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Douglas C. Marchion

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Activation of Connective Tissue Cells in Vital Pericardium, Relevance to the Behavior of Vital Autologous Pericardial Implants in Cardiovascular Surgeries

Douglas C. Marchion

M.S., University of Montana, Missoula, MT 2001

Presented in partial Fulfillment of the Requirements for the degree of Doctor of Philosophy

The University of Montana 2001

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The use of vital autologous pericardium as a cardiovascular repair biomaterial has produced mixed results. Autologous pericardium was found to behave favorably as a patch for left ventricle repair and atrial septal defect closure and as a conduit for pulmonary artery and right ventricular outflow-tract reconstruction. In contrast, fresh autologous pericardium became fibrotic when used for mitral valve chordae and leaflet repair. Our laboratory investigated healing reactions of vital autologous pericardium implanted as a flap in the descending aorta of sheep (a model that simulated a heart valve leaflet, Cheung et al., 1999). In this in vivo model the pericardium became fibrotic and was characterized by cellular accumulation and tissue retraction. Histological analysis of recovered implants indicated that activation of cells endogenous to the pericardium contributed to the detrimental healing outcome.

Here we report an in vitro model of pericardial tissue healing using living pericardium. In this in vitro model, pericardial tissue contracted and the cells in situ expressed the proliferative marker PCNA and procollagen. These physical and cellular changes of cultured pericardium were stimulated by blood serum in a dose dependent manner and could be modulated by chemical inhibition of protein synthesis and protein function. In these studies, serum-stimulated pericardial tissue contraction was dependent upon the synthesis and deposition of Type I collagen. The mechanism of tissue contraction through the deposition of collagen is unknown. In addition, serum-stimulated cellular proliferation was independent of both tissue contraction and collagen synthesis. These data suggest that fibrosis of vital autologous pericardium in vivo may occur in response to the activation of cells in situ to the implant. One mechanism of cellular activation was through the exposure to blood serum. Data acquired from this in vitro study indicated that the microenvironment associated with cells and tissues is unique to those tissues and changes in tissue microenvironment may result in cellular activation and possible detrimental healing reactions.
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I dedicate this dissertation to my parents, Theodore and Louise Marchion. Their continuing love and support was an inspiration throughout my college career.
INTRODUCTION

Pericardium is a fibro-serous membrane that surrounds the heart functioning to reduce friction as the heart beats. It is composed of two distinct surfaces, the fibrous layer, and a serous layer. The serous layer faces the heart and is covered by mesothelial cells; a type of endothelial cell, while the fibrous layer faces away from the heart and is covered by loose tissue and fat. When pericardium is used in cardiovascular surgeries, the loose tissue, fat, and mesothelium are removed.

Autologous pericardium has many advantages as a cardiovascular repair material. It is non-immunogenic, free of donor-derived pathogens, shows excellent hemostatic qualities, and is easy to access [1,2]. However, autologous pericardium was found to behave differently depending upon its usage. When used as a patch, vital autologous pericardium was found to behave favorably for left ventricle repair [3, 4] and atrial septal defect closure [2]. When used as a conduit, vital autologous pericardium functioned well for pulmonary artery [5] and right ventricular outflow tract reconstruction [6, 7].

In contrast, vital autologous pericardium became fibrotic when used for mitral valve chordae and leaflet repair [8]. Aneurysmal changes were observed following patch reconstruction of large ventricular defects, or right ventricular outflow tract repair with distal pulmonary hypertension [5, 9]. To avoid these changes, some authors have suggested fixation of the pericardium with glutaraldehyde [10].

Understanding the behavioral variability of vital pericardial implants would allow for the development of strategies to control the outcome of the implant. For this reason, Cheung et al. implanted vital autologous pericardium as a patch, conduit, and...
flap in the descending aorta of sheep [11]. The implants were placed in the same location and differed only by configuration and therefore hemodynamic stresses. Thirty days after implantation, the patch and conduit showed organized layers of α-smooth muscle cell actin (α-actin)-expressing cells covered by a surface layer of cells expressing von Willebrand Factor (vWF). In contrast, α-actin-expressing cells in the flap were randomly oriented. The flap was retracted, fibrotic, and never acquired a vWF-expressing surface layer of cells.

To determine if vital cells of the pericardium contributed to the fibrotic reactions of the flap, Cheung et al. killed the intrinsic pericardial cell population by washing the tissue with 50% ethanol for 5min [11]. The ethanol-treated autologous pericardium was implanted as a patch, conduit, and flap in the descending aorta of sheep.

Thirty days after implantation, the ethanol-treated pericardial flap was found to be repopulated by host cells and had a continuous surface layer of vWF-expressing cells, indicative of an endothelium. There were no signs of fibrosis. The reasons for the observed differences are unknown but are thought to involve normal wound healing responses.

**Wound Healing**

The process of wound healing has been extensively characterized in the skin. Cutaneous wound healing can be described in three phases; inflammation, granulation tissue formation, and extracellular matrix remodeling [12, 13]. Inflammation is triggered by injury and is designed to restore structure and function to the tissue [12].
while preventing systemic infection [13]. Damage to blood vessels results in denudation of the endothelium, exposing collagen types IV and V on the subendothelium [12]. Exposure of collagen promotes binding and subsequent activation of platelets. Activated platelets degranulate and interact with fibrinogen, fibronectin, vWF, and thrombospondin to prevent blood loss by forming a platelet plug [14]. Platelet degranulation results in the release of factors including transforming growth factor α (TGFα) and β1, platelet derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), serotonin, fibronectin, adenosine diphosphate, thromboxane, platelet activating factor (PAF), platelet factor-3 and platelet factor-4 into the local environment [12]. Platelet factor-3 interacts with blood coagulation proteins resulting in the formation of a fibrin matrix. The fibrin matrix strengthens the platelet plug and provides binding sites for cellular infiltration [14].

The first cell type to migrate into the wound site is the neutrophil [12]. Neutrophils clean the wound, removing invading microorganisms by phagocytosis, and degrading damaged tissue by the release of elastase and collagenase [12]. Subsequent to the neutrophil, the macrophage becomes the dominant cell type at the wound site in response mainly to TGFβ1, but also bacterial products and complement components [12]. Macrophages remain at the wound site for several weeks and mediate wound repair by cytokine secretion [12].

The second phase of wound healing is the granulation tissue formation. The formation of granulation tissue is coordinated by macrophage which recruit fibroblasts
and other cell types to the wound site. Fibroblasts are attracted and activated by PDGF and TGFβ₁. Activated fibroblasts migrate to the wound and secrete PDGF and TGFβ₁ with autocrine activity [13]. TGFβ₁ induces fibroblast synthesis of collagen Types I and III. The fibroblasts then acquire a smooth muscle cell phenotype characterized by the synthesis of α-actin, and participate in wound contraction [13].

After contraction, macrophage and fibroblasts release the growth factors TGFα, TGFβ₁, EGF, and VEGF that activate and recruit endothelial cells [13]. The endothelial cells form capillaries through the fibrin matrix. Pericytes migrate along the capillary tubes and encircle them. Together, endothelial cells and pericytes deposit collagen type IV to form a basal lamina around the vessel [13].

The final phase of wound healing is extracellular matrix remodeling [13]. During this phase, the total cell number continually decreases while collagen type III is degraded and replaced by collagen type I [13]. TGFβ₁ plays a major role in this phase of wound healing by having both positive and negative control of collagen synthesis [12]. TGFβ₁ and PDGF induce the synthesis of procollagenase by fibroblasts [12]. Procollagenase is cleaved to collagenase by metallo-proenzymes and inhibited by tissue inhibitor of metallo-proteinases (TIMP) [12]. In contrast, TGFβ₁ has been shown to block the production of procollagenase induced by other growth factors and induce the production of TIMP [12]. In concert with PDGF, TGFβ₁ stimulates fibroblast production of glycosaminoglycans and proteoglycans and induces the synthesis of collagen and fibronectin [13]. TGFβ₁ modulates a decrease in cell density by inhibiting endothelial cell
proliferation. In addition, TGFβ, blocks the effects of PDGF on fibroblasts and the effects of bFGF on endothelial cells [13].

In terms of arterial wound healing, where pericardium is used to reconstruct heart valve leaflets, or patch cardiac defects, the cell types of concern are endothelial cells, smooth muscle cells, and fibroblasts.

**Endothelial cells**

Endothelial cells form the friction reducing lining of the heart, blood vessels, and lymphatic vessels [15-17]. The endothelium maintains blood flow and controls the trafficking of circulating cells out of the vascular system into the surrounding tissue [18]. The endothelium is involved in numerous metabolic functions such as angiogenesis [19], [20], coagulation [21], thrombolysis [22], basement membrane synthesis [22, 23], production of growth factors [21, 24], and regulation of vascular tone [25-27].

The endothelium has a critical function in the maintenance of the vascular system. By secretion of soluble factors, such as nitric oxide and heparin-binding epidermal growth factor-like growth factor, the endothelium is thought to regulate the function of smooth muscle cells (SMC) in the underlying media [25].

Arterial wound healing models suggest loss or disfunction of the endothelium may result in activation of SMC [28]. Several laboratories have noted the importance of endothelial cell-SMC interaction in relation to arterial wound healing. This research indicated that hyperplasia of SMC in the intima occurred specifically in areas where the endothelium was absent. In other areas, where a rapid regeneration of the
endothelium occurred, SMC migration and proliferation in the intima was not observed [29-31].

**Smooth Muscle cells**

Smooth muscle cells are specialized cells of the arterial media that regulate blood pressure and blood flow [32]. In the developing organism, SMC are in a synthetic state characterized by increased proliferation, large endoplasmic reticulum, large golgi complex, β-actin synthesis, and secretion of extracellular matrix components [32]. In the fully developed organism, SMC differentiate from a synthetic to a contractile activation state. This differentiation is characterized by a switch from β-actin production to α-actin production [33], a size reduction of the endoplasmic reticulum and golgi complex, increased myosin and myofilament synthesis, and a slower growth rate [32]. It is not known what induces SMC to differentiate into a contractile state of activation. However, it is known that this differentiation is not terminal. In certain pathological situations SMC are stimulated to revert back to a synthetic state. Reversion of SMC activation state has been documented in pathological situations involving atherosclerosis [34, 35], restenosis after coronary angioplasty [36], restenosis after coronary and peripheral artery bypass using autologous saphenous vein grafts [37-39], angiogenesis [25], and experimental wound healing [28].

Several factors may influence SMC transition to a synthetic state of activation. Nitric oxide (NO) produced by endothelial cells was shown to inhibit SMC growth [40], whereas soluble factors released from platelet α-granules were shown to have a
stimulatory effect on SMC mitogenesis [41]. Among the SMC stimulatory factors released from platelets are PDGF [41], thrombin [42], bFGF [43], TGFβ, [44, 45], and IGF-1 [46]. Taken together, reduction of local NO and stimulation of platelet aggregation and degranulation due to the loss of endothelium may be responsible for SMC reversion to a synthetic state. Reversion to a synthetic state would allow for SMC migration into the intima and SMC proliferation resulting in many disease states of the cardiovascular system.

**Fibroblasts / Myofibroblasts**

Fibroblasts are the dominant cell type of arterial adventitia and connective tissues. Towards the conclusion of the inflammation phase during cutaneous wound healing, factors released from platelets and macrophage recruit cellular infiltrates to the wound site [13, 47]. Of these cellular infiltrates, fibroblasts are attributed with the majority of new matrix deposition [13]. Fibroblasts in the wound secrete collagen and differentiate into myofibroblasts [48, 49].

