In Vitro Repair of the Wounded Porcine Mitral Valve

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An organ culture of the anterior leaflet of the porcine mitral valve was developed and characterized in order to study the early events in the repair of small endocardial wounds. A linear superficial denuding endocardial injury was made with a nylon filament attached to a stereo turnarm. The repair process was studied by scanning and transmission electron microscopy over a 6-day period. By day 2 in culture, flattened endocardial cells at the wound edge extended processes out onto the wound edge. By 4 and 6 days, the wound was bridged over by spindle-shaped cells although gaps still remained between cells. In some regions, multilayering of cells beneath the surface was present. The results indicate that the initial events in in vitro endocardial repair involve both surface endocardial cells and interstitial cells. (Circulation Research 1988;62:833-845)

Although there has been considerable work done on the repair of the injured vascular endothelium, little is known about the repair of the injured valvular endocardium. Studies have suggested that injury to the endocardial cells lining the surface of the heart valves is one of the important initial steps in the pathogenesis of several pathological conditions affecting the valve. Furthermore, the increasing use of balloon catheter and surgical valvuloplasty for treatment of stenotic valvular disease requires that we understand how the endocardium responds to direct mechanical injury.

The purpose of our experiments was to study the early events in the repair of a small superficial endocardial wound using an organ culture system of the anterior leaflet of the porcine mitral valve. No attempt was made to model a specific disease state. The pig was chosen as a model since its cardiovascular system is similar to that of man. In addition, the histological findings reported below show that the valve has similarities to that of man.

Linear superficial mechanical wounds were made on the atrial surface of the valve, and repair was studied over a period of 6 days. Scanning and transmission electron microscopy were used to study the cell types involved in repair and the distribution and orientation of the cells. Part of this study was presented previously in abstract form.

Materials and Methods

Organ Culture

Pig hearts were obtained from a local slaughterhouse within 15 minutes of death. The anterior leaflet of the mitral valve with adjacent myocardium was rapidly dissected out and transported to the lab in sterile cold phosphate buffered saline (PBS). The anterior leaflet was then trimmed, leaving 3 mm of left atrial myocardium along the proximal edge of the leaflet in order to manipulate the tissue without damaging the endocardial surface. The proximal half of the leaflet was cut into two rectangles, 1 cm by 1.5 cm, with the long axis perpendicular to the direction of in vivo blood flow. These pieces were incubated individually in 100-mm tissue culture dishes (Falcon Plastics Company, Oxford, California), atrial surface uppermost, in Medium 199 with 25 mM HEPES buffer and Earle's salts and l-glutamine with 10 units/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml amphotericin B (all from GIBCO, Grand Island, New York) supplemented with 10% fetal bovine serum, in a humidified atmosphere of 95% air-5% CO₂ at 37°C. Cultures were fed every 2 days.

Wounding

A wounding machine was assembled, consisting of a turntable (CSS14 belt-driven semiautomatic, Dual GmBH, Schwarzwal, FRG) driven by a synchronous low-speed motor (Sigma Instruments, Braintree, Massachusetts) via a friction plate consisting of a metal rod and O-ring. A platform-type manual jack attached to the motor allowed movement of the friction plate into place. The cartridge of the stereo turntable was replaced by a holder for a wounding device, a 200-μm wide nylon filament (Ethilon monofilament nylon 000, Ethicon Sutures Ltd., Peterborough, Ontario). The force exerted by the wounding device was controlled by the counterweight of the tone arm and was adjusted to 1.5 g in order to have a reproducible tension. This machine was a modification of one used by Pederson and Bowyer in which a steel stylus and a counterweight of 0.5 g was used to wound rabbit aortae.

Each valve culture was secured with needles to a piece of dental wax fixed to the turntable platform, atrial surface uppermost, and kept moist with sterile PBS at all times to prevent drying. The wound was made perpendicular to the direction of in vivo blood flow (i.e., along the long axis of the organ culture) and
the valve was rinsed gently. Culturing was performed in the same manner as for unwounded cultures.

**Histology**

Tissue for light microscopy was fixed in 10% buffered formalin and processed for embedding in paraffin. Three unwounded and three wounded organ cultures were fixed at 0, 2, 4, and 6 days. In addition, the entire anterior mitral leaflet from four pigs was embedded on edge in seven blocks to study the normal anatomy in more detail. Sections were stained with hematoxylin phloxine safran, van Gieson elastic, and Alcian blue at pH 2.5.

