

Cellular Expression of PCNA and Procollagen in Vital and Ethanol-treated Autologous Pericardial Implants in Sheep

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Background and aims of the study: Cardiovascular surgeries involving repair or reconstruction of heart valve leaflets with vital autologous pericardium have shown detrimental healing outcomes, mainly fibrosis with retraction. It is proposed that cells intrinsic to the pericardial implants may contribute to this fibrosis by becoming activated to proliferate and synthesize type I collagen.

Methods: Vital and ethanol-treated autologous pericardium were implanted as rectangular flaps bisecting the lumen in the descending aorta of sheep to simulate a heart valve leaflet. Implants recovered at 5, 10, 15, and 30 days were evaluated immunohistologically for expression of PCNA and procollagen.

Results: In ethanol-treated pericardium, concentra-

The use of vital autologous pericardium as a cardiovascular repair biomaterial is limited due to the unpredictability of implant healing. Pericardial implants used in heart valve reconstruction became fibrotic and retracted (1,2), sometimes the pericardium became thinned and dilated (3-5) or was accepted into the surrounding tissue (3,6,7). Sauvage et al. (8) reported tissue thickening in a dog model where mitral leaflets were enlarged with pericardium. Pericardial tissue thickening was observed near sutures, with greater pliability being found in the central portion of the grafts. Also using a dog model, Bortolotti et al. (1) reported that chordae fashioned from vital autologous pericardium became stiff at the ends but had greater pliability in the center. Frater and Bodnar (9) used vital autologous pericardium to replace dog posterior mitral leaflet and chordae, and implants recovered after three weeks showed thin fibrin deposition with tissue thickening, though the implant remained pliable. However, after two months this pliability was

lost and tissue thickening and retraction were observed. tions of activated cells shifted from the fibrin layers on the periphery of implants at days 5 and 10 to cells internal to the implant at days 15 and 30. In contrast, concentrations of activated cells in vital pericardium shifted from cells within the implants at days 5 and 10 to the fibrin deposits overlaying the implants at days 15 and 30.

Conclusion: Different distributional patterns of activated cells were observed between vital and ethanol-treated pericardial flap implants. These different patterns may be important in understanding the cause of the detrimental healing outcome observed with vital autologous pericardial flap implants.

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lost and tissue thickening and retraction were observed.

Previously, an in-vivo model of pericardial tissue healing was reported which accentuated the differential healing reactions of vital (living) and ethanol-treated (void of living cells) autologous pericardium (10). In this model, autologous pericardium was implanted in the descending aorta of sheep as an intraluminal flap with both surfaces exposed to the blood flow. After 30 days, ethanol-treated implants were integrated into the surrounding tissue, were totally repopulated by host cells, and had regained an endothelium. In contrast, the vital flap implant showed signs of retraction and thickening by day 15. Continual fibrin clot deposition and subsequent tissue build-up characterized the vital fibrotic flap, and it never regained an endothelium. By day 30, this implant was a mass of fibrotic tissue that no longer resembled the original pericardial tissue. Although limited in its capabilities, this in-vivo flap model was designed to simulate the usage of vital autologous pericardium in heart valve leaflet reconstruction and replacement. In fact, the observed healing reactions were similar to those seen in clinical studies where vital autologous pericardium was used for this purpose (9,11). The healing response observed in this study resembled a chronic wound

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where the healing process was incomplete resulting in loss of functional integrity of the implant. Histologic analysis of the recovered ethanol-treated and vital flap implants indicated that activation of cells intrinsic to the vital flap had contributed to the observed fibrotic healing outcome.

In the present study, the differences between ethanol-treated and vital pericardial flap implants were further characterized in order to gain insight into the observed differential healing patterns. In this time-dependent study, the spatial distribution patterns of cellular activation relating to the immunohistologic detection of cells activated to divide and cells activated to synthesize type I collagen are described.

