FN as described in the text. Brown staining indicating the presence of FN can be seen in the endothelial regions of thoracic aorta* **(A)**, *pulmonary artery* **(B)**, *and coronary artery* **(C)** *tissues. In contrast, the aortic valve* **(D)** *demonstrated comparatively weakly positive staining for FN. Scale bar = 10 μ.*

endothelial cells. Both cultured endothelial cells and endothelium *in vivo* exist in an environment relatively rich in fluid-phase FN. This is particularly so for cells *in vivo*, since plasma contains substantial quantities of FN.[20] Although fetal bovine serum has lesser amounts of FN than does plasma, the cultured endothelial cells would still have their apical surfaces in contact with FN or FN fragments. Therefore differences in FN synthesis by endothelial cells might be rendered insignificant with respect to surface expression of FN if the cells bound the plasma form of the molecule from the fluid phase. Last, our previous experiments left unanswered the important question of whether differences in *in vitro* synthesis of FN reflected distinctions truly found *in vivo*. Cultured cells are well known to have the ability to alter their phenotype as a result of artificial culture conditions, raising the possibility that our previous observations represented an *in vitro* artifact.

The present studies offer significant new information on these questions. We have now shown that differences in *in vitro* synthesis of FN by porcine endothelial cells extend beyond our previous comparison of aortic valve to thoracic aorta. Figure 3 indicates that aortic valve endothelial cells are unusual among porcine vascular endothelia, releasing virtually no FN into the culture media. Since extracellular matrix is typically rich in FN, we hypothesized that the matrices of the various cell types could also differ in FN content, particularly the aortic valve matrix. Figures 4 and 5 suggest that this hypothesis was correct. However, it is also apparent that the relative deficiency of FN in the aortic valve matrix (Figure 5) was not as prominent as the much greater deficiency noted in secreted proteins (Figure 3). This suggests that the aortic valve cells may synthesize sufficient FN for their matrix requirements. Interactions between matrix and endothelial cells are partly mediated by structures termed stress fibers: bundles of actin, myosin, and α-actinin.[21] Observations made *in vivo* noted marked variability in the presence of stress fibers in bovine aortic valve endothelial cells,[22] suggesting that there may exist uniquely distinct microenvironments *in vivo* that influence cell-matrix adhesion in the cardiac valve.

Our data have shown a striking difference between cardiac (coronary artery, mitral valve, aortic valve) and non-cardiac (thoracic aorta, pulmonary artery) endothelial cells in their surface expression of FN. Overall, the endothelial cells differed little in the repertoire of proteins surface-labeled by the technique used in these studies (Figure 1). However, one difference in surface protein expression was apparent on the gel of total protein samples (Figure 1), and this difference was shown by immunoprecipitation (Figure 2) to be FN. The difference was striking, with cardiac endothelial cells expressing little or no FN detectable by this technique. A very long exposure of the autoradiogram shown in Figure 2 did demonstrate a faint band at 220 kd corresponding to FN in the coronary artery sample, whereas the mitral and aortic valve samples still had no band present (data not shown). The antibody used in these studies did not distinguish between bovine and porcine FN, suggesting that the cells did not bind FN from the media. It is possible that cell-surface FN was not uniformly accessible between the cell types to the radiolabeling reaction, but overall these data show that, irrespective of biosynthesis of FN, cardiac valve endothelial cells *in vitro* exhibited substantial differences from non-cardiac endothelial cells in surface FN.

The differences that we observed between cells in FN biosynthesis and surface expression depended upon the specificity of our anti-porcine FN antibody. Since FN occurs in different isoforms that result from alternative splicing of the primary transcript,[18] the possibility must be considered that our antibody discriminated between isoforms. This could lead to differences in antibody signal that did not in fact reflect overall total FN biosynthesis, but were rather the result of cellular differences in the species of FN synthesized. Potential evidence for such an effect was noted in the cell matrix samples in the form of a 206-kd band that was weakly reactive with the antibody (Figure 5). In all other experiments (Figures 2 and 3) the antibody identified a 220-kd FN species exclusively. It is possible that matrix FN differed from that found on the apical cell surface and from FN secreted into the media.

Our data on FN cell-surface expression led us to hypothesize that the cells might differ in their expression of integrin $\alpha_5\beta_1$ (VLA-5), a molecule identified as a principal FN receptor on many cell types,[23] including endothelial cells.[24] The availability of a commercial monoclonal antibody to the α_5 component of the human heterodimer allowed us to assess this possibility. We selected for ELISA assay two endothelial cell types: aortic valve and thoracic aorta. We first determined that the antibody recognized the porcine antigen. The ELISA results, normalized to cellular mass, showed that thoracic aorta cells did express more of this integrin than did aortic valve cells. However, this difference, although statistically significant, was modest when compared with the striking differences in cell-surface FN demonstrated in Figure 2. These data suggest that the differences in surface FN between cardiac and non-cardiac endothelial cells could not be totally explained by differences in FN receptor density.

The immunocytochemistry studies shown in Figure 6 are an important adjunct to the experiments performed on cultured cells. The aortic valve had minimal amounts of FN present in the endothelial region. This was in distinct contrast to thoracic aorta, pulmonary artery, and coronary artery tissues, where substantial amounts of FN were observed. It is interesting that the coronary artery tissue, in contrast to what might be expected from our observations on cultured cells, showed substantial quantities of FN present *in vivo*. It should be noted that the immunocytochemical studies do not indicate the origin of the FN, so it is unclear whether the positive staining represented FN synthesized by endothelial or by subendothelial cells, such as smooth muscle cells or fibroblasts. We have also not excluded the possibility that, in contrast to the *in vitro* situation, the endothelial surface *in vivo* may bind plasmatic FN. Even with these caveats, our data taken together suggest that the relative deficiency of FN synthesis and FN surface expression by aortic valve endothelial cells *in vitro* is paralleled by differences in FN found *in vivo*.

These findings have potential implications for our understanding of the pathophysiology of cardiac valvular disease. Of particular interest is infective endocarditis, in which damage to the cardiac valve leads to deposition of platelets and fibrin.[25] In experimental animal models this lesion is subsequently colonized by circulating micro-organisms, leading to active infection.[26] The actual binding events that establish this infection are unknown, although tissue FN has been proposed as a mediator of this process.[27] The FN-binding ability of some micro-organisms has been reported to correlate with the ability of the bacteria to bind to damaged cardiac tissues.[28] Our studies suggest that experiments carried out using non-cardiac endothelial cells may not be appropriate models for examining these binding events. The present studies also suggest that various endothelial cells may differ in their regulation of the FN gene. Further studies will be needed to determine if this is the case, and if such

differences alter endothelial cell phenotype in ways that affect the pathogenesis of cardiac valve disease.

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