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GLYCOPROTEINS SYNTHESIZED BY CULTURED CARDIAC VALVE ENDOTHELIAL CELLS: UNIQUE ABSENCE OF FIBRONECTIN PRODUCTION

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We have previously reported that cultured porcine cardiac valve endothelial cells released less fibronectin into the culture supernatant when compared to other porcine endothelial cells. In this report we compared the spectrum of glycoproteins synthesized by cardiac valve endothelial cells to glycoproteins synthesized by comparison endothelial cells derived from the ascending thoracic aorta. The cells were endogenously radiolabeled and extracted with detergent. Glycoproteins in the cell extracts were then isolated on wheat germ lectin-agarose and compared using autoradiography following polyacrylamide gel electrophoresis. Fibronectin was identified by immunoblotting with specific antibody. The results showed that the outstanding difference between the endothelial cell types was the virtual absence of fibronectin in the cardiac valve endothelial cell extract.

Fibronectin is a major biosynthetic product of cultured vascular endothelial cells (1). Other glycoproteins synthesized and released *in vitro* by endothelial cells include von Willebrand factor (2) and thrombospondin (3,4). It has been proposed that these three adhesive glycoproteins have importance for endothelial interactions with platelets (5,6), as well as serve functions related to intercellular communications between endothelial cells and other cells of the vessel wall (7). Of particular importance for the cardiac valve, it has been proposed that endothelial cell fibronectin may serve as an attachment site for circulating bacteria, thereby initiating the lesion of infective endocarditis (8). We have previously reported that cultured porcine cardiac valve endothelial cells, when compared to endothelial cells derived from the nearby ascending aorta, released only 10% of the quantity of fibronectin into the culture supernatant (9). In this study we wished to determine if expression of other glycoproteins differed between these cell types, since such differences may be important in understanding the pathophysiology of diseases unique to the cardiac valve.

MATERIALS AND METHODS

Cell Isolation and Culture

Porcine endothelial cells derived from the aortic valve and ascending thoracic aorta were isolated as previously described (9). All cells were grown in medium 199 (Sterile Systems, Logan, UT) supplemented with 10% fetal bovine serum (Sterile Systems) and were used in these experiments at fourth passage.

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Endogenous Radiolabeling

Confluent monolayers of endothelial cells were radiolabeled with [^{35}S]methionine (Amersham, Arlington Heights, IL) as follows. The cells were incubated for 24 hours with RPMI 1640 medium, containing 10% fetal bovine serum, which had been depleted to 20% of its normal concentration of methionine (Grand Island Biological Company, Grand Island, NY). The cells were then incubated for 24 hours with 75 μCi/ml of radiolabel in RPMI 1640 otherwise without methionine; the medium also contained 10% fetal bovine serum and 50 kallikrein units/ml of Aprotinin A (Sigma, St. Louis, MO). At the end of the labeling period the cells were washed with phosphate-buffered saline (PBS) and extracted for 20 minutes with 1% (w/v) 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (Sigma) in PBS containing 1 mM phenylmethylsulfonyl flouride.

Lectin Chromatography

Wheat germ lectin-agarose columns (Sigma) were equilibrated in the above extraction buffer. The two endothelial cell extracts were then applied to the columns and allowed to bind as a slurry. The columns were then washed with extraction buffer until no further radioactivity eluted, following which they were eluted with 100 mg/ml of n-acetylglucosamine in the extraction buffer (10).

Immunoprecipitation

The total cellular extracts were first precleared by incubation with normal rabbit IgG, followed by Protein A-agarose (Sigma). The supernatants were then incubated with the IgG fraction of our previously described rabbit anti-porcine fibronectin (9), followed by Protein A-agarose. The resins were then washed with PBS and the radioactivity bound determined by liquid scintillation counting.