Materials and methods

Animals

Targhee sheep (n = 20; age range 4 to 10 months; mean bodyweight 36 ± 7.6 kg; range: 28 to 52 kg) were used in this study. Eight sheep received vital flap implants and 12 sheep received ethanol-treated implants. The sheep were treated perioperatively with broad-spectrum antibiotics. All animals were cared for in accordance with the *Principles of Laboratory Animal Care* formulated by the National Society of Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication # 85-23, revised 1996). The use of the animals for this research was also reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Montana.

Surgical technique

General anesthesia was induced with intravenous ketamine (1.0 mg/kg) and propofol (4.0 mg/kg) and maintained with oxygen (4 l/min) and isoflurane gas 1.5-2.5%. The electrocardiogram and systemic arterial blood pressure were monitored during surgery. The chest wall was opened through a left lateral thoracotomy, and the pleural cavity through the 4th intercostal space. The overlying parietal pleura were dissected from the descending thoracic aorta and a 10 cm segment mobilized. A 6×10 cm rectangular piece of pericardium was harvested anterior to the left phrenic nerve and placed in physiological saline. Markers were placed to help maintain the original craniocaudal pericardial orientation. A 1.5×2 cm segment was resected from the pericardium to be used as an intraluminal flap, bisecting the aortic lumen. For animals receiving ethanol-treated implants, the tissues were treated with 50% ethanol for 5 min and rinsed in sterile phosphate-buffered saline (PBS).

When pericardial preparation was complete, the descending thoracic aorta was isolated with cross-clamps placed distal to the arch and proximal to the first major intercostal or spinal artery branch. The animals were heparinized to maintain an accelerated clotting time (ACT) above 300 s. An apical-femoral shunt was then created to bypass the isolated segment of the descending thoracic aorta that received the implants. The distal perfusion pressure through the retrograde femoral flow was maintained at >35 mmHg. The aorta was incised longitudinally along its anterior margin for a distance of 2 cm. One edge of the intraluminal flap was sutured under tension to the aortic wall directly opposite the longitudinal incision and the opposite edge sandwiched between the walls of the aortic incision as the aorta was being closed with continuous running 5-0 polypropylene sutures. The long axis of the flap was also oriented parallel to the long axis of the harvested pericardium (Fig. 1).

Implant harvesting

In order to harvest the implants, the animals were fully heparinized (300 U/kg) prior to sacrifice in order to prevent post-mortem intraluminal clotting. Sheep were sacrificed by injection of 1% propofol and 20 ml KCl (USP 20 mEq/ml). The descending aorta was excised en bloc to include pericardial implants as well as a margin of normal aortic tissue extending 1 cm beyond the sutures. The aorta was rinsed in physiological saline and split longitudinally for gross observation from the luminal side. Gross observations were made on the degree of fibrosis, dilatation or thinning, thickening, and retraction. Vital samples were harvested at days 5 (n = 2), 10 (n = 2), 15 (n = 2) and 30 (n = 2). Ethanol-treated samples were harvested at days 5 (n = 3), 10 (n = 3), 15 (n = 3) and 30 (n = 3).

Histology

Recovered implants were fixed in Histo-Choice (Amresco Inc., Solon, OH, USA), embedded, and sec-

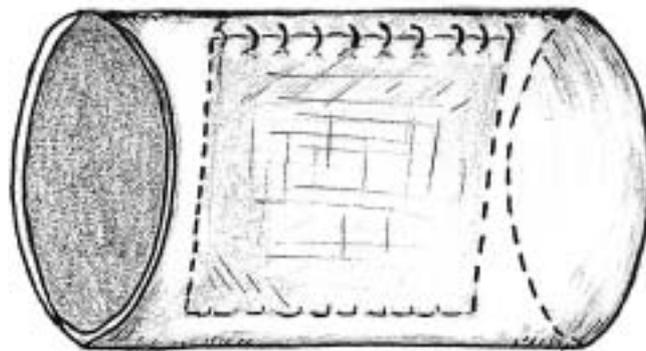


Figure 1: Depiction of the autologous pericardial flap implanted in the descending aorta of sheep

tioned for histology. Cross-sections of the flap and aortic wall were made perpendicular to the blood flow in order to observe the aortic wall and edges of the flap. Sections were stained with hematoxylin and eosin (H&E).