To test for the presence of factor VIII–related antigen, paraffin sections of five organ cultures fixed at 0 and 4 days were examined by immunoperoxidase staining. The slides were deparaffinized in xylene, washed with absolute alcohol, incubated for 30 minutes in 0.6% hydrogen peroxide in methanol, and washed with PBS. Sections were then incubated for 10 minutes with 0.05% protease P-5147 (type XIV, Sigma Chemical, St. Louis, Missouri) in PBS and then for 30 minutes in 10% normal goat serum (Vector, Burlingame, California) and overnight with rabbit antibody to human factor VIII–related antigen (Dako Ltd., Copenhagen, Denmark) diluted 1:100 in 3% normal goat serum. The sections were then washed in PBS and flooded with biotinylated antibody to rabbit IgG (Vector) for 30 minutes, washed with PBS, and then exposed to avidin-biotinylated horseradish peroxidase complex (Vectastain ABC kit, Vector) for 45 minutes. After PBS washing, the sections were placed in 0.05% diaminobenzidine-0.015% hydrogen peroxide solution (Sigma) for 5 minutes, washed in running tap water, counterstained with hematoxylin, dehydrated with absolute alcohols, dipped in xylene, and mounted in Permount. For negative controls, normal rabbit serum (Cappel, Cooper Biomedical, Malvern, Pennsylvania) was substituted for the primary antibody.

**Scanning Electron Microscopy**

Five wounded and five unwounded organ cultures were fixed at 0, 2, and 4 days for scanning electron microscopy. Cultures were washed twice in 37° C PBS, and fixed in 1.5% glutaraldehyde in 0.1 M Sorenson’s phosphate buffer at pH 7.3 for 24 hours. Dehydration was carried out through a graded series of absolute alcohols, dipped in xylene, and mounted in Epon-araldite, and then sputter coated with 0.05% gold coating in a critical point dryer (Polaron, Watford, England). The gold coating was about 50–60 nm thick. The specimens had been dissected initially, elongated to polygonal-shaped multilayered cells appeared at 3–6 days. Focal stromal cell necrosis was identified by day 2 in the fibrosa layer. Although there was little necrosis of stromal cells in the spongiosa, Alcian blue staining decreased by day 6. The myocardial cells at the proximal edge of the organ culture underwent degeneration in culture.

**Transmission Electron Microscopy**

Three unwounded and three wounded organ cultures were taken for transmission electron microscopy at 0, 2, 4, and 6 days. They were washed twice in PBS at 37° C and then stained with Trypan blue and washed with PBS. The Trypan blue staining of the wounded specimens allowed for identification of the wound edge and the wound. Specimens were fixed in 2.5% glutaraldehyde in 0.1 M Sorenson’s buffer, pH 7.3. After fixation at 4°C, they were changed to 0.1 M phosphate buffer, postfixed with 1% osmium tetroxide, and stained en bloc with 1% aqueous uranyl acetate. They were dehydrated in a graded series of acetones, infiltrated with Epon-araldite, and sectioned. Semithin sections were stained with toluidine blue and thin sections with uranyl acetate and lead citrate. A Philips 300 transmission electron microscope (Philips Electronic Instruments, Mount Vernon, New York) was used at 60 kV.

**Results**

**Anterior Leaflet, Mitral Valve**

The atrial and ventricular surfaces of the leaflet were covered by a flat layer of endocardial cells. Left atrial myocardium extended into the base of the leaflet. A few bundles of smooth muscle, confined to the proximal half of the valve, were sometimes present beneath the endocardium. Loose myxoid tissue, the spongiosa, which was more prominent on the atrial surface of the leaflet (especially distally), was present beneath the elastic fibers. There were sparse randomly arranged elastic fibers present within the spongiosa. Interstitial cells were randomly dispersed throughout the spongiosa, and in focal areas, they were close to the endocardium. In the spongiosa, capillaries and arterioles were more numerous on the atrial than the ventricular side of the leaflet. The core of the leaflet, the fibrosa, which was surrounded by the spongiosa, consisted of dense fibrous connective tissue that was sparsely cellular and extended directly into the chordae tendineae as they emerged from the ventricular side of the leaflet. While the fibrosa contained streaks of Alcian blue positive material, the spongiosa was diffusely and strongly Alcian blue positive. The fibrosa was essentially devoid of blood vessels.