The myofibroblast is a specialized phenotype of fibroblasts that functions in wound contraction [13, 50]. The myofibroblast has features common to both fibroblasts and smooth muscle cells such as the synthesis of α-actin, desmin, and vimentin [51]. Although the differentiation between myofibroblasts and smooth muscle cells is at best subtle, myofibroblasts may be distinguished from other cell types by ultrastructure. Schurch *et al.*, 1998, define the myofibroblast as having isolated bundles of α-actin stress fibers, a feature not distinguishable in smooth muscle.
cells [52]. In addition, the myofibroblast may be identified by distinct cell to stroma attachment sites, and intracellular gap junctions [52].

*In vitro* experimentation has suggested that TGFβ1-stimulation is responsible for inducing the phenotypic change of fibroblasts into myofibroblasts. Jessen and Petersen, 1993, reported that α-actin-negative breast fibroblasts became α-actin positive upon addition of TGFβ1 in culture [53].

In terms of wound healing, the myofibroblast participates in wound contraction, collagen deposition, and scar formation [48]. Although the myofibroblast has a valuable function in wound healing, it has been associated with various pathologies that result in deformation of tissues [50]. These pathologies include idiopathic pulmonary fibrosis [54], fibrosis of the breast after radiation [55], renal fibrosis [56], liver fibrosis [57], etc. Studies of experimental wounds have shown that scarring may be reduced by addition of TGF-β3 [58], which has been shown to prevent fibroblast transition into myofibroblasts [59]. In addition, experimental tubulointerstitial fibrosis was prevented by the addition of the angiotensin converting enzyme inhibitor enalapril [60]. Enalapril was shown to prevent fibrosis by decreasing the interstitial accumulation of myofibroblasts. This suggests that pathologies involving fibrosis may be a direct result of myofibroblast persistence. In fact, it has been documented that myofibroblasts are induced into apoptosis soon after wound contraction [61]. The apoptotic signal has not been identified, but may involve re-epithelialization of the wound [62, 63].
Cardiovascular Wound Healing

Whereas wound healing has been studied in the skin, wound healing is not well documented in the cardiovascular system. Our laboratory investigated cardiovascular wound healing as it related to vital autologous pericardial transplants. Although the clinical use of vital autologous pericardium has been extensively reported, the healing variability associated with these implants has not been studied. We explored the healing behavior of pericardial implants using the juvenile sheep model. Vital and ethanol-treated autologous pericardium was implanted as a patch, conduit, and flap in the descending aorta of Targhee sheep [11]. Thirty days after implantation, there was an orderly invasion and subsequent repopulation of all ethanol-treated implants by cells from the surrounding host tissue. The endothelium was restored and the implants essentially became an extension of the native host tissue. In short, the healing process appeared controlled and was completed by the conclusion of the experimental time period of 30 days.

In contrast, much more activity was seen during the healing process of vital autologous transplants. Significantly more clot deposition, fibrin accumulation, and cellular disorganization was observed with the vital patch and conduit than the ethanol-treated counterparts. However, these tissues eventually regained an endothelium and were also incorporated into the surrounding tissue. Successful healing was not observed when vital autologous pericardium was implanted directly in the bloodstream as an intraluminal flap. With this implant, continual clot deposition was seen through the experimental time period. This deposition resulted in a build-up of granulation tissue with cellular invasion, matrix synthesis, and capillary formation.
Proliferating cells were concentrated at the interface between the granulation tissue and newly deposited clot, which suggested a self-renewing cycle of tissue accumulation. The tissue of the original pericardial implant, which showed signs of retraction by day 15, was thickened by day 30 from collagen synthesis by \textit{in situ} cells. In fact, by day 30, some sections of the original implant were indistinguishable from the surrounding granulation tissue. By conclusion of the experimental time period, the vital pericardial flap was a mass of fibrotic tissue that no longer resembled the original implant and never regained an endothelium. In this respect, the vital flap resembled a chronic wound where the healing process was incomplete resulting in the loss of functional integrity of the implant.

It is not known why the vital patch and conduit were accepted into the surrounding tissue while the vital flap was not. Furthermore, all ethanol-treated implants were incorporated regardless of the implant configuration. Taken together, these data suggested that cellular activation contributed to the detrimental healing outcomes of the vital pericardial flap. However, the question remains as to which cells were activated and what was the activation signal. The above \textit{in vivo} experiment confirmed that cells \textit{in situ} to the implants were required for the development of fibrosis. However, these cells may interact and therefore be activated by cells from the host aortic wall and blood stream as well as soluble factors within the blood.

\textbf{The First Hypothesis} of this study is:

Fibrosis of vital autologous pericardial transplants is promoted by activation of \textit{in situ} cells.
The Second Hypothesis is:

Soluble blood factors may stimulate cells within cultured pericardium to contract, proliferate, and synthesize new matrix resulting in tissue contraction and thickening.

The Third Hypothesis is:

Tissue contraction, cellular proliferation, and matrix synthesis are independent events.

To test these hypotheses, the following Specific Aims were investigated:

1. An in vitro model of pericardial tissue contraction that simulated the healing responses associated with the vital fibrotic pericardial flap was developed. These healing responses included tissue contraction, cellular proliferation, and collagen deposition.

2. Cells in situ to cultured pericardium were activated with blood serum and the ability of activated cells to contract pericardial tissue, proliferate, and synthesize collagen was investigated. The mechanism of serum-stimulated cellular activation was evaluated using chemical inhibition of protein synthesis and function, and cellular proliferation.

3. The stimulatory component of serum capable of inducing cultured pericardial tissue contraction was characterized in terms of molecular weight range, heat sensitivity and protease sensitivity.

4. The mechanism of serum-stimulated tissue contraction was investigated using time related SDS-PAGE protein profiles of cultured pericardial tissues stimulated with serum with and without the presence of inhibitors of protein synthesis.

5. The relationship between serum-stimulated tissue contraction and cellular proliferation, collagen synthesis and cellular proliferation, and tissue contraction and collagen synthesis using chemical inhibition of protein synthesis and function, and cellular proliferation.

I. In Vitro Modelling of Wound Healing

This study focuses on understanding the activation of cells within cultured pericardial tissue to gain insight into the development of fibrosis observed in the vital autologous pericardial flap. Although in vivo models of fibrosis would best represent
actual clinical situations, these studies are expensive, time consuming, and do not allow for the isolation of variables, which may be responsible for the observed results. The approach used in this investigation was the development of an in vitro model of fibrosis and tissue contraction that simulated healing outcomes associated with the fibrotic pericardial flap in vivo.

Whereas the majority of in vitro models are composed of a homogenous matrix seeded with a particular cell type, this model incorporated living tissue complete with native cell types and extracellular matrix. Recently, Kratz et al., 1998, reported two in vitro wound healing models using viable human skin explants [64]. These models allowed for the interaction of mixed cell types native to the tissue and the cells maintained natural morphology and proliferative activity after 14 days of incubation. Furthermore, each of these models showed distinctive healing responses characteristic of incision and burn wounds that were similar to healing responses observed in clinical cases. Here we offer a living tissue model of pericardial wound healing. The pericardial tissue used in this model was processed analogously to the pericardium used in vivo, with the mesothelium being removed prior to the experiment. This in vitro model allowed components of the cardiovascular system to be systematically screened for the ability to activate cells within vital pericardial tissue sections to contract the tissue, proliferate, and deposit de novo collagen.

In order to adequately screen cardiovascular factors for the ability to activate cells within cultured pericardium, a comparison needed to be made between stimulated tissue and native tissue. Pericardium was harvested from normal sheep and immediately placed in fixative. Although this harvesting was designed to minimize
mechanical stimulation through handling, significant differences, both macroscopically and histologically, were observed between pericardial sections from different sheep as well as pericardial sections from the same sheep. Macroscopically, these differences were in tissue thickness and loose tissue adhesions. Microscopically, these differences were in cellular density and proliferative activity. For this reason, harvested pericardial tissue sections were cultured for 48hr in serum-deprived conditions. Serum deprivation was used to induce pericardial cells into a quiescent state [65] allowing comparisons to be made between stimulated and unstimulated samples. Furthermore, the proliferative index used in this study was proliferating cell nuclear antigen (PCNA). PCNA has a half-life of approximately 20hr [66]. Therefore, 48hr serum deprivation was used to prevent PCNA staining of cell just leaving the cell cycle.

In this in vitro model, cardiovascular factors, such as serum, were screened for the ability to activate cells within serum-starved pericardial tissue sections. Activated cells were evaluated for the ability to contract the tissue, proliferate, and synthesize Type I collagen as observed during the fibrotic development of the in vivo vital autologous pericardial flap.

Materials and Methods

Harvesting of Pericardial Tissue

Parietal pericardium was harvested from sheep and the loose tissue, adhesions, and the mesothelium were removed using sterile gauze. The tissue was dissected into approximately 1cm² sections and serum starved by incubation with Dulbecco’s modified Eagle’s medium (DMEM, Gibco BRL, Gaithersburg, MD) containing 0.5%
fetal bovine serum (FBS, HyClone, Logan, UT) for 48hr prior to experimentation. Tissues were then incubated with DMEM containing 20% FBS (DMEM/20% FBS) for up to 30 days. Control tissues included pericardium killed by freezing at \(-20^\circ\text{C}\) for 1 week prior to use and pericardium killed by a 5min wash in 50% ethanol shown previously to kill all cells within the tissue [11].

**Histology**

Specimens were fixed in Histo-Choice (Amresco Inc., Solon, OH) for a minimum of 12hr. The sections were dehydrated and embedded in PolyFin (Polysciences, Inc.) wax for sectioning. Wax blocks were sectioned at 5\(\mu\text{m}\) and collected on poly-L-lysine coated slides. Histological stains included hematoxylin and eosin (H&E) for visualization of cell nuclei and tissue matrices respectively, as well as Masson's Trichrome for collagen density.

**Immunohistochemistry**

Tissue samples were analyzed by immunohistology for the synthesis of proliferating cell nuclear antigen (PCNA) (Sigma Chemical Co., St. Louis, MO). Sections were deparaffinized and the tissue was unmasked by 10min microwaving on high power in 0.01M citrate. Unmasked tissues were treated with 3\% \(\text{H}_2\text{O}_2\) for 5min to remove endogenous peroxidases. The sections were blocked with 0.05\% Tween-20 (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline (PBS-T) with 10\% normal sheep serum (NSS) for 30min and incubated with an appropriate concentration of primary antibody in PBS-T, 10\% NSS for 30min. Sections were rinsed with PBS-T and incubated with the LSAB-2 strepavidin-biotin amplification kit (DAKO,
All incubations were performed in a moist chamber at room temperature. Sections were rinsed with PBS-T and 100μl of diaminobenzine (DAB) substrate (Sigma Fast DAB, Sigma Chemical Co., St. Louis, MO) added to each section for development. For some tissues, development was performed using the ImmunoPure metal enhanced DAB substrate kit (Pierce, Rockford, IL). The development was stopped with water and the slides counter stained 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**

Pericardial tissues incubated in 0.5% FBS for 48hr showed greater than 95% cell viability by Trypan Blue staining. The cells *in situ* to cultured pericardium became activated after incubation in DMEM, 20%. This activation resulted in cell-mediated tissue contraction (Figure 1), cellular synthesis of PCNA (Figure 2), and cellular collagen synthesis and subsequent deposition (Figure 3). Cellular activation was the result of serum stimulation since serum-free DMEM did not induce physical changes to cultured pericardium (not shown). Furthermore, these physical and cellular changes observed during the culture of living pericardium were absent in freeze-killed and ethanol-treated tissues (Figure 1).