Immunohistochemistry

Tissue sections were deparaffinized and the antigen was unmasked. To evaluate the cellular expression of proliferating cell nuclear antigen (PCNA; Sigma Chemical Co, St. Louis, MO, USA), tissues were heated in 0.01 M citrate for 10 min on high power using a microwave oven. For evaluating the cellular expression of procollagen, tissues were first incubated at 45°C in 4 M NaCl for 40 min followed by 0.01 M citrate for 7 min in a rice steamer (Sunbeam Products Inc., Delray Beach, FL, USA). Unmasked tissues were treated with 3% H₂O₂ for 5 min to remove endogenous peroxidases. Tissues were blocked with 0.05% Tween-20 in PBS (PBS-T) containing 10% normal sheep serum (NSS) for 30 min. Tissues were incubated for 30 min with antibody specific for PCNA (indicating dividing cells) and with a monoclonal antibody specific for sheep type I procollagen (SP1.D8; Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA, USA) diluted in PBS-T containing 10% NSS. Sections were rinsed with PBS-T and incubated

with the LSAB-2 streptavidin-biotin amplification kit (DAKO, Carpinteria, CA, USA). All incubations were performed in a moist chamber at room temperature. Sections were rinsed with PBS-T, and antibody recognition of antigen was visualized using diaminobenzidine (DAB; 100 µl) staining (Sigma Fast DAB; Sigma Chemical Co.), which resulted in the formation of a brown precipitate. The development was stopped with water and the slides counterstained with H&E. Negative controls included using an irrelevant primary antibody or replacing the secondary antibody with PBS-T. A positive control, such as a tissue known to express the marker in question, was used for every immunohistochemical stain.

Results

Vital implants and cellular proliferation

In vital flaps, cells within the pericardial implant expressing PCNA were observed throughout the implant and were present during the entire experimental time frame of 30 days. At early time points (days 5 and 10), proliferating cells were concentrated at the junctions of the implant and aortic wall (Fig. 2A). PCNA expression at the junctions subsequently diminished in the later time points of days 15 and 30. Starting by day 10, proliferating cells were seen in fibrin deposits that overlaid the pericardial implants (Fig. 2B). There was a progressive decrease in PCNA-expressing cells in these fibrin layers as the fibrin

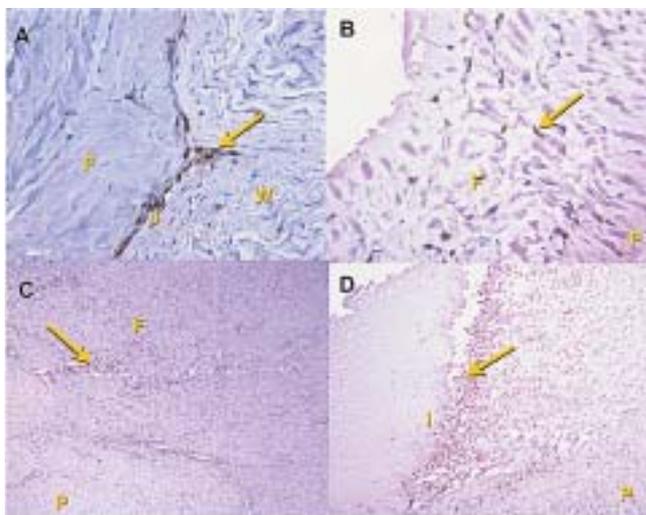


Figure 2: Expression of proliferating cell nuclear antigen (PCNA) in vital autologous pericardial flap implants. Cells expressing PCNA were observed at day 5 at the junction (A, original magnification, $\times 400$) of the implant and aortic wall and in the fibrin (B, $\times 400$) that covered the implanted tissue. At day 30, PCNA expression was observed in the fibrin (C, $\times 100$) and at the interface (D, $\times 100$) of the remodeled fibrin and the newly deposited fibrin clot. F: Fibrin; I: Interface of remodeled fibrin and new fibrin clot; J: Junction of aortic wall and implant; P: Implanted pericardial tissue; W: Aortic wall.