**Unwounded Organ Cultures**

**Histology.** At day 4, there was very focal multilayering of surface cells, and this was more extensive at day 6. At the cut ends of the organ cultures, where the specimens had been dissected initially, elongated to polygonal-shaped multilayered cells appeared at 3–6 days. Focal stromal cell necrosis was identified by day 2 in the fibrosa layer. Although there was little necrosis of stromal cells in the spongiosa, Alcian blue staining decreased by day 6.

The myocardial cells at the proximal edge of the organ culture underwent degeneration in culture.

**Factor VIII R antigen.** The endothelium of small vessels within the intact valve was factor VIII R antigen positive. The atrial surface endocardium was only focally positive, and positive staining was weaker than that of the small vessel endothelium. Following 4 days in culture, the endothelium of small vessels within the
valve was weakly positive, but the endocardium was negative.

Scanning electron microscopy. Specimens fixed at the slaughterhouse had a wrinkled surface (Figure 1), and the endocardial cells were polygonal. Specimens fixed after preparation for organ culture that had been in PBS for 2.5 hours had flatter endocardial cells than those fixed at the slaughterhouse. Gaps between endocardial cells were rare. By day 2 in culture, mild focal endocardial cell retraction and gap formation was apparent, and occasional rounded cells were present on the surface (Figure 2). By days 4 and 6, there were more rounded cells than at earlier time points, focal endocardial cell retraction was more prominent, and occasional spindle-shaped cells were present on the endocardial cell surface (Figure 3).

Transmission electron microscopy. Noncultured specimens were lined by flattened endocardial cells that had small amounts of rough endoplasmic reticulum, small bundles of thin filaments, and plasmalemmal vesicles (Figure 4). Some had luminal cell processes. Various forms of endocardial cell degeneration were seen in about 4% of the cells characterized by cytoplasmic blebs, vacuoles, and partial cell detachment. The endocardial cells were separated from the stroma by a basal lamina that was often reduplicated. The valve stroma consisted of collagen with elastic fibers. The number of interstitial cells directly beneath the endocardial cell layer varied from site to site. The stromal cells had oval nuclei and were spindle-shaped with long cell processes. They had varying proportions of thin filaments, rough endoplasmic reticulum, and Golgi apparatus. The rough endoplasmic reticulum was sometimes dilated, containing granular material. Some cells had vacuoles. The stromal cells had plasmalemmal vesicles, but basal lamina was only rare and focal. Cell junctions were present. A rare stromal cell contained a lipid droplet. In addition to stromal cells, some of the specimens had small nerves, bundles of smooth muscle, capillaries, and arterioles.

The day 2 cultures had endocardial cells with more prominent rough endoplasmic reticulum and Golgi apparatus than at day 0. There was focal layering of two to three surface cells. More stromal cells had lipid droplets and large cytoplasmic vesicles than at day 0. There was focal stromal cell necrosis.

On day 4, while in some areas the endocardial cells were flattened and still formed a monolayer, in other areas there was multilayering (three to four layers) of surface cells. These surface cells were elongated and attached to each other by junctions. They had rough endoplasmic reticulum, thin filaments, and plasma-cell processes.
FIGURE 2. Scanning electron micrograph of unwounded culture at 2 days. Note the focal gap formation (arrow). Magnification: ×1,782.

FIGURE 3. Scanning electron micrograph of unwounded organ culture at 6 days. Note rounded (arrowhead) and spindle-shaped cells (arrow) present on the endocardial surface. Magnification: ×1,710.
FIGURE 4. Transmission electron micrograph of organ culture at day 0. The endocardial cell has dilated endoplasmic reticulum, Golgi apparatus, and plasmalemmal vesicles. Portions of underlying interstitial cells are present (arrow). L, lumen. Magnification: \( \times 19,110 \).

Some of the endocardial cells had vacuoles and single lipid droplets. On day 6, the endocardial cells were still single-layered in some areas while in other areas there were up to seven layers of cells beneath the surface cells. Interstitial cell necrosis was present in the central portion of the organ cultures. Occasional stromal cells contained many lipid droplets.