**Conclusions**

*In vitro* models are necessary for the thorough investigation of complex biological processes because they allow selective manipulation of the testing
Figure 1. Comparison of vital and killed pericardial tissue sections within the *in vitro* model of retraction and fibrosis. Vital tissue sections incubated in the presence of fetal bovine serum resulted in the activation of cells within the pericardium and subsequent contraction of the tissue (yellow arrow). Tissues killed by freeze-thaw or by a 5min wash in 50% ethanol did not contract (blue arrow).
Figure 2. Immunohistological detection of PCNA. Antibody recognition of proliferating cell nuclear antigen was detected by DAB development resulting in a brown precipitate (yellow arrows) and indicates dividing cells. Tissues were counter stained with Hematoxylin, which stains nuclei blue, and Eosin, which stains collagen pink. The pericardial tissue was incubated in DMEM medium containing 20% fetal bovine serum. 400x
Figure 3. Collagen deposition by cells within pericardial tissue. Masson’s Trichrome collagen density stain showing new collagen deposition (light blue, yellow arrow) in the folds of the original pericardial tissue (dark blue). Collagen production was observed 15 days after incubation in DMEM containing 10% fetal bovine serum. 100x
environment. The popular model for studying tissue retraction and its relation to wound healing is fibroblast populated type I collagen gels (FPCG). Bell et al., 1979, first developed FPCG to study fibroblast function [67]. Since then, FPCG were used to explore fibroblast response to chemical mediators [68-72] and were modified to accommodate other cell types such as chondrocytes [73], keratocytes [70] H29 colon carcinoma cells [74], etc.

Through the use of FPCGs, three main schools of thought have arisen regarding the contraction of wounds. The first, as occurs in skin wound healing, involves the transformation of fibroblasts into myofibroblasts [12, 13]. In this theory, fibroblasts acquire a contractile phenotype expressing α smooth muscle cell actin [72, 75, 76] and compress the collagen filaments of the gel. The other theories suggest that locomotion and intracellular tension forces, which are interrelated in the aspect that both are modulated by adhesion molecules and cytoskeletal actin reorganization, are responsible for wound contraction. Nunohiro et al., 1999, showed angiotensin II or osteopontin-induced gel contraction was blocked by antibodies specific for β3 integrins and RGD peptide [69]. Other researchers showed α2 β1 integrins [70, 77] may mediate collagen gel contraction suggesting more than one signalling pathway might be involved. Furthermore, Tomasek et al., 1992, reported two phases of gel contraction, rapid and slow, and that an organized cytoskeleton was required for contraction to occur [78], a process later shown to result in an increase in f-actin production [68]. Lee et al., 1996, also reported the requirement of an organized cytoskeleton, showing
the addition of Cytochalasin, an inhibitor of f-actin polymerization, to the culture medium suppressed fibroblast-mediated gel contraction [79].

In addition to exploring the mechanism of contraction, researchers have investigated the ability of soluble blood factors to stimulate FPCG contraction. Since the development of the collagen gel model, serum has been known to induce collagen gel contraction by fibroblasts [80], and that the rate of gel contraction was proportional to serum concentration [73]. Recently, researchers have been examining the components of serum that may modulate gel contraction by fibroblasts. Growth factors such as TGF-\(\beta\), PDGF [73, 79], and IGF-1 [71, 79, 81] were shown to augment FPCG contraction whereas bFGF [82] was shown to suppress contraction. Furthermore, compounds like glucocorticoids [68, 83] and indomethacin [68] also modulate gel contraction by regulating fibroblast synthesis of prostaglandin E\(_2\) (PGE\(_2\)).

However, FPCG may be overly simplistic to accurately reflect cardiovascular wound healing. First, these models do not allow for the multiple events that occur during natural wound healing with interactions between different cell types, proteases, and chemical messengers [80]. Secondly, it is well known that the extracellular matrix has significant influences on cell morphology and function [79, 80, 84, 85] suggesting cells *in vitro* do not always reflect cells *in vivo*. Finally, FPCGs were developed to model wound healing as it relates to skin wounds where there is a low density of cells and a loosely organized matrix.

In our *in vitro* model of pericardial tissue contraction and fibrosis, living tissue was used to simulate healing outcomes associated with autologous pericardial implants.
*in vivo.* This tissue was composed of the native cells and complex extracellular matrix with the exception of the mesothelium. Using this model, pericardial tissue was serum starved to induce internal cells into a quiescent state and subsequently activated using stimulants. Activation of the cells *in situ* to pericardial tissue resulted in tissue responses similar to those observed during the fibrotic healing reactions of the *in vivo* vital autologous pericardial flap implant including tissue contraction, cellular proliferation, and collagen deposition.

II. Activation of Cells within Cultured Pericardium

The fibrotic reactions of the vital autologous pericardial flap included tissue retraction, cellular accumulation, and collagen deposition. Analogous reactions were observed in living pericardial tissue sections cultured within an *in vitro* model of tissue contraction and fibrosis. Within this model, pericardial tissue sections contracted, evidenced by tissue curling, and the cells *in situ* expressed the proliferative marker, PCNA, and synthesized collagen. Common to both the *in vivo* and *in vitro* models was the presence of serum. For this reason, in this section, FBS was analyzed for the ability to activate cells within cultured pericardial tissue. Dose and time dependency experiments were used to relate stimulants and cellular activation using tissue contraction, PCNA production, and collagen synthesis as activation indicators. In addition, the stimulatory component within FBS was categorized by molecular weight range as well as heat and protease sensitivity using tissue contraction as an indicator of cellular activation. The mechanism of serum-stimulated tissue contraction, cellular proliferation and collagen synthesis was evaluated using chemical inhibitors of protein synthesis and protein function, and SDS-PAGE generated protein profiles.
Materials and Methods

Stimulation of Cultured Pericardium

Pericardial tissue was harvested and processed as described previously. Tissue sections were transferred to Costar 24-well tissue culture plates (Corning Inc., Corning, NY) and incubated as free-floating sections with DMEM (1ml) containing 0.5%, 5%, 10%, 20%, or 50% FBS for 7 days with the media being replaced every other day. Media were purchased at 1X concentration and mixed with the appropriate volume of FBS to achieve the desired percentage of FBS with the exception of 50% FBS which was mixed 1:1 with 2X DMEM to insure an appropriate buffering capacity of the medium. Controls included ethanol-treated pericardial tissues.

The degree of tissue contraction was determined using circular pericardial disks (1cm in diameter) generated using a standardized punch. Disks were incubated in DMEM/20% FBS for 7 days and fixed in Histo-Choice (Amresco Inc., Solon, OH). Pericardial disks immediately fixed in Histo-Choice were used as the control. Contracted and control samples were traced on transparency film and the weights of cut out tracings were compared to determine the decrease in 2 dimensional surface area. The degree of contraction was thus defined as the average percent decrease in weight of cut out tracings of contracted samples when compared to control tissues.

Inhibition of pericardial tissue contraction

Various inhibitors were added to DMEM containing 20% FBS (DMEM/20% FBS) for up to 30 days to determine the ability of these compounds to prevent pericardial tissue contraction. Inhibitors included Mitomycin C and Actinomycin D.
Ipg/ml, Sigma Chemical Co., St. Louis, MO), inhibitors of nucleic acid synthesis; Aphidicolin (1 μg/ml, Alexis Biochemicals, San Diego, CA) and cytosine α-D arabinofuranoside (AraC, 1 μg/ml, Sigma Chemical Co., St. Louis, MO), inhibitors of DNA synthesis; Cytochalasin B and Cytochalasin D (1 μg/ml, Sigma Chemical Co., St. Louis, MO), inhibitors of contractile actin filament formation; Colchicine and Nocozadole (0.0078 μg/ml, Sigma Chemical Co., St. Louis, MO), microtubule depolymerizers; Cycloheximide (1 μg/ml, Sigma Chemical Co., St. Louis, MO) an inhibitor of protein synthesis; and Halofuginone (100 ng/ml, Collgard Biopharmaceuticals, LTD, Petach Tikva, Israel), an inhibitor of collagen type I synthesis. Inhibitors were used at the maximum concentration that showed no cytotoxicity of fibroblast monolayers. Pericardial tissue sections were incubated as free-floating sections in Costar 24-wells tissue culture plates with DMEM/20% FBS containing the above chemical inhibitors. The media (1 ml) were replaced every other day and the sections were maintained at 37°C with 5% CO₂. Controls included tissues incubated in DMEM/20% FBS without inhibitors. Inhibitor toxicity was evaluated using fibroblast monolayers isolated from vital pericardium and was defined as the concentration of inhibitor that induced rounding and loss of adherence of fibroblasts cultured in monolayers.

Characterization of the Stimulatory Component of Serum

In order to determine characteristics associated with the stimulatory component within serum, FBS was fractionated using Centricon centrifugal filters (Millipore, Bedford, MA). Molecular weight fractions, ≥ 100kDa, 30-99kDa, and 5-29kDa, were
obtained by centrifugation for 15 min at 4,000 x g (20°C) using a Beckman J2-21 centrifuge (Beckman instruments Inc., Palo Alto, CA). Concentrated fractions were diluted in 10 ml PBS resulting in 5x the original concentration in whole serum and added to serum-free DMEM media at 20% of the total volume. Fractions were incubated with serum-starved pericardial tissue sections in Costar 24-well tissue culture plates for up to 14 days at 37°C with 5% CO₂ and compared to those incubated in DMEM/20% FBS.

In addition, the stimulatory capacity of FBS was characterized in terms of heat and protease stability. Whole serum was heat treated at 56°C and 70°C for 30 min and enzyme treated with 5 units/ml of proteinase K linked to sephadex beads (Sigma Chemical Co., St. Louis, MO) for 30 min at room temperature. The digested FBS was separated from the enzyme using a Beckman J2-21 centrifuge at 1000 x g. Heat-treated and protease-digested FBS were added separately to serum-free DMEM for a final concentration of 20% total volume. Treated media (1 ml) were used to maintain pericardial tissues as free-floating sections in Costar 24-well tissue culture plates. Tissues were incubated for up to 14 days at 37°C with 5% CO₂, with the media being replaced every other day.

**Protein synthesis**

Pericardial tissue sections were evaluated for *de novo* protein synthesis during the process of tissue contraction. The pericardium was processed as described previously. Pericardial sections were serum starved for 48 hr, transferred to Costar 24-well tissue culture plates and incubated with DMEM/20% FBS for up to 12 days at
37°C with 5% CO₂. The media were replaced every other day. Pericardial tissue sections were harvested at day 1, 2, 3, 4, 5, 7, 10 and 12 and minced in the presence of protease inhibitors. Samples were solubilized and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [86]. Protein banding patterns were visualized by Coomassie Brilliant blue staining. Controls included tissue incubated with DMEM/20% FBS containing the protein synthesis inhibitor, Cycloheximide (1μg/ml). Samples incubated with Cycloheximide were harvested at day 12. Protein banding patterns were compared between samples at different stages of contraction and samples treated with Cycloheximide.

**Stimulation of Cellular Proliferation**

Pericardial tissue sections were transferred to Costar 24-wells tissue culture plates and incubated as free-floating sections with DMEM containing 0.5, 5, 10, 20, or 50% FBS. Since the DMEM used here was high glucose (300mg/lt), glucose-free RPMI 1640 culturing medium (Gibco BRL, Gaithersburg, MD) containing 50% FBS was also used to determine if excess energy aided serum in the stimulation of pericardial cells. The carbonate level in the medium was adjusted in order to compensate for the differing volumes of serum. The medium was changed daily for 4 days, and the sections were fixed in Histo-Choice, dehydrated, and embedded.

Immunohistological analysis using an antibody specific to proliferating cell nuclear antigen (PCNA) was performed on 5μm sections. Percentage of cells staining positive for PCNA was calculated from 4 replicate tissue samples counting 500 cells per replicate in random fields\[ (cells expressing PCNA / total cells) \times 100 \].