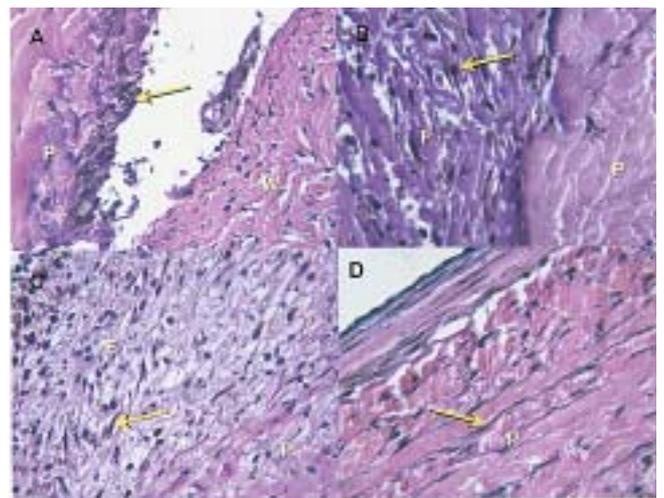


Figure 3: Expression of PCNA in ethanol-treated autologous pericardial flap implants. Cells expressing PCNA were observed at the junction (A, original magnification, $\times 400$) and in the fibrin layers (B, $\times 400$) that covered the implants at day 10. Expression of PCNA remained in the fibrin (C, $\times 400$) at day 15 and was observed throughout the implanted pericardium (D, $\times 400$) at day 30. Abbreviations are as in Figure 2.

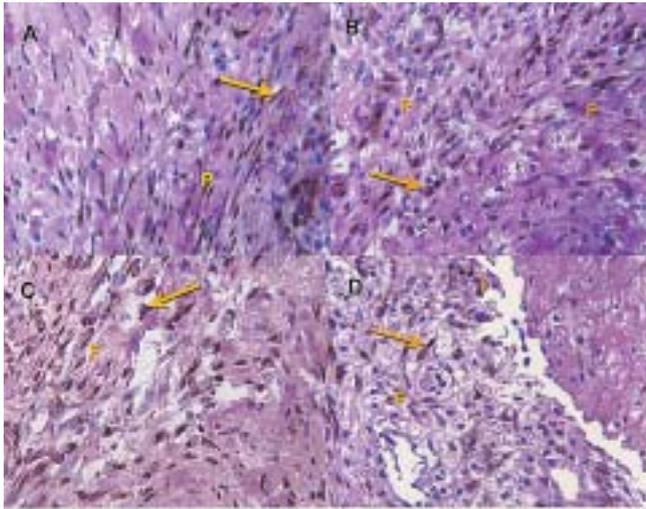


Figure 4: Cellular expression of procollagen in vital autologous pericardial flap implants. At day 10, procollagen was expressed by cells within the implanted tissue (A, original magnification, $\times 400$) and in the fibrin near the junction of the implant and aortic wall (B, $\times 400$). By day 15, procollagen expression was observed throughout the thickening fibrin layers (C, $\times 400$) with focal concentration at the interface (D, $\times 400$) of the remodeled fibrin and newly deposited fibrin clot. Abbreviations are as in Figure 2.