Repair of Wounded Organ Cultures

Scanning electron microscopy. The wounds measured 150–240 \( \mu \text{m} \) in width (15–25 endocardial cells wide). The endocardial cells were denuded from the wound area, and only rare damaged endocardial cells remained on the wound surface (Figure 5). By day 2, endocardial cells at the wound edge were flattened and extended prominent lamellipodia out onto the wound surface (Figure 6). On days 4 and 6, spindle-shaped cells covered the wound (Figures 7 and 8). These cells overlapped each other and extended numerous filopodia between cells. These cells were generally oriented parallel to the wound edge. Subendothelial matrix was present between cells. Examination of wounds that were made parallel to the direction of blood flow also showed that the cells were orientated parallel to the long axis of the wound at day 4.

Transmission electron microscopy. Immediately after wounding, the surface of the wound was covered by a very thin layer of cellular debris (Figure 9). Occasional necrotic endocardial cells remained on the surface of the wound. The underlying stroma was not disrupted. On day 2, flattened lamellipodia extended from the edge. At days 4 and 6, some of the wound was covered by a thin single layer of cells (Figure 10), while in other areas multiple layers of cells were present on the wound surface (Figure 11). These cells had thin filaments, abundant rough endoplasmic reticulum, and Golgi apparatus (Figure 12). Most of these cells contained vacuoles or one or two lipid droplets. They were connected to each other by a few junctions that had features of tight junctions. There were still some areas in which the wound was bare.

Discussion

Our studies are the first to describe the in vitro repair of a small superficial endocardial wound. Three important findings are noted related to cell types and orientation of cells. First, although flat endocardial cells extended onto the surface of the wound from the wound edge by 4 and 6 days, there were elongated cells within the wound that overlapped each other and did not reform the flat polygonal monolayer that had been removed. Second, in focal areas, layers of overlapping cells were observed within the wound. These cells had
FIGURE 5. Scanning electron micrograph of wounded organ culture at day 0. The endocardium in the wound (W) is totally denuded. Magnification: ×306.

FIGURE 6. Scanning electron micrograph of wounded organ culture at 2 days. Endocardial cells at the wound edge are flattened and extend processes out onto the wound (W). Magnification: ×1,620.
thin microfilaments, abundant rough endoplasmic reticulum, and Golgi apparatus. There was no basement membrane or dense bodies, suggesting that these cells were not smooth muscle cells but instead were stromal interstitial cells, which we have shown to be normally located focally just beneath the endocardium. Third, the elongated cells on the surface of the wound were oriented parallel to the wound edge, although the wound was oriented perpendicular to the direction of in vivo blood flow across the mitral valve.

In addition to multilayering in the wound itself at days 4 and 6, both single-layered endocardium and multiple surface cell layers were seen in the nonwounded cultures as well. This multilayering in the presence of endothelium is not unique to valvular organ cultures. Merrilees and Scott, in organ culture of the rat carotid artery, found that multilayering of cells in the subendothelial intima was prominent when 2% or 10% fetal bovine serum was used but less apparent when 0 or 0.2% serum was used. They suggested that the multilayered cells were endothelial by demonstrating factor VIII-related antigen positivity. However, Pederson and Bowyer used 30% fetal bovine serum for organ culture of rabbit aorta because they found that using 5% or 10% led to patchy denudation of the endothelial cells by 1–2 weeks in culture. With use of 30% fetal bovine serum in the media, the endothelial cells remained a monolayer. Gotlieb and Boden showed that multilayering occurred in porcine thoracic aortic organ cultures incubated in 5% serum and was more prominent in the presence of surface endothelium. It would seem that multilayering of surface cells in vascular organ culture may vary from species to species, from site to site, and with the concentration of serum in the media.