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Inhibition of Proliferation

The ability of chemical inhibitors to prevent serum stimulated cellular proliferation was investigated using isolated pericardial fibroblast monolayers. Pericardial fibroblasts were seeded on Costar 96-well tissue culture plates (Costar, Corning, NY) at a concentration of 5,000 cells per well and allowed to adhere in DMEM containing 0.5% FBS overnight at 37°C, 5% CO₂. Mitomycin C, Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, and Cycloheximide (1μg/ml) as well as Nocozadole (0.0078μg/ml) and Halofuginone (0.1μg/ml), were incubated separately with fibroblast monolayers for 4 days at 37°C, 5% CO₂. The media were replaced every other day and cellular proliferation was assayed using Celltiter 96 (Promega, Madison, WI) according to the manufacturer’s protocol. Briefly, the tetrazolium compound, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, 20μl) was added to each well containing 100μl DMEM/20% FBS and the plate was incubated for up to 4hr at 37°C, 5% CO₂. In this assay, MTS is metabolized by fibroblasts to the colored end-product formazan and secreted into the medium. Formazan concentration in the medium was assayed by optical density recorded at 490nm. Controls included fibroblasts (5000 cells/well) incubated with DMEM containing 0.5% and 20% FBS. All assays were performed in duplicate.

Pericardial tissue sections were evaluated for the cellular synthesis of PCNA in the presence of proliferative inhibitors. Tissues were incubated as free-floating sections in Costar 24-wells tissue culture plates with DMEM/20% FBS containing

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either, Mitomycin C (1µg/ml), Aphidicolin (1µg/ml) AraC (1µg/ml), Cytochalasin B,
Cytochalasin D (1µg/ml), Nocozadole (0.0078µg/ml), Cycloheximide (1µg/ml) or
Halofuginone (0.1µg/ml). Tissues were incubated for 4 days with the medium being
replaced every other day. The sections were fixed in Histo-Choice, dehydrated, and
embedded. The synthesis of PCNA was assessed on 5µm sections as previously
described. Percentage of cells staining positive for PCNA was calculated from 4
replicate tissue samples counting 500 cells per replicate in random fields.

**Collagen Synthesis**

Pericardial tissues were incubated as free-floating sections in Costar 24-wells
tissue culture plates with DMEM/20% FBS for up to 30 days. The tissues were
maintained at 37°C with 5% CO₂ and the medium was replaced every other day.
Samples were fixed in Histo-Choice at day 2, 4, 5, 6, 10, 15, 25, and 30, embedded in
wax, and sectioned onto slides. Sections were stained with Masson’s Trichrome for
collagen density and probed with a monoclonal antibody specific for sheep procollagen
(SP1.D8, Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City,
IA). Immunohistochemistry using the monoclonal SP1.D8 was performed as
previously described with the exception that the antigen was first unmasked using 4M
NaCl at 45°C for 40min followed by 0.01M citrate for 7min in a Sunbeam rice steamer
(Sunbeam Products Inc., Delray Beach, FL).

**Inhibition of Collagen Synthesis**

Pericardial tissue sections were incubated as free-floating sections in
DMEM/20% FBS containing inhibitors of protein synthesis and function.
Halofuginone (0.1μg/ml), Cytochalasin B (1μg/ml), Cytochalasin D (1μg/ml), Aphidicolin (1μg/ml), AraC (1μg/ml), Nocozadole (0.0078μg/ml), and Cycloheximide (1μg/ml) were made fresh and replaced every 48hrs. Samples were harvested at day 5, 10, 20, and 30, fixed in Histo-Choice, and embedded. Embedded samples were sectioned onto slides and stained with Masson’s Trichrome stain and probed with SP1.D8 as previously described.

Results

Tissue Contraction

Pericardial tissue contraction occurred in a dose-dependent manner to the percentage of serum with a peak at 20% (Figure 4). All tissues incubated with 20% FBS contracted by day 6 while tissues maintained with 10% and 50% FBS had just begun to curl. In contrast, tissues incubated with 0.5% FBS did not contract.

Figure 5 shows a comparison of pericardial disks before and after tissue contraction. Experimental pericardial disks incubated with DMEM/20% FBS showed an average reduction in 2 dimensional surface area of 71% when control tissues were normalized to 100% (Figure 6).

Inhibition of Tissue Contraction

Serum-stimulated tissue contraction was evaluated in the presence of inhibitors of DNA synthesis, protein synthesis, and protein function. Tissue contraction was evaluated in the presence of Mitomycin C, Actinomycin D, Aphidicolin, AraC, Cytochalasin B, Cytochalasin D, Colchicine, Nocozadole, Cycloheximide, and Halofuginone. Figure 7 compares pericardial tissue contraction in the presence of
Figure 4. Dose-dependent tissue contraction in response to serum. Pericardial tissues (1 cm², n=4) were incubated with DMEM containing 0.5, 5, 10, 20, or 50% FBS for 7 days at 37°C, 5% CO₂. The tissues contracted in a dose-dependent manner in response to serum with a peak at 20% FBS. Tissues incubated with DMEM containing 0.5% FBS did not contract.
Tissue Contraction with Increasing Percentages of Serum

0.5% FBS 5% FBS 10% FBS 20% FBS 50% FBS

Day 7
**Figure 5.** Contraction of vital pericardial disks. Tissue disks (1 cm in diameter) were generated using a standard punch. Contracted disks were incubated with DMEM/20% FBS for 0 and 7 days at 37°C, 5% CO₂. Disks incubated for 0 days did not contract.
Figure 6. Degree of tissue contraction. Pericardial tissue disks (1cm in diameter, n=8) were generated using a standardized punch and incubated in DMEM/20% FBS for 7 days at 37°C, 5% CO₂ until tissue contraction was observed. A reduction in the 2 dimensional surface area of contracted tissues was determined by tracing the tissue perimeter onto transparency film and weighing cut-outs of the tracings. Weighed tracings were compared between tissues incubated for 0 days (control, n=4) and those incubated for 7 days (n=4). The degree of contraction was thus defined as the average decrease in 2 dimensional surface area of contracted tissues with the control tissue disks set at 100%. Tissue contraction stimulated by serum resulted in a 71% decrease in surface area. Error bars indicate the range of replicate samples.
Figure 7. Tissue contraction in the presence of inhibitors. Pericardial tissue sections (1cm² n=4) were incubated for 14 days at 37°C, 5% CO₂ with DMEM/20% FBS (1ml) in the presence of inhibitors. Inhibitors included, Mitomycin C (1μg/ml); an inhibitor of nucleic acid synthesis, Cytochalasin B (1μg/ml); an inhibitor of f-actin polymerization, Cycloheximide (1μg/ml); a protein synthesis inhibitor, Aphidicolin (1μg/ml); an inhibitor of DNA synthesis, and Colchicine (0.0078μg/ml); an inhibitor of tubulin polymerization. Mitomycin C, Cycloheximide, and Colchicine effectively prevented serum stimulated tissue contraction whereas Cytochalasin B and Aphidicolin did not. Colchicine was later found to be cytotoxic.
Tissue Contraction with Inhibitors

- DMEM 20% FBS
- Mitomycin C
- Cytoskeleton B
- Cycloheximide
- Aphidicolin

Day 14

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Mitomycin C, Cytochalasin B, Cycloheximide, Aphidicolin, and Colchicine for 14 days. Mitomycin C, Cycloheximide, and Colchicine effectively inhibited serum-induced tissue contraction whereas Cytochalasin B and Aphidicolin delayed, but did not prevent tissue contraction. In addition, Actinomycin D but not Cytochalasin D, AraC, and Nocozadole that have analogous functions to Cytochalasin B, Aphidicolin, and Colchicine respectively, prevented tissue contraction. Furthermore, Halofuginone, a Type I collagen synthesis inhibitor prevented tissue contraction out to 30 days (Figure 8). Actinomycin D and Colchicine were shown to be toxic causing cell rounding and loss of adhesion of fibroblast monolayers. These inhibitors were removed from the rest of the study. Control tissues, incubated with DMEM/20% FBS without inhibitors, were contracted by day 7.

**Characterization of Serum Stimulation**

Figure 9 shows pericardial tissue sections incubated with DMEM/20% FBS and those incubated in molecular weight fractions ≥100kDa, 30-99kDa, and 5-29kDa for 14 days. Tissue sections incubated with DMEM/20% FBS and those incubated with DMEM containing the serum fraction ≥100kDa were completely contracted by day 7. At this time, tissues incubated with DMEM containing the serum fraction 30-99kDa had just began to contract, evidenced by curling of the edges of the tissues toward the center. These tissues reached maximum contraction by day 10. Tissues incubated with DMEM containing the serum fraction 5-29kDa began contracting by day 10. By day 14, 50% of the tissues incubated with this serum fraction had contracted. Serum fractions under 5kDa did not induce tissue contraction (not shown).
Figure 8. Tissue contraction in the presence of Halofuginone. Pericardial tissue sections (1cm², n=8) were incubated for 30 days at 37°C, 5% CO₂ with DMEM/20% FBS (1ml) in the presence of the Type I collagen synthesis inhibitor, Halofuginone. Halofuginone (100ng/ml) effectively prevented serum-stimulated tissue contraction out to 30 days. Control tissues incubated with DMEM/20% FBS only, contracted within 6 days.
Tissue Contraction in the Presence of Halofuginone

DMEM
20% FBS

50 ng/ml

100 ng/ml

Day 30

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Figure 9. Tissue contraction in the presence of fractionated serum. Fetal bovine serum was fractionated into molecular weight ranges of \( \geq 100\text{kDa} \), 30-99kDa, and 5-29kDa and added separately to DMEM at 20% total volume. Pericardial tissue sections (1cm\(^2\), n=4) were incubated with 1ml DMEM/20% FBS or DMEM containing different serum fractions for 14 days at 37°C, 5% CO\(_2\). Tissue contraction was observed with all serum fractions above 5kDa. Fractions of serum under 5kDa did not induce tissue contraction (not shown).
Tissue Contraction and Fractionated Serum

DMEM 20% FBS

≥ 100kDa

30-99kDa

5-29kDa

Day 14

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Figure 10. Tissue contraction in the presence of treated sera. Fetal bovine serum was proteinase K-treated or heat-treated at 56°C and 70°C and added separately to DMEM at 20% total volume. Pericardial tissues (n=4) were incubated with 1ml DMEM/20% FBS, or DMEM containing proteinase K-treated or heat-treated sera for 14 days at 37°C, 5% CO₂. Tissue contraction was observed with all treated sera, but was significantly delayed with proteinase K-treated serum.
Tissue Contraction and Treated Serum

DMEM 20% FBS
Heat-Treated 56C
Heat-Treated 70C
Proteinase K-Treated

Day 14

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The stimulatory component contained within serum was evaluated in terms of heat and protease stability. Figure 10 shows pericardial tissue sections incubated with DMEM/20% FBS, serum heated to 56°C and 70°C, and serum treated with proteinase K for 14 days. Control tissues incubated in DMEM/20% FBS whole serum and tissue incubated in DMEM containing heat-treated sera contracted by day 7. In contrast, pericardial tissues incubated with proteinase K-treated serum began contracting at day 9 but were not completely contracted by termination of the experiment.

**Protein synthesis**

Pericardial tissue sections were evaluated for protein synthesis during the process of tissue contraction. SDS-PAGE protein profiles of tissue sections incubated with DMEM/20% FBS were compared to sections incubated in the presence of Cycloheximide. Figure 11 is a Coomassie Brilliant blue stained 12.5% polyacrylamide gel showing a protein band of approximately 60kDa in day 10 samples that was absent in day 2 samples and absent in samples incubated in the presence of Cycloheximide.

**Stimulation of Proliferation**

Fetal bovine serum stimulated the proliferation of cells within pericardial tissue sections. This stimulation was not the direct result of mechanical stimulation from handling since the tissue serum deprived for 48hr prior to use. Serum stimulation resulted in the cellular production of PCNA and was visualized by DAB development resulting in a brown precipitate (Figure 2). In a preliminary experiment maximal proliferation occurred at day 4 in all samples, suggesting an early proliferative
response to serum stimulation. Due to data acquired from this preliminary study, samples in this experiment were harvested at day 4.