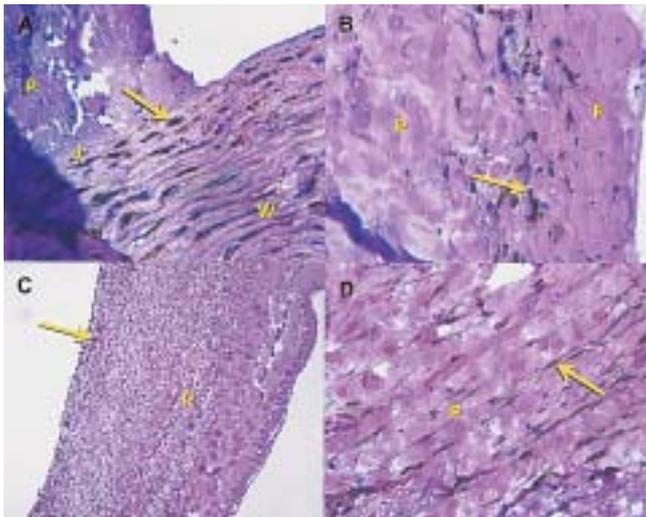


Figure 5: Cellular expression of procollagen in ethanol-treated autologous pericardial flap implants. Procollagen expression was observed in the aortic wall at the junction of the wall and the implant (A, original magnification, $\times 400$) by day 5 and by some cells within the implant (B, $\times 400$) by day 10. By days 15 (C, $\times 100$) and 30 (D, $\times 400$), most cells within the implant expressed procollagen. Abbreviations are as in Figure 2.

became more organized. At days 15 and 30 after implantation, PCNA expression was concentrated both in the fibrin (Fig. 2C) and at the interface of reorganized fibrin and the newly deposited fibrin clot (Fig. 2D). In addition, very few PCNA-expressing cells were observed within the aortic wall during this study.

Ethanol-treated implants and cellular proliferation

In ethanol-treated flaps, few proliferating cells were observed within the pericardial implants at days 5 and 10. Cellular expression of PCNA was concentrated at the junctions of the implant and the aortic wall (Fig. 3A) and observed in the fibrin layers covering the implants (Fig. 3B). By days 15 and 30, PCNA expression was reduced at the junctions and in the fibrin deposits (Fig. 3C). Instead, proliferating cells were concentrated within the pericardium of the implant (Fig. 3D).

Vital implants and procollagen

Vital implants recovered at days 5 and 10 showed procollagen expression throughout the pericardial tissue (Fig. 4A), and fibrin deposits on the implant (Fig. 4B) as well as under the endothelium of the aortic wall by day 10. In the later time points (days 15 and 30), cellular expression of procollagen was reduced in the pericardial tissue of the implant and absent in the aortic wall. Instead, cellular procollagen expression at days 15 and 30 shifted to the remodeled fibrin layers (Fig. 4C) and the interface of the reorganized fibrin tissue and newly deposited fibrin clot (Fig. 4D).

Ethanol-treated implants and procollagen

Throughout this study period, cellular procollagen expression was not observed in the aortic wall of ethanol-treated pericardial flap implants. Procollagen expression was observed at the junctions of the implants and the aortic wall (Fig. 5A) as well as in the thin fibrin deposits that overlaid the implant surface at days 5 and 10 (Fig. 5B). By days 15 and 30, procollagen expression remained associated with the implant-aortic wall junction, but was also expressed by the majority of cells that repopulated the pericardial implant (Figs. 5C and D).

Figure 6 clarifies the time course and differences of cellular activation in PCNA and procollagen expression within the ethanol-treated and vital tissue. In ethanol-treated pericardium, cellular activation began outside the implant and progressed inward. In vital pericardium, cellular activation began within the implant and progressed outward.

