The effects of cardiotonic steroids on dermal collagen synthesis and wound healing

Nasser El-Okdi

Medical University of Ohio

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation

http://utdr.utoledo.edu/theses-dissertations/1181

This Thesis is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository’s About page.
The Effects of Cardiotonic Steroids on Dermal Collagen Synthesis and Wound Healing

Submitted by:
Nasser El-Okdi

In partial fulfillment of the requirements for the degree of Master of Science in Biomedical Sciences

Examination Committee

Major Advisor: Joseph Shapiro, M.D.

Academic Advisory Committee: Sonia Najjar, Ph.D.
Zi-Jian Xie, Ph.D.
Deepak Malhotra, M.D., Ph.D.

Senior Associate Dean
College of Graduate Studies
Michael S. Bisesi, Ph.D.

Date of Defense: May 8, 2008
The Effects of Cardiotonic Steroids on
Dermal Collagen Synthesis and Wound Healing

By

Nasser El-Okdi

From

Departments of Medicine and Physiology/Pharmacology
University of Toledo College of Medicine
2008
DEDICATION

Totus tuus.
ACKNOWLEDGEMENTS

With extreme appreciation I would like to recognize the sacrifice, support, encouragement, counsel, and, most of all, love of my Mother and Father.

I am inclined to emphasize the devotion, concern, kindness, generosity, graciousness, wisdom, guidance, and profound support Dr. Joseph Shapiro has so eloquently provided me. I must also recognize with my deepest sincerity the insight, aid, support, astuteness, direction, and friendship supplied to me by Dr. Sonia Najjar. I would also like to sincerely thank Drs. Zi-Jian Xie, Jiang Liu, Larisa Fedorova, KV Chin, and Deepak Malhotra for their support and encouragement.

I am bound to acknowledge the support of my good friends and co-workers in and around the laboratory over the years: Dr. Sankaridrug Periyasamy, Vanamala Raju, Amjad Shidyak, Jihad Elkareh, Adnan Alsaka, Imad Hariri, Liang Wu, Haiping Cai, David Kennedy, Sleiman Smaili, Steven Haller, Sandeep Vetteth, Shalini Gupta, Carol Woods, Tomasa Guerrero; Other faculty and mentors who have devoted solicitous time and effort helping me to grow personally and professionally: Drs. Sandrine Pierre, Bashar Kahaleh, Eric Morgan, Deepak Malhotra, Sadik Khuder, Julie Westerink, Dorthea Sawicki, Merle Hienke, Kathy Goans, and Randall Worth.
<table>
<thead>
<tr>
<th>Title</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Literature</td>
<td>3</td>
</tr>
<tr>
<td>Manuscript 1: The Effects of Cardiotonic Steroids on Dermal Collagen Synthesis and Wound Healing</td>
<td>23</td>
</tr>
<tr>
<td>Summary</td>
<td>53</td>
</tr>
<tr>
<td>Conclusions</td>
<td>55</td>
</tr>
<tr>
<td>Abstract</td>
<td>56</td>
</tr>
<tr>
<td>References</td>
<td>57</td>
</tr>
</tbody>
</table>
INTRODUCTION

Chronic and fibrotic wounds significantly impair quality of life and prolong hospital stays, burdening both individuals and society with an estimated $25 billion in health costs. Understanding the mechanisms that accelerate wound healing, but prevent chronic wound formation and fibrosis is an incredibly important step towards resolving these issues.\(^{(1,2,3,4)}\) The cardiotonic steroid, marinobufagenin (MBG), has recently been described to be strongly associated with cardiac fibrosis in experimental renal failure models.\(^{(7, 13)}\) Specifically, MBG infusion in normal animals achieved plasma concentrations similar to the levels observed in experimental renal failure models, which induced cardiac fibrosis. Moreover, immunization against MBG prior to induction of experimental renal failure markedly attenuated the development of cardiac fibrosis.\(^{(7)}\) We also observed that relatively low concentrations of MBG and other cardiotonic steroids such as ouabain and digoxin induced cardiac fibroblasts to produce greater amounts of collagen as assessed by radiolabeled proline incorporation and Western blot analysis. Furthermore, we noted that the induction of collagen production by cardiotonic steroids depended on the integrity of signaling through the Na/K-ATPase-Src-ROS cascade.\(^{(13)}\)

Because low concentrations of cardiotonic steroids had dramatic effects on collagen production and since systemic cardiotonic steroids are extensively used clinically, we examined the effect of these compounds on collagen synthesis and wound healing. Because the rate of collagen formation plays a major role in the wound healing process\(^{(43)}\), we tested the effect of cardiotonic steroids on an \textit{in vitro}, and on \textit{in vivo}
wound healing models. Additionally, microarray analysis was performed to elucidate any further genes implicated in the process.
LITERATURE

I. Epidemiology of Chronic and Fibrotic Wounds

Chronic wounds are increasingly becoming a major burden for many elderly and disabled people, as they significantly impair quality of life and increase health costs for people across the world.\(^{(1)}\) Prolonged hospital stays and treatment of chronic wounds cost the United States over $25 billion annually.\(^{(2)}\) Additionally, chronic wounds are becoming more prevalent in people with diabetes, neuropathic foot ulcers, and pressure ulcers.\(^{(1)}\) Stimulating accelerated and efficient wound healing is an essential step towards successful treatment, improving quality of life, decreasing healthcare costs, and reducing morbidity.\(^{(3)}\)

Fibrotic diseases result in chronic illness, not only increasing morbidity and mortality, but also placing an immense financial burden, which has equal affects on both the individual and society. Thermal injury alone yields greater than 100,000 hospital admissions annually, for which treatment (via skin grafts) results in hefty increases of fibrosis and hypertrophic scarring, thus limiting functionality post-injury. Vital organs, such as the kidney, heart, liver, and brain, undergo progressive fibrosis post injury, making them a primary cause of illness and death.\(^{(4)}\) Hence, a balance must be maintained between accelerating wound closure and averting chronic healing that may result in fibrosis.
II. Renal Failure, Uremic Cardiomyopathy, and the onset of Fibrosis

The link between renal disease and cardiac failure due to cardiac dysfunction has been common knowledge since the beginning of the 20th century. Numerous changes are produced in cardiac structure and function that account for the high levels of morbidity and mortality due to cardiac issues. Structural changes associated with renal disease include left ventricular hypertrophy, coronary artery disease, valvulopathies, and pericarditis. Cardiac disease due to renal failure is termed uremic cardiomyopathy, and is generally characterized by ventricular hypertrophy and diastolic dysfunction in a setting of systemic oxidant stress. Left ventricular hypertrophy is not only characterized by increased myocardial fiber mass, but more importantly, by interstitial cardiac fibrosis. The pathogenesis of uremic cardiomyopathy is still a major source of controversy, and several factors are believed to play a role, including hypertension, anemia, hyperthyroidism, and oxidant stress.

Around the 1980s, circulating levels of digitalis were found in both the urine and the serum of patients with uremic cardiomyopathy. Clinical and animal