The percentage of cells producing PCNA increased in a dose-dependent manner relative to the percentage of FBS present in the medium (Figure 12). Percentage of cells staining for PCNA increased from 19% to 61% when the FBS was increased from 0.5% to 50%. Changes in glucose concentration of the medium did not alter cellular production of PCNA since glucose free RPMI 1640 medium showed similar results as DMEM medium containing the same concentration of FBS.

Inhibition of Proliferation

The ability of chemical inhibitors to prevent proliferation was evaluated on isolated pericardial fibroblast monolayers. Figure 13 shows the recorded optical densities relating to the production of formazan through fibroblast metabolism of MTS and relates to cell numbers. Figure 13 shows formazan production by fibroblasts incubated with DMEM/20% FBS in the presence of Mitomycin C, Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, Cycloheximide, Nocozadole and Halofuginone. Formazan production in the presence of inhibitors was compared to formazan production by fibroblasts incubated with DMEM containing 0.5% and 20% FBS. A decrease in MTS metabolism was observed in the presence of all inhibitors when compared to cells incubated with DMEM/20% FBS only (red arrow). In fact, fibroblasts incubated with DMEM containing 0.5% FBS (OD=1.8) showed greater MTS metabolism than those incubated with DMEM/20% FBS in the presence of all concentrations of Mitomycin C, Aphidicolin, AraC, and Cycloheximide.

Cycloheximide, a protein synthesis inhibitor, showed the greatest inhibition of MTS
Figure 11. Separation of pericardial tissues proteins in a 12.5% SDS-PAGE gel stained with Coomassie Brilliant blue. Tissues (n=3) were incubated with DMEM/20% FBS for 2 and 10 days and DMEM/20% FBS containing 1μg/ml Cycloheximide for 12 days (C12) at 37°C, 5% CO₂. Tissues were diced and boiled in SDS-PAGE solubilizing solution. Molecular weight markers (MW) were acquired from BioRad. A protein band of approximately 60kDa (red arrow) that appeared during the process of tissue contraction is shown at day 10. This protein band was absent at day 2 and absent in samples treated with Cycloheximide.
Figure 12. Percent cellular production of proliferating cell nuclear antigen (PCNA).

Cells staining for PCNA increased in a dose-dependent manner relative to increasing serum concentration in DMEM. Pericardial tissues (n=4) were incubated with 1ml DMEM containing 0.5, 5, 10, 20, or 50% serum for 4 days at 37°C, 5% CO₂. Tissues were embedded in wax and assayed immunohistologically for the production of PCNA. Error bars indicate ranges from replicate tissue samples counting 500 cells per replicate in random fields. Percent cellular production of PCNA was calculated as follows: \[
\left( \frac{\text{number of cells staining for PCNA}}{\text{total number of cells counted}} \right) \times 100
\]
Figure 13. MTS metabolism in the presence of inhibitors. Pericardial fibroblast metabolism of MTS into the colored end-product formazan was assayed in the presence of inhibitors. Fibroblast monolayers (5000 cells/well, n=4) were incubated for 4 days at 37°C, 5% CO₂ with 1μg/ml of Mitomycin C, Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, Cycloheximide, 0.0078μg/ml Nocozadole, or 0.1μg/ml Halofuginone in DMEM/20% FBS and evaluated for the production of formazan using Celltiter 96 proliferation kit (Promega). Formazan production is directly related to the number of living cells and was evaluated by absorption at 490nm. Mitomycin C, Aphidicolin, AraC, Cycloheximide, and Halofuginone greatly reduced MTS metabolism by cultured fibroblasts when compared to control samples (red bar), while Cytochalasin B, Cytochalasin D, and Nocozadole did not. Error bars indicate the range of data from replicate samples.
MTS Metabolism

Absorption (490nm)

- 1µg/ml
- 0.0078µg/ml
- 0.1µg/ml

DMEM/20%
Mitomycin C
Cytochalasin B
Cytochalasin D
Aphidicolin
AraC
Cycloheximide
Nocozadole
Halofuginone
metabolism. Chemical toxicity indicated by cell rounding and loss of adherence was not observed.

Cellular proliferation was also evaluated by cellular PCNA production in the presence of chemical inhibitors of DNA synthesis, protein synthesis, and protein function. Figure 14 shows percentages of cells staining positive for PCNA in tissues incubated with DMEM/20% FBS containing Cycloheximide, Mitomycin C, Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, Nocozadole, and Halofuginone. Tissues incubated with DMEM/20% FBS averaged approximately 45% cellular PCNA production. Percent cells staining positive for PCNA was significantly reduced in the presence of all inhibitors used except Cycloheximide.

**Collagen Synthesis**

Serum stimulated the synthesis of Type I collagen by cells in situ to cultured pericardium. Figure 15 shows tissue incubated with DMEM/20% FBS for 0 days (A) and 30 days (B) and stained with Masson’s Trichrome. Masson’s Trichrome, a collagen density stain, revealed the deposition of new collagen, which stains light blue (yellow arrows), in the folds of contracted tissues. In some samples, new collagen deposition was also observed on the surface of contracted tissues. In comparison, tissues harvested at day 0 stained only dark blue indicating no new collagen deposition.

Pericardial tissues were also evaluated for the synthesis of Type I procollagen. Tissues harvested during the process of contraction were evaluated by immunohistology using the sheep specific procollagen antibody SP1.D8. Figure 16 shows cellular production of procollagen (yellow arrows) as early as day 2 after
Figure 14. Chemical inhibition of cellular PCNA production. Cellular proliferation, evaluated by the production of proliferating cell nuclear antigen (PCNA), was investigated in the presence of inhibitors. Pericardial tissues (n=4) were incubated for 4 days at 37°C, 5% CO₂ with 1ml DMEM/20% FBS containing 1μg/ml Mitomycin C. Cycloheximide, Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, 0.0078μg/ml Nocozadole, or 0.1μg/ml Halofuginone. Tissues were embedded in wax, sectioned, and assayed by immunohistochemical techniques for the production of PCNA. Percentages of cells staining positive for PCNA was determined by the following formula:

\[(\text{number of cells positive for PCNA} / \text{total number of cells}) \times 100\]

Error bars indicate standard deviation from replicate tissue samples counting 500 cells per replicate in random fields.
PCNA Synthesis in the Presence of Inhibitors

Chemical Inhibitors

- DMEM
- Cycloheximide
- Mitomycin C
- Cytochalasin B
- Cytochalasin D
- Aphidicolin
- Ara C
- Nocodazole
- Halofuginone
Figure 15. Serum-stimulated collagen deposition. Pericardial tissues were incubated for 30 days at 37°C, 5% CO₂ with DMEM/20% FBS, embedded, sectioned, and stained with Masson’s Trichrome for collagen density. Control tissues, harvested at day 0 (A) showed no new collagen deposition. In contrast, tissues harvested at day 30 (B) showed new collagen deposition, which stained light blue (yellow arrows) in the folds of the original pericardium (dark blue). 400x
**Figure 16.** Immunohistological staining for procollagen. Pericardial tissue sections were incubated with DMEM/20% FBS for 0-10 days at 37°C, 5% CO₂ and evaluated for the cellular production of procollagen using the monoclonal antibody, SP1.D8, specific for sheep procollagen. Antibody recognition was visualized by DAB staining resulting in a brown precipitate (yellow arrows). Tissues were counterstained with Hematoxylin (blue) and Eosin (pink). Procollagen production was observed as early as day 2 after FBS stimulation. 400x
incubation with DMEM/20% FBS. At this time, cells staining positive for procollagen were randomly distributed throughout the tissue. By day 30, procollagen production (A) was concentrated within the folds of contracted tissues and correlated with new collagen deposition observed using Masson's Trichrome staining (B) (yellow arrows) (Figure 17). Tissues incubated with DMEM containing 0.5% FBS for 48hrs and ethanol-treated tissues did not show cellular procollagen production.

Inhibition of Collagen Synthesis

Figure 18 compares type I collagen deposition in tissues incubated with 100ng/ml Halofuginone (A) and DMEM/20% FBS (B) for 30 days. Masson's Trichrome staining for collagen density revealed the deposition of new collagen in control tissue incubated with DMEM/20% FBS. An accumulation of new collagen, which stains light blue (yellow arrow), was observed in the folds and on the outer surface of the original pericardium (dark blue). In contrast, tissues incubated in DMEM/20% FBS containing Halofuginone stained dark blue only indicating that there was no new collagen deposition.

In addition, Halofuginone and Cycloheximide, but not Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, and Nocoazadole prevented serum-stimulated cellular procollagen synthesis. Figure 19 shows cellular procollagen production in pericardial tissues incubated with DMEM/20% FBS (A) and Halofuginone (B). Control tissues incubated with DMEM/20% FBS showed procollagen production concentrated in the folds of the contracted tissues (yellow arrows). In contrast, procollagen synthesis was absent in tissues incubated in the presence of Halofuginone.
Figure 17. Comparison of procollagen synthesis and collagen deposition. Pericardial tissues were incubated for 30 days at 37°C, 5% CO₂ with DMEM/20%, embedded, sectioned, and evaluated for the production of collagen by immunohistological and histological techniques. Cellular synthesis of procollagen, evaluated using the monoclonal antibody, SP1.D8, specific for sheep procollagen, was observed in the folds of contracted tissues. Procollagen synthesis (A) correlated with new collagen deposition observed by Masson's Trichrome staining for collagen density (B) (yellow arrows). Masson's Trichrome stains new collagen light blue and is indicated by the yellow arrow. Procollagen production was visualized by DAB development resulting in a brown precipitate. 400x
**Figure 18.** Inhibition of collagen deposition by Halofuginone. Pericardial tissues were incubated for 20 days at 37°C, 5% CO₂ with DMEM/20% FBS containing 100 ng/ml Halofuginone. Tissues were embedded, sectioned, and stained with Masson’s Trichrome for collagen density. Tissues incubated with Halofuginone (A) stained dark blue only indicating no new collagen production. Control tissues (B) showed the deposition of new collagen (light blue, yellow arrow) in the folds of the original collagen of the pericardium (dark blue). 400x
**Figure 19.** Inhibition of procollagen synthesis by Halofuginone. The cellular synthesis of procollagen was compared between tissues incubated with and without Halofuginone. Pericardial tissues were incubated for 15 days at 37°C, 5% CO₂ with DMEM/20% FBS containing 100ng/ml Halofuginone. Procollagen synthesis, detected immunohistologically using the sheep specific monoclonal antibody, SP1.D8 was observed in control tissues (A) indicated by the yellow arrows. In contrast, tissues incubated in the presence of Halofuginone (B) did not show cellular procollagen production. Procollagen production was visualized by DAB development resulting in a brown precipitate. Tissues were counterstained with H&E. 400x
Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, and Nocozadole, shown previously to reduce serum-stimulated PCNA synthesis, did not prevent serum-stimulated procollagen production. Figure 20 shows procollagen production in tissues incubated with DMEM/20% FBS containing Cytochalasin D.

**Conclusions**

Tissue retraction, cellular accumulation, and matrix deposition are the three healing outcomes associated with the vital fibrotic flap implants *in vivo*. Histological and immunohistological analysis of these implants suggested that activation of the cells *in situ* to the implants contributed to the detrimental healing. However, this *in vivo* study was limited in the ability to identify the contribution of specific cardiovascular factors in relation to the observed fibrosis. For this reason, an *in vitro* model of tissue contraction was developed using living pericardium. In this model, serum induced tissue contraction, cellular synthesis of PCNA, and new collagen deposition in cultured pericardial tissue.