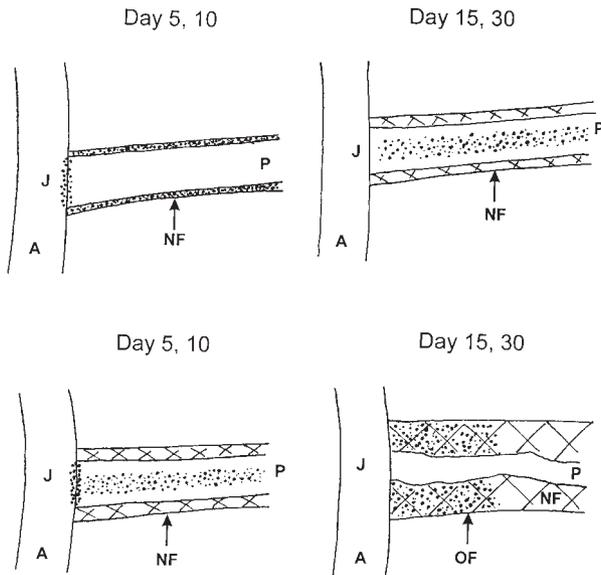


Figure 6: Schematic illustration of PCNA and procollagen expression in ethanol-treated (top) versus vital (bottom) pericardium implanted as a flap in the descending aorta of sheep. In the ethanol-treated group, staining was apparent at the junction and in the new fibrin deposited on the surface of the pericardium at days 5 and 10, and transitioned to expression in the center of the pericardial implant by days 15 and 30. Cellular activation in vital pericardial flaps began within the implant at days 5 and 10, and progressed outwardly in a time-dependent manner by days 15 and 30. A: Aortic wall; J: Junction of implanted pericardium and aortic wall; NF: New fibrin; OF: Organized fibrin; P: Pericardium.

Discussion

Vital autologous pericardium has limited uses as a cardiovascular repair material due to the unpredictable healing responses associated with these implants. When vital pericardium was used to repair or replace heart valve leaflets, retraction and fibrosis were the most commonly observed sequelae during healing. Previously, the implantation of vital autologous pericardium as a flap in the descending aorta of sheep has been reported, wherein the intraluminal flap represented a heart valve leaflet in the open position. Although this model was limited in its ability to simulate the turbulence and dynamic stresses experienced by heart valves, the observed healing reactions were similar to those seen in clinical studies where vital autologous pericardium was used to replace or reconstruct heart valve leaflets (9,11). In this study, vital and ethanol-treated pericardium were implanted in this model for 30 days and evaluated histologically and immunohistologically for the presence of endothelial cells (von Willebrand factor) and the presence of

smooth muscle cells/myofibroblasts (smooth muscle cell α -actin).

Analysis of recovered implants showed that ethanol-treated tissues were repopulated in an orderly manner by host cells expressing α -smooth muscle cell actin, and that these implants were covered by a surface layer of cells expressing von Willebrand factor. Ethanol-treated implants showed no signs of fibrosis. In contrast, vital implants were retracted and fibrotic. These implants showed continual deposition of fibrin that resulted in a build-up of granulation tissue characterized by cellular invasion, matrix synthesis, and capillary formation. The vital flap implants never regained an endothelial layer.

To better understand how cellular activation affected vital pericardial implant healing, the spatial distribution of dividing cells and cells producing type I procollagen was evaluated immunohistologically in a time-dependent study. Dividing cells, identified by the cellular expression of PCNA, were observed in the same areas of the implants where cells expressing type I collagen were seen. However, the spatial pattern of these activated cells was distinct between vital and ethanol-treated implants. In ethanol-treated flaps, the cells were concentrated at the periphery of the implants at early time points (days 5 and 10), but at later times (days 15 and 30) the concentration of activated cells shifted to cells that had repopulated the pericardial implant. This shift seemed to coincide with a re-establishment of the endothelium by day 15 (10). In contrast, cellular activation in vital flaps was concentrated internal to the implant (days 5 and 10) and subsequently shifted to the fibrin deposits overlying the implant (days 15 and 30). Vital implants recovered after 30 days showed cellular expression of PCNA and procollagen concentrated at the interface of reorganized fibrin and newly deposited clot, indicating a self-renewing cycle of tissue accumulation. In this respect, the vital flap resembled a chronic wound.

The differential patterns of cellular activation between vital and ethanol-treated flap implants reflected the healing outcomes of these implants previously reported by the present authors' laboratory (10). In ethanol-treated flap implants, as healing progressed, a general shift of cellular activation from cells external to the implant to cells internal to the implant was found. This shift coincided with a cessation in fibrin deposition and the re-establishment of an endothelium. In contrast, concentrations in cellular activation of vital flap implants shifted from cells internal to the implant to cells external to the implant. The deposition of fibrin did not diminish by day 30, and an endothelium was never re-established. It is the present authors' consideration that activation of cells intrinsic to the vital flap resulted in a cycle of cellular activation leading to the

observed fibrotic developments. Strategies to reduce cellular activation on implantation of vital autologous pericardium may beneficially affect the healing outcomes of this useful biomaterial.

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