Electrophoresis and Immunoblotting

The radioactive fractions eluting from the lectin columns were processed for polyacrylamide gel electrophoresis on 5-15% gradient slab gels according to Laemmli (11). All samples were reduced with 1% (v/v) 2-mercaptoethanol. Care was taken to include equal amounts of radioactivity in comparison lanes between aortic and valvular cells. The gels were treated with Enhance (New England Nuclear, Boston, MA), dried, and exposed in cassettes. Gels for immunoblots were transferred to nitrocellulose electrophoretically (12), blocked with bovine serum albumin, and probed with the above rabbit anti-porcine fibronectin. Control blots received normal rabbit IgG (Sigma). Bound antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit IgG, followed by development with fast violet dye (Sigma).

RESULTS AND DISCUSSION

The glycoproteins eluting from the lectin columns are shown in Figure 1, along with the total cellular proteins present in the stock endothelial extract. It can be seen that, overall, the endogenously labeled proteins in the total extracts did not differ between the cell types. However, there were two differences noted in the populations of glycoproteins present in aortic and valvular cells. The valvular sample showed a minor, but distinct, band at an apparent molecular weight of 73,000 that was not seen in the ascending aortic sample. Much more impressive was the band corresponding to an apparent molecular weight of 220,000, the molecular weight of reduced porcine fibronectin (9): this glycoprotein was a major biosynthetic product of the aortic endothelial cells, whereas it was not seen in the valvular endothelial sample. We then used immunoblotting to identify this protein as fibronectin. Figure 2 shows the results of a nitrocellulose blot processed for both autoradiography and probed with the anti-fibronectin antibody. The overall intensity of the exposure of the blot autoradiogram was substantially less than the results shown in Figure 1, even though the

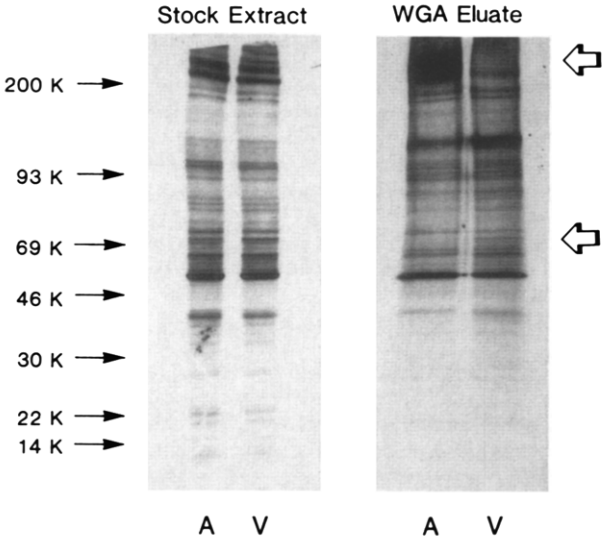

Figure 1— Isolation of endothelial glycoproteins. Shown are autoradiograms of endogenously labeled samples from ascending aortic (A) and cardiac valvular (V) cells. The left two lanes show the total cellular extracts (stock extract); the two lanes on the right of the figure display the samples eluted from wheat germ lectin-agarose (WGA eluate). Migration distances of standard proteins are indicated at the far left of the figure. Equal amounts of radioactivity were applied to each pair of comparison lanes. It can be seen that the aortic eluate contained a major protein band at an apparent molecular weight of 220,000 (top open arrow) that was not seen in the valvular sample. Additionally, the valvular sample contained a band at an apparent molecular weight of 73,000 that did not appear in the aortic sample (lower open arrow).