What is the nature of these layered surface cells in our day 4 and day 6 organ cultures? These cells are connected to each other by junctions; have plasma-lamellar vesicles, Golgi apparatus, rough endoplasmic reticulum, and thin filaments; and have similar features to the multilayered cells in the wound. It is known that cultured porcine endothelial cells are not always factor VIII positive, and this may vary with the vessel of origin. We found that the mitral endocardium was only focally positive for factor VIII R antigen, while that of organ cultures was negative. Thus, this technique is not useful for identification of cultured porcine mitral valve endocardium. Another characteristic feature of the endothelial cell, the Weibel-Palade body, is very rare in porcine endothelium in vitro, and we did not observe Weibel-Palade bodies in the endocardium. The presence of angiotensin-converting enzyme is not always specific for endothelium. There are a few bundles of smooth muscle in the proximal one third of the anterior leaflet of the porcine mitral valve, but the major cell population in the spongiosa consists of cells that ultrastructurally are neither typical fibroblasts nor typical smooth muscle cells but have features of both.
This cell type, the interstitial cell, has been recently studied by Filip et al. and may be the cell that responds to injury beneath the endocardial surface. Our ultrastructural findings in the pig are consistent with theirs. The fact that the multilayering is focal may indeed reflect the finding that these cells are normally focally distributed in the subendocardium.

The orientation of the cells that heal the wound is of interest, especially since the endocardial cells lining this portion of the valve are not oriented parallel to blood flow and are not elongated at all. While the endocardial cells at the edge of the wound extend their processes out onto the wound more or less perpendicular to it, by day 4 the spindle-shaped cells that cover the wound surface are oriented almost parallel to the wound. Jackman used organ cultures of rat aorta to examine the orientation of the endothelial cells during repair of denuding injuries. He found that, in axial wounds made circumferentially, by 48 hours the endothelial cells at the original wound edge were oriented circumferentially while the cells at the edge of the regenerating sheet were oriented axially. When axial wounds were made axially, very limited healing took place. Since the aortic endothelial cells orientated axially (elongated parallel to the direction of blood flow), he concluded that there may be cues in the subendothelium that determine endothelial cell orientation.

In our study, the wound length exceeds the wound width, and it is possible that this determines the orientation of the spindle cells. They may elongate to cover as much of the wound as possible, and since there is more space to elongate parallel to the long axis of the wound, this could determine their orientation. To investigate this possibility, some organ cultures were wounded parallel to in vivo blood flow. The spindle cells that repaired the wound were oriented parallel to the long axis of the wound in these as well, suggesting that the subendothelial cues may not be important in mitral endocardial repair. The role of blood flow on orientation awaits further study using in vitro or in vivo systems.

The endocardial cells lining the proximal portion of the atrial surface of the anterior leaflet of the porcine mitral valve are polygonal and are not oriented uniformly with respect to blood flow. This is in contrast to endothelial cells lining the aorta away from branch points, which are elongated parallel to the direction of blood flow. Observations similar to our own in the porcine mitral valve have been made in the canine mitral valve and in the atrioventricular valves of rabbits and rats. Deck found that aortic valve endothelial cells, rather than being oriented parallel to the direction of blood flow, were in fact oriented circumferentially, parallel to the free edge of the leaflet.
FIGURE 9. Transmission electron micrograph of wound at day 0. Some cellular debris remains on the wound surface. The underlying stroma is not disrupted. L, lumen. Magnification: ×28,800.
FIGURE 10. Transmission electron micrograph of wounded culture at 4 days showing thin cell processes extending onto the surface of the wound. L, lumen. Magnification: ×10,000.
FIGURE 11. Transmission electron micrograph of wound at 6 days showing multiple layers of elongated cells. L, lumen. Magnification: x13,600.
He suggested that the endocardial cells followed the alignment of the collagen bundles in the valve. In our organ culture model, the wrinkling of the organ culture pieces is likely related to loss of tension on the valve once its chordal attachments are cut24-26 and is similar to the longitudinal folds seen in immersion fixed aortas with loss of axial tension.27-28 We did not, however, see a relation between folds and orientation of cells within the wound.

Thus, our in vitro studies suggest that endocardial repair is a function of both surface endocardial cells and subendocardial interstitial cells. Studies on cell proliferation in the wound are underway to distinguish between proliferation and cell migration. The precise role that the endocardial and interstitial cells have in the pathogenesis of valvular disease was not addressed in this study. However, our data do suggest that future studies on the role of blood elements, soluble factors, and hemodynamic stress in regulating this repair should consider both endocardial cells and subendocardial interstitial cells. A consideration is that the frequent early restenosis reported to occur by 3 to 6 months following catheter balloon valvuloplasty9 may involve, at least in part, subendocardial interstitial cell proliferation and subsequent fibrosis.

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