Serum stimulated cells *in situ* to cultured pericardium to contract the tissue, evidenced by tissue curling. Dose-dependency experiments indicated that serum-induced tissue contraction was related to serum concentration. DMEM containing 20% FBS proved to be the optimal concentration of serum to induce tissue contraction in the shortest time frame. It is worth noting that tissues stimulated with 2% serum will eventually contract (approximately 14-20 days). This observation would suggest a threshold level of stimulation must be reached for the reaction to occur. Most tissues stimulated with 20% FBS contracted between 6 and 8 days. However, variability was observed in the length of time required for tissues to contract. This variability was not
Figure 20. Procollagen synthesis in the presence of Cytochalasin D. Pericardial tissues were incubated for 4 days at 37°C, 5% CO₂ with DMEM/20% FBS containing 1μg/ml Cytochalasin D and evaluated for the synthesis of procollagen using the sheep specific monoclonal antibody, SP1.D8. Cytochalasin D, which inhibited cellular proliferation, did not prevent procollagen synthesis (yellow arrows). Procollagen production was visualized by DAB development resulting in a brown precipitate. Tissues were counterstained with H&E. 400x
investigated but may reflect intrinsic differences in pericardial tissue. In preliminary experiments, differences were observed in tissue thickness and cellular density between pericardium from different sheep as well as pericardial tissue sections from the same sheep. These differences may be related to the ability of cultured pericardium to respond to serum stimulation.

Serum-stimulated tissue contraction was effectively prevented by inhibitors of protein synthesis. Mitomycin C, an inhibitor of nucleic acid synthesis; Cycloheximide, an inhibitor of protein synthesis; and Halofuginone, an inhibitor of Type I collagen synthesis, prevented serum-stimulated tissue contraction. The ability of Cycloheximide to prevent tissue contraction indicated that the contraction process was dependent upon de novo protein synthesis, an observation reported in the inhibition of FPGC contraction [68]. Whereas Mitomycin C, which prevents all nucleic acid synthesis, also prevents protein synthesis, the ability of Halofuginone to inhibit tissue contraction suggested that Type I collagen production was related to tissue contraction.

Serum stimulated cells in situ to cultured pericardium to proliferate. Cellular proliferation was evaluated by PCNA synthesis. Cellular production of PCNA was variable in harvested pericardium from different sheep. For this reason, pericardium was serum starved for 48hrs prior to experimentation. Serum starvation was used to induce cells within the cultured pericardium into a quiescent state allowing for uniform stimulation between pericardial sections.

In a preliminary experiment, cellular PCNA synthesis (approximately 3%) was observed in pericardial samples incubated with serum-free DMEM for 48hr indicating a low stimulatory capacity of the medium. Furthermore, tissue sections stimulated

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with 2% FBS in DMEM, showed PCNA production of approximately 4% after 4 days of incubation. In this study, tissues incubated with 0.5% FBS showed 19% cellular synthesis of PCNA. The reason for this discrepancy may be the result of procedural differences. In this experiment, the mesothelium was removed prior to serum stimulation, whereas in the preliminary experiment, the mesothelium was never removed. Mesothelial cells are a type of endothelial cell. Endothelial cells are known to control the passage of nutrients to cells within the subendothelium as well as the activity of those cells [87]. It is therefore reasonable to consider that a lower serum concentration may result in a higher percent PCNA synthesis in pericardial samples where the mesothelium was removed. In fact, percent PCNA production was greater in all pericardial samples during this experiment than during the preliminary experiment even when the same volumes of FBS were used in both.

In this model, percent cellular PCNA synthesis increased in a dose-dependent manner relative to the concentration of FBS. Concentrations of FBS greater than 50% were not evaluated due to the limited buffering capacity of the medium and nutritional requirements of the tissue. In addition, when glucose-free RPMI was used in replacement of DMEM, no differences were observed in percent cellular PCNA synthesis within tissues incubated in 50% FBS. This observation suggested that serum-stimulated PCNA synthesis was not completely dependent on energy production.

Inhibition of serum-stimulated cellular proliferation was evaluated by MTS metabolism on fibroblast monolayers and by cellular PCNA production in cultured pericardium. Formazan production from MTS metabolism was greatly reduced in the
presence of all inhibitors. In fact, treatment with Mitomycin C, AraC, and Cycloheximide resulted in less formazan production than cells incubated with 0.5% FBS. The protein synthesis inhibitor Cycloheximide, shown to prevent tissue contraction, showed the greatest inhibition of MTS metabolism, whereas Cytochalasin B, an inhibitor of cytoskeletal actin polymerization, showed the least inhibition of MTS metabolism.

Serum-stimulated PCNA synthesis was significantly reduced in the presence of Mitomycin C, Cytochalasin B and D, Aphidicolin, AraC, and Halofuginone. All these inhibitors showed a 30% or greater reduction in cellular PCNA synthesis when compared to control samples. Although Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, and Nocozadole reduced serum-stimulated PCNA production, these inhibitors did not prevent serum-stimulated tissue contraction. In contrast, tissue treatment with Cycloheximide, which prevented tissue contraction, did not reduce serum-stimulated PCNA synthesis.

Serum stimulated cells within cultured pericardium to synthesize and deposit new collagen. Masson’s Trichrome staining revealed collagen deposition in the folds and on the surface of pericardial tissue stimulated with 20% FBS for 30 days. Parallel control samples, fixed at day 0, showed no new collagen staining by Trichrome. Furthermore, immunohistological staining showed cellular procollagen production in cultured pericardial tissues as early as day 2 after serum stimulation, indicating an early response to FBS. Tissue samples analyzed after tissue contraction showed procollagen production was concentrated in the folds of the tissue which correlated with Masson’s Trichrome staining.
Serum-stimulated collagen synthesis was evaluated in the presence of Halofuginone, Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, Nocozadole, and Cycloheximide. Cultured pericardial tissues, which contracted in the presence of Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, and Nocozadole, showed cellular production of procollagen. In contrast, pericardial tissues stimulated with 20% FBS in the presence of Halofuginone and Cycloheximide did not contract and the cells in situ to the tissue did not synthesize procollagen. These data suggested that serum-stimulated tissue contraction was related to the synthesis of Type I collagen.

III. Relationship Between Tissue Contraction, Cellular Proliferation, and Collagen Deposition

The fibrotic reactions of the in vivo autologous pericardial flap implant were characterized by tissue contraction, cellular accumulation, and new matrix deposition. An in vitro model of tissue contraction was developed to study the fibrotic reactions of the in vivo flap in a controlled environment. In this model, cultured pericardium contracted, and cells within the tissue expressed the proliferative marker, PCNA, and synthesized Type I collagen. These reactions were analogous to the healing response of the in vivo pericardial flap implant and were mediated by the activation of in situ cells. Furthermore, cellular activation was in response to serum stimulation. In this chapter, we explore the relationship between serum-stimulated tissue contraction, proliferation, and collagen synthesis. Time dependency experiments and chemical inhibitors were used to evaluate the relationship between tissue contraction and proliferation, collagen synthesis and proliferation, and tissue contraction and collagen synthesis.
The interdependency of serum-stimulated tissue contraction and proliferation were also evaluated using chemical inhibitors. As stated previously, all inhibitors reduced MTS metabolism in comparison to the same number of cells stimulated with 20% FBS (Figure 13). In addition, Mitomycin C, Cycloheximide, (Figure 7) and Halofuginone (Figure 8) effectively prevented serum-stimulated tissue contraction. Figure 21 compares formazan production by cells incubated with DMEM/20% FBS with and without inhibitors shown to prevent tissue contraction. In this experiment, Mitomycin C, Cycloheximide, and Halofuginone greatly reduced MTS metabolism to formazan. In contrast, Cycloheximide, which reduced formazan production, did not prevent serum-stimulated PCNA synthesis (Figure 22). Furthermore, concentrations of Cycloheximide (0.25μg/ml) that did not prevent tissue contraction still showed a significant reduction in MTS metabolism (not shown). Although the data relating to Cycloheximide appears contradictory, the question remains as to which assay more accurately expresses prevention of cellular proliferation. Whereas PCNA is an accessory protein to the DNA polymerase and therefore required for cellular proliferation, changes in formazan production by MTS metabolism directly represent cell numbers. However, data acquired during this study and by other researchers suggested that MTS metabolism may relate as much to the metabolism of cultured cells as it does to the number of cells [88]. Preliminary experimentation in this laboratory investigated MTS metabolism by fibroblast stimulated with increasing concentrations of serum. In this experiment, increases in optical density reflecting
Figure 21. MTS metabolism in the presence of inhibitors that prevented tissue contraction. Pericardial fibroblasts (5000 cells/well, n=4) were incubated for 4 days at 37°C, 5% CO₂ with DMEM/20% FBS containing Mitomycin C, Cycloheximide, or Halofuginone in concentrations that prevented tissue contraction. All inhibitors reduced formazan production when compared to control samples. Formazan production was evaluated by optical density recorded at 490nm. Error bars indicate the range of data from replicate samples.
MTS Metabolism with Inhibitors that Prevent Tissue Contraction

Absorption (490nm)

- DMEM, 20%
- Mitomycin C
- Cycloheximide
- Nocozadole
- Halofuginone

- 1ug/ml
- 0.0078ug/ml
- 0.1ug/ml
Figure 22. Percent cellular PCNA synthesis within cultured pericardium in the presence of the protein synthesis inhibitor, Cycloheximide. Pericardial tissues (n=4) were incubated with DMEM/20% FBS with 1μg/ml Cycloheximide for 4 days at 37°C, 5% CO₂. The cellular synthesis of PCNA was detected immunohistologically and visualized by DAB development resulting in a brown precipitate. Error bars indicate standard deviation from replicate tissue samples counting 500 cells per replicate in random fields.
PCNA Synthesis in the Presence of Cycloheximide

DMEM

Cycloheximide (1ug/ml)
formazan production, were observed with increased serum concentration in fibroblast monolayers seeded with the same numbers of cells. Furthermore, Cytochalasin B, which effectively prevented PCNA production (Figure 14), did not greatly reduce MTS metabolism (Figure 13). It is therefore not unlikely that certain inhibitors that prevent PCNA production do not greatly effect the metabolism of cultured cells and in concert, inhibitors that reduced MTS metabolism may not effect cellular PCNA synthesis. Taken together, these data indicate that serum-stimulated tissue contraction and serum-stimulated cellular proliferation are independent events.

**Collagen Synthesis and Cellular Proliferation**

The interdependency of serum-stimulated collagen synthesis and serum-stimulated cellular proliferation were investigated by experiments involving the chemical inhibition of collagen synthesis and cellular proliferation. As shown earlier, Halofuginone, an inhibitor of Type I collagen synthesis, prevented serum-stimulated cellular procollagen production in tissues incubated with DMEM/20% FBS (Figure 19). Figure 23 shows a dose-dependent reduction in MTS metabolism by fibroblast monolayers in the presence of Halofuginone. This reduction was not due to cell death since cell rounding and loss of adhesion were not observed. Figure 24 shows a reduction in serum-stimulated cellular PCNA production in tissue incubated in 100ng/ml Halofuginone when compared to tissue stimulated with DMEM/20% FBS. Although these data suggest serum-stimulated collagen synthesis and serum-stimulated cellular proliferation are linked events the mechanism by which Halofuginone prevents cellular proliferation is not known [89]. In contrast, the chemical inhibitors Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, and Nocozadole, shown
Figure 23. MTS metabolism in the presence of the collagen synthesis inhibitor, Halofuginone. Fibroblasts (5000 cells/well, n=4) were incubated for 4 days at 37°C, 5% CO$_2$ with DMEM/20% FBS containing serial dilutions (0.25µg/ml-0.0078µg/ml) of Halofuginone and assayed for the production of formazan by absorption at 490nm. Halofuginone (blue) reduced formazan production in a dose-dependent manner. Controls included fibroblasts incubated with DMEM/20% FBS (gold). Error bars indicate range of data from replicate samples.
MTS Metabolism in the Presence of Halofuginone

Absorption (490nm) vs. Halofuginone Concentration (µg/ml)

Concentrations: 0.25, 0.125, 0.0625, 0.0313, 0.0156, 0.00781, DMEM/20%
Figure 24. Percent of cells staining positive for PCNA synthesis were evaluated in the presence of Halofuginone. Tissues (n=4) were incubated with DMEM/20% FBS in the presence of 100ng/ml Halofuginone for 4 days at 37°C, 5% CO₂. The production of PCNA was detected immunohistologically and visualized by DAB development resulting in a brown precipitate. Error bars indicate standard deviation from replicate tissue samples counting 500 cells per replicate in random fields.
PCNA Synthesis in the Presence of Halofuginone
previously to reduce MTS metabolism by fibroblast monolayers (Figure 13) and to
inhibit serum-stimulated PCNA synthesis (Figure 14), did not prevent cellular
production of procollagen (Figure 20). Procollagen synthesis was also observed in
tissue treated with Cytochalasin B, Aphidicolin, and AraC. These data confirmed that
serum-stimulated collagen synthesis and serum-stimulated cellular proliferation were
mutually exclusive events.

**Tissue Contraction and Collagen Synthesis**

The interdependency of serum-stimulated tissue contraction and cellular
collagen synthesis were evaluated by time-related protein synthesis and by chemical
inhibition of protein synthesis. As stated previously, serum stimulated production of
Type I collagen by cells within cultured pericardium. In fact, procollagen
production by randomly distributed cells was observed as early as day 2 after serum
stimulation (Figure 16) and subsequently concentrated in the folds of contracted tissues
by day 30 (Figure 17). Taken together with the observation that inhibition of protein
synthesis by Cycloheximide prevented serum-stimulated tissue contraction (Figure 7),
these data suggested that collagen production was related to tissue contraction. In
support of this observation, Halofuginone, an inhibitor of Type I collagen synthesis,
effectively prevented serum-stimulated tissue contraction through the experimental
time frame of 30 days (Figure 8). In addition, collagen deposition was not seen in
tissues stained by Masson’s Trichrome (Figure 18) and cellular procollagen production
was not observed in tissues treated with Halofuginone (Figure 19). These observations
suggest that serum-stimulated tissue contraction and collagen deposition are
interdependent.
DISCUSSION

It is not known why fibrosis occurs in some clinical cases where vital autologous pericardium has been used as a repair biomaterial in the cardiovascular system. In most cases where fresh autologous pericardium was used as a patch, favorable outcomes were observed. Even in cases where the tissue became fibrotic, it maintained its structural integrity. However, in cases where vital autologous pericardium was used to reconstruct heart valve leaflets, tissue retraction and fibrosis were the most commonly observed healing outcomes. When heart valve leaflets are concerned, even minimal fibrosis can have detrimental effects necessitating reoperation and the implantation of a mechanical valve.

To better understand the differential healing responses of vital autologous pericardium, Cheung et al., 1999, implanted vital and ethanol-treated autologous pericardium as a patch, conduit, and intraluminal flap in the descending aorta of sheep [11]. Implants were recovered on day 5, 10, 15, and 30 and evaluated for cellular activation by histological and immunohistological techniques. Microscopically, at day 5, both vital and ethanol-treated implants looked similar with fibrin deposits overlaying the pericardial implants. However, at later time points, differential healing responses were observed between vital and ethanol-treated implants. In ethanol-treated implants, there was a cessation in fibrin deposition and an orderly repopulation of the pericardial tissues by cells from the aortic wall that expressed α-smooth muscle cell actin. In contrast, vital implants showed greater fibrin accumulation populated with randomly oriented cells, some of which also expressed α-actin. The origin of these cells was
unknown but may have come from the implant, the aortic wall, or the blood stream. In addition, all ethanol-treated implants were covered by a surface layer of cells expressing von Willebrand Factor (vWF), indicative of an endothelium. The vital autologous patch and conduit showed some thickening and fibrosis but also eventually regained a vWF-expressing surface layer of cells. However, the vital autologous flap did not. The vital autologous flap retracted, became fibrotic, and was characterized by a continual deposition of clot that did not cease by termination of the experiment (30 days). The reason for the differential healing outcomes between the vital patch and conduit and the vital flap are unknown but may reflect the configuration of the implanted tissue. Whereas the vital patch and conduit were sutured directly into the wall of the aorta with one surface exposed to the blood stream, the vital flap bisected the lumen of the aorta with both surfaces exposed to the flow of blood. In this configuration, the vital flap simulated a heart valve leaflet and showed healing characteristics similar to clinical cases where vital autologous pericardium was used to reconstruct or repair heart valves. Further analysis of vital and ethanol-treated pericardial flap implants showed different patterns in cellular activation that led to the detrimental healing response of the vital flap. These implants were evaluated by immunohistological techniques for the synthesis of proliferating cell nuclear antigen (PCNA) and procollagen. Like the previous in vivo study, the early time points of day 5 and 10 showed similar patterns of cellular activation between vital and ethanol-treated flap implants. Cellular production of PCNA and procollagen were observed in the fibrin layers covering the pericardial implant as well as at the junction of the implants and the aortic wall. In contrast, by day 15 fibrin deposition had ceased in the
ethanol-treated flaps that seemed to correspond with the establishment of the endothelium. At later time points, day 15 and 30, concentrations of cells staining positive for PCNA and procollagen shifted from the junction of the implant and aortic wall to the cells that had repopulated the pericardial tissue. In vital pericardial implants, the opposite was observed. Concentrations of cells, staining positive for PCNA and procollagen in these flaps shifted from cells within the implant to cells in the fibrin layers covering the implants. In other words, there was a gradual shift of cellular activation from outside to inside the implanted tissue in ethanol-treated flaps, and from inside to outside the implanted tissue in vital flaps. In ethanol-treated flap implants, this shift in cellular activation patterns resulted in a cessation of fibrin deposition, reestablishment of the endothelium, repopulation of the implanted tissue, and an integration of the implant into the surrounding tissue. In vital flap implants, this shift cellular activation patterns resulted in continual fibrin deposition, a build up of granulation tissue, cellular accumulation, and eventual loss of the functional integrity of the implant. By day 30, vital implants showed cells staining positive for PCNA and procollagen concentrated at the interface of granulation tissue and newly deposited fibrin clot, which indicated a continual cycle of tissue accumulation. In this aspect, incomplete healing of the vital autologous pericardial flap resembled a chronic wound.

The above in vivo studies accentuated the healing variability of vital autologous pericardium used in cardiovascular surgeries, but did not indicate the mechanisms by which these healing responses occurred. Questions still remain as to which cells became activated and what signal induced the activation. In terms of the vital

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autologous pericardial flaps, the activated cells within the fibrin layers may have originated from the implant, the aortic wall, or from the blood stream. Although *in vivo* models of fibrosis would best represent actual clinical situations, these studies do not allow for the isolation of variables responsible for the observed results.

Our approach was the development of an *in vitro* model of pericardial tissue fibrosis and contraction. The most common *in vitro* model of tissue contraction is fibroblast populated Type I collagen gels (FPCG). Although FPCGs provide useful information to relate tissue contraction and wound healing, these models may be too simplistic to adequately reflect most clinical wound healing situations. In fact, the FPCG model probably does not reflect cardiovascular wound healing of vital autologous pericardial implants. First, FPCGs are matrices composed of one type of tissue that is loose and unorganized. Secondly, FPCGs only contain one cell type. It is well known that wound healing involves the interaction of multiple cell types and that the extracellular matrix may regulate cell function, behavior, and morphology. Our *in vitro* model of tissue contraction was composed of living pericardial tissue with a complete extracellular matrix and the native cell population left intact. The only alteration to the tissue was the removal of the mesothelial layer. This tissue processing was analogous to our previous *in vivo* study where vital autologous pericardium was implanted as a flap that bisected the descending aorta of sheep. In this *in vivo* model, healing responses of the vital autologous flap resulted in retraction and fibrosis and was similar to clinical cases where vital autologous pericardium was used to reconstruct heart valve leaflets. Fibrosis of the vital autologous pericardial flap was characterized by tissue retraction, cellular accumulation, and matrix deposition. In
turn, pericardial tissue reactions observed in our \textit{in vitro} model were analogous to the observed healing responses of the \textit{in vivo} vital autologous pericardial flap. Living pericardium cultured in our \textit{in vitro} model, contracted, evidenced by tissue curling, and the cells within the tissue stained positive for the proliferative marker PCNA and deposited collagen. Therefore, our \textit{in vitro} model of tissue contraction is a valid model of \textit{in vivo} pericardial tissue healing and may reflect healing responses of vital autologous pericardium clinically used to repair or reconstruct heart valve leaflets.

The observation that cultured pericardial tissue in our \textit{in vitro} model showed the similar healing characteristics as the vital autologous \textit{in vivo} flap, not only validated our model, but also supported our primary hypothesis: cells \textit{in situ} to the pericardial implant contributed to the detrimental healing outcome of the vital autologous flap.

We attempted to characterize the stimulatory component(s) within serum capable of inducing tissue contraction in terms of molecular weight, heat stability, and protease stability. We established that the stimulatory component(s) of serum was $>5$kDa. The fact that the stimulatory activity was observed in all fractions above 5kDa suggested that stimulatory component(s) is either a small macromolecule, which can be aggregated, a large macromolecule that could be broken into active fractions, or a small molecule that was capable of binding to a carrier protein. Interestingly, the stimulatory component(s) was partially sensitive to proteinase K treatment suggesting at least part of the active component was a protein, but resistant to heat denaturation up to 70°C where most proteins undergo conformational change.
Using our *in vitro* model, we investigated serum-induced cellular activation and the relation of activated cells to cultured pericardial tissue contraction, cellular proliferation, and collagen deposition. Dose-dependency experiments and chemical inhibitors as well as histological and immunohistological techniques were used to evaluate cellular activation and subsequent pericardial tissue responses to stimulants found in the cardiovascular system.

In this *in vitro* model, cultured pericardium contracted when stimulated with FBS. Contraction of cultured pericardium, evidenced by tissue curling, was dose-dependent in response to FBS and was effective at 20% FBS. This serum-stimulated response was a function of cellular activation, since ethanol-treated and freeze-killed tissues did not contract. Furthermore, cultured pericardium stimulated with low concentration of FBS (0.5%) did not contract suggesting a threshold level of stimulation must be reached for the reaction to occur. Serum-stimulated tissue contraction was evaluated in the presence of Mitomycin C, Actinomycin D, Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, Cycloheximide, Colchicine, Nocodazole, Prostaglandin E2, and Halofuginone. Of these compounds, Mitomycin C, Actinomycin D, Cycloheximide, Colchicine, and Halofuginone effectively prevented tissue contraction. Actinomycin D and Colchicine were later shown to be cytotoxic, again indicating that living cells were required for contraction to occur. Mitomycin C inhibits nucleic acid synthesis, which suggested that tissue contraction required cellular activation relating to DNA synthesis and/or gene expression. The ability of Cycloheximide to prevent serum-stimulated tissue contraction indicated the requirement *de novo* protein synthesis in the contraction process. This observation was
consistent with FPCG studies. However, in FPCGs, the de novo synthesized protein was shown to be f-actin, the actin isoform of the cytoskeleton. This observation was supported by inhibitor studies where treatment with Cytochalasin prevented FPCG contraction. In our in vitro model, neither Cytochalasin B nor Cytochalasin D prevented serum-stimulated pericardial tissue contraction, indicating that the mechanism of tissue contraction did not require f-actin polymerization. Instead, in our study, tissue contraction was prevented with the Type I collagen synthesis inhibitor Halofuginone. Therefore, one mechanism of serum-stimulated tissue contraction required the deposition of de novo synthesized Type I collagen.

Serum induced proliferation by cells within cultured pericardial tissues. Immunohistological detection of proliferating cell nuclear antigen (PCNA) indicated that cellular proliferation was directly proportional to the concentration of serum. Serum-stimulated cellular proliferation was not dependent upon glucose concentration since tissues maintained in glucose-free media showed similar levels of PCNA staining. This observation suggested that cellular proliferation was not completely energy dependent.

Fibroblast metabolism of MTS and cellular production of PCNA were used to evaluate serum-stimulated cellular proliferation in the presence of chemical inhibitors. MTS is a tetrazolium salt that is metabolized in the mitochondria to the colored end-product formazan, which is released into the medium. Formazan production, analyzed by optical density, is then related to cell numbers. Fibroblasts isolated from pericardial tissue were stimulated with 20% FBS with and without the presence of Mitomycin C, Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, Cycloheximide, Nocozadole, and...
Halofuginone, Mitomycin C, Cytochalasin D, Aphidicolin, AraC, Cycloheximide, and Halofuginone greatly reduced MTS metabolism by fibroblast monolayers stimulated with 20% FBS. Cytochalasin B and Nocozadole did not. These data suggested that most inhibitors used in this study prevented cellular proliferation, however, formazan production may reflect cellular metabolism to a greater extent than cell density.

Preliminary studies involving MTS metabolism showed that formazan production by fibroblasts was dose-dependent upon serum concentration within consistent cell populations. Although the same concentration of serum (20%) was used in this experiment, it is possible reduction of MTS metabolism by the inhibitors may reflect reduction in cellular metabolism and not cell density. This theory is supported by differences in fibroblast metabolism of MTS in the presence of like inhibitors. For example, Cytochalasin B and Cytochalasin D both prevent the polymerization of f-actin and Cytochalasin B has an added function of blocking cellular uptake of glucose. However, Cytochalasin D showed a greater inhibition of formazan production than Cytochalasin B in the same concentration. These data suggest that Cytochalasin D may have an added effect on cellular metabolism of MTS.

Serum-stimulated cellular proliferation was evaluated by the cellular synthesis of PCNA in cultured pericardial tissues with and without the presence of inhibitors. All inhibitors reduced cellular staining for PCNA in cultured pericardium with the exception of Cycloheximide. These data suggests that PCNA is not synthesized de novo. In relation to Cycloheximide and cellular proliferation, data acquired from MTS metabolism are contradictory to cellular PCNA staining. The MTS experiment indicated that Cycloheximide adversely affected cellular proliferation whereas the
PCNA production data did not. It is possible that PCNA is not synthesized *de novo* whereas complete cellular division does require gene expression. If this was the case, then the data are accurate. However, it is also possible that Cycloheximide did not interfere with DNA replication, but had an added effect of altering MTS metabolism.

MTS metabolism and PCNA synthesis in the presence of inhibitors that prevented tissue contraction were used to evaluate the relation between serum-stimulated tissue contraction and cellular proliferation. As stated above, Mitomycin C, Cycloheximide, and Halofuginone effectively prevented serum-stimulated tissue contraction. Of these inhibitors, Cycloheximide did not alter serum-stimulated cellular staining for PCNA in cultured pericardial tissues suggesting that serum-stimulated tissue contraction and cellular proliferation are independent events.

Serum-stimulated collagen deposition by cells within cultured pericardium. Masson's Trichrome staining for collagen density showed collagen deposition in the folds of contracted pericardial tissues cultured in our *in vitro* model. In addition, immunohistological staining with the sheep specific procollagen antibody SP1.D8 showed cellular synthesis of procollagen as early as day 2. This indicated an early response of cells to serum stimulation. Histological analysis of tissues harvested at later time points colocalized Type I procollagen synthesis with Type I collagen deposition observed by Masson's Trichrome.

Serum-stimulated cellular collagen production was evaluated in the presence of chemical inhibitors. Tissues stimulated with 20% FBS in the presence of Cytochalasin B, Cytochalasin D, Aphidicolin, AraC, Nocozadole, and Halofuginone were analyzed for the cellular synthesis of procollagen. Halofuginone, which inhibited serum-
stimulated tissue contraction, effectively prevented serum-stimulated procollagen synthesis. Cytochalasin B, which reduced serum-stimulated PCNA synthesis, and Cytochalasin D, Aphidicolin, AraC, and Nocozadole, which reduced serum-stimulated PCNA synthesis and serum-stimulated MTS metabolism, did not prevent tissue contraction or the production of Type I procollagen. These data indicated that cellular collagen production was not related to cellular proliferation, but was related to serum-stimulated tissue contraction.

Finally, we identified the molecular mass of a newly synthesized protein that appeared during the process of serum-stimulated pericardial tissue contraction. SDS-PAGE protein profiles of incubated pericardial tissues showed the appearance of at least one newly synthesized protein during the process of tissue contraction. This protein of approximately 60kDa appeared at day 4 of incubation and was absent in earlier days and in Cycloheximide-treated samples. The identity of this protein has not yet been determined.

In summary, an in vitro model of fibrosis and tissue contraction was developed. Pericardial tissues cultured in this in vitro model simulated healing reactions observed when vital autologous pericardium was implanted as flap in the descending aorta of sheep when stimulated by FBS. Serum stimulated cultured pericardium to contract, and the cells in situ to synthesis PCNA and Type I collagen. Studies involving chemical inhibitors indicated that serum-stimulated tissue contraction was dependent upon collagen deposition. The mechanism of tissue contraction through the deposition of collagen is unknown, but may involve the formation of substrate adhesions. In
addition, serum-stimulated cellular proliferation was shown to be independent of both tissue contraction and collagen synthesis.

The data reported here indicated that fibrosis of the vital autologous pericardial flap occurred in response to the activation of cells in situ to the implant. These data also indicated that one mechanism of cellular activation was through exposure to blood serum, which resulted in cellular proliferation. Preliminary data involving the immunohistological detection of PCNA showed reduced cellular staining of PCNA when pericardial tissues were covered by mesothelial cells than when the mesothelium was completely removed. In this preliminary study, the mesothelium was restored in approximately 5 days after serum stimulation. In these tissues, PCNA staining was maximal at day 4 resulting in an average percentage of cells staining positive for PCNA of 12% within cultured pericardium stimulated with 10% FBS. In addition, the connective tissue cells synthesizing PCNA were located directly under the mesothelial layer. In fact, the further away from the surface, less PCNA production was observed. In comparison, when new techniques were employed to completely remove the mesothelium, average percentage of cells staining positive for PCNA was approximately 50% and these cells were randomly distributed throughout the tissue. These data suggested a regulation of connective tissue cell activation by the overlaying mesothelium. This was not surprising since mesothelial cells are a type of endothelial cell, and the endothelium is known to regulate the activity and function of cells within the subendothelium. Knowing the regulatory function of endothelial cells, a correlation may be proposed relating to healing responses of the vital and ethanol-treated pericardial flap implants. As stated previously, a shift in patterns of cellular

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activation and a cessation in fibrin accumulation coincided with the establishment of an endothelium on the ethanol-treated in vivo pericardial flap. In addition, a cessation in fibrin deposition on the vital autologous patch and conduit also seemed to coincide with establishment of an endothelium. In contrast, fibrin deposition was continuous and an endothelium was never established on the vital in vivo pericardial flap. Therefore, it is possible that the chronic wound healing responses observed in the vital fibrotic flap were a consequence of the absence of an endothelium. Of course, the opposite may also be true that the inability of the vital flap to establish an endothelium may be the result of a chronic wound-healing situation.

In addition to cellular proliferation, tissue contraction and collagen deposition were observed in the healing responses of the in vivo vital pericardial flap and in vitro cultured pericardial tissue. These healing responses in vitro were the result of serum stimulation. Therefore, it may be assumed that one mechanism of cellular activation in vivo that led to tissue contraction and collagen deposition was cellular stimulation by blood serum. Serum-stimulated pericardial tissue contraction could be observed by day 4 in vitro and was evidenced by tissue curling. In comparison, retraction of the vital in vivo pericardial flap was not observed until day 15. Data acquired in this in vitro study showed an early response (day 2) of cellular activation in terms of procollagen synthesis when the tissue was exposed to serum. Cellular synthesis of Type I procollagen was also observed in the early time points (day 5) by cells within the in vivo vital pericardial flap. Furthermore, chemical inhibition of collagen synthesis in vitro alleviated serum-stimulated cultured pericardial tissue contraction indicating that tissue contraction and collagen deposition are linked events.
Although this *in vitro* study indicated the mechanism of vital autologous pericardial implant fibrosis was related to activation of cells *in situ* to the pericardium, it did not reveal why these cells became activated. As stated above, cessation of fibrin accumulation on ethanol-treated implants and fibrotic reactions of the vital patch and conduit seemed to coincide with the establishment of an endothelium on the surface of these *in vivo* implants. In turn, cellular activation in relation to cellular PCNA staining was significantly lower in *in vitro* cultured pericardium where the mesothelial layer was intact compared to samples where the mesothelium was removed. This data suggested that not only was cellular activation related to serum-stimulation but cellular activation within pericardial implants may also be related to the removal of the mesothelial and subsequently the repressive factors secreted by this type of endothelial cell (see *Endothelial cells*).

In addition, pericardium is a fluid filled sack that surrounds the heart functioning as a cushion. It is compartmentalized in the body to the extent that pericardium is not exposed to blood factors or the mechanical forces generated by the flow of blood. Although steps were taken *in vitro* to counteract cellular activation due to mechanical stimulation, it is possible that transference of pericardium from an environment of low serum to an environment of high serum, aided in the activation of pericardial cells.

In conclusion, pericardium is an attractive biomaterial for cardiovascular repair surgeries. It is non-immunogenic, has excellent hemostatic qualities, and its intrinsic function is not needed for survival. Unfortunately, the clinical healing outcomes associated with the use of autologous pericardium are unpredictable and therefore limit
its usage. Depending upon its usage, autologous pericardium was shown to become thin and dilated, retracted and fibrotic, or incorporated into the surrounding tissue. Our previous in vivo study and this in vitro study indicated that activation of cells in situ to pericardial tissue contributed to the detrimental healing outcomes associated with some vital autologous pericardial implants. In this in vitro study, we developed a model of pericardial tissue contraction. Within this model, we investigated the stimulatory characteristics of serum, the mechanism by which serum-stimulated cellular activation resulted in tissue contraction, cellular proliferation, and collagen synthesis, and the relationships between these serum-induced phenomena. We demonstrated the ability of serum to stimulate the activation of cells in situ to cultured pericardium resulting in tissue contraction, cellular proliferation, and collagen synthesis and deposition. This stimulatory capacity of serum was resistant to moderate heat treatment but sensitive to proteinase K digestion. Furthermore, the stimulatory capacity of serum could not be classified into a molecular weight range suggesting more than one protein or an interaction of two or more proteins of different masses conveyed the stimulatory signal. In addition, serum-stimulated pericardial tissue contraction and cellular proliferation were dose-dependent in relation to serum concentration and immunohistological staining of cellular PCNA was not dependent upon glucose concentration. Finally, experiments involving chemical inhibition of DNA synthesis and protein synthesis indicated that cellular proliferation within cultured pericardium was independent of both tissue contraction and collagen synthesis. However, these experiments showed tissue contraction was dependent upon collagen deposition.
Overall, data acquired from this *in vitro* study and our previous *in vivo* study indicated that the microenvironment associated with cells and tissues is unique to those tissues and changes in tissue microenvironment may result in cellular activation. This seemingly simplistic statement has significant inferences on cardiovascular repair surgeries involving the use of autologous tissue. Foremost, it suggests that the source of the autologous tissue has dramatic effects on healing outcome and conversely, the healing outcome of autologous tissue transplants is influenced by the microenvironment in which the tissue is implanted. According to this *in vitro* study, cardiovascular surgeries involving the use of vital autologous pericardium as a repair biomaterial should include strategies to minimize cellular activation. These strategies may involve procedures designed to maintain the mesothelium, however, since the mesothelial may be removed by mere handling of the tissue, methods should be designed to aid in the establishment of an endothelium after pericardial tissue implantation. Future experiments involving the above mentioned strategies with studies relating to tissue treatments before implantation may alleviate the development of fibrosis observed with vital autologous pericardial transplants.
LITERATURE CITED