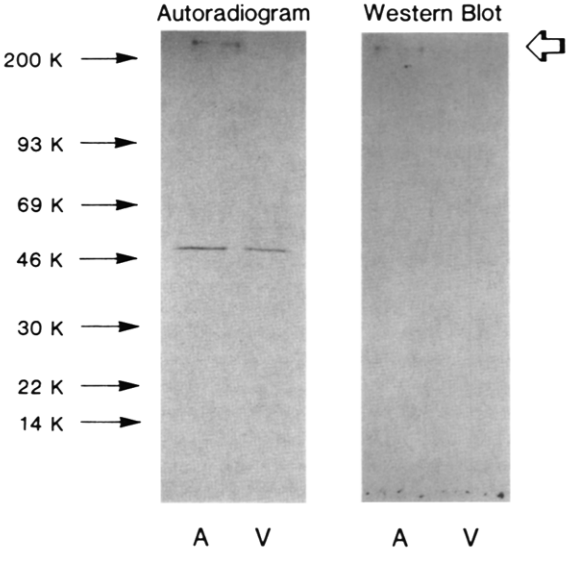

Figure 2— Immunoblot of wheat germ lectin-agarose eluate. This figure shows the same samples displayed in the right hand lanes of Figure 1 (WGA eluate), processed both as an immunoblot (Western blot) probed with anti-fibronectin, and as an autoradiogram. Following development of the enzyme-linked antibody, the blot was exposed as an autoradiogram. It can be seen that the protein species present at an apparent molecular weight of 220,000, present only in the aortic sample (A), is identifed by anti-fibronectin antibody (open arrow); no fibronectin was detected in the valvular (V) sample by this technique. Equal amounts of radioactivity were applied to each lane. Migration distances of standard proteins are shown at the left of the figure.

samples were the same, because the Figure 1 gel was treated with Enhance. Additionally, the efficiency of transfer is not good for high molecular weight proteins (13). Even so, sufficient signal was present on the Figure 2 autoradiogram to identify on the accompanying immunoblot that the 220,000 molecular weight protein was fibronectin. Control blots (not shown) probed with non-immune rabbit IgG showed no reactivity for either endothelial cell type.

We have previously reported results of a specific immunoassay for fibronectin released into the supernatant by cultured endothelial cells (9). In those experiments we found that ascending aortic endothelial cells released eight to ten times more fibronectin into the medium than did cardiac valve endothelial cells. The present results with immunoprecipitations of the total cellular extracts support our previous data: overall, 11% of the total radioactivity present in the aortic extract was precipitated by anti-fibronectin; in contrast, only 1.0% of the total radioactivity present in the valvular sample was precipitated by this antibody. However, the data in Figure 1, although not quantitative, suggest that the difference between the two cell types in newly synthesized fibronectin present in the cellular extract may be more marked than this; no fibronectin at all was detected in the valvular sample on the immunoblot shown in Figure 2. It should be noted that the fibronectin identified in these figures was isolated by means of lectin chromatography, whereas the immunoprecipitation used a specific anti-fibronectin antibody; it is possible that the valvular cells differed from aortic cells in glycosylation of their fibronectin, thereby altering the amount that was recovered on the lectin column.

As we have noted, fibronectin is a major secreted protein for previously described endothelial culture systems (1). In this report we have shown that the endothelial cells of the porcine cardiac valve, although in other respects sharing the attributes of glycoprotein synthesis with ascending aortic endothelial cells, exhibited a specific and profound difference regarding fibronectin synthesis. In addition, the valve cells synthesized a glycoprotein with an apparent molecular weight of 73,000 that was not present in the aortic samples. These data show that, in spite of their close proximity *in vivo* (the aortic cells were isolated from a region only one cm away from the cardiac valve), the two cell types expressed different phenotypes under identical culture conditions *in vitro*. Moreover, this phenotypic difference involved an adhesive glycoprotein for which important functions have been postulated regarding cellular adhesion to substrata and intercellular communication (7). There are several pathologic processes of particular importance for the cardiac valve, including infective endocarditis and calcific aortic stenosis. The former of these is believed to become established by means of microbial attachment to platelets adherent to a previously damaged cardiac valve (14). The ability of endothelial cells to synthesize fibronectin may affect their capacity to maintain attachment to subendothelial structures, thereby resisting potentially denuding injury. Thus our observations may have importance for understanding the pathogenesis of disease processes unique to the cardiac valve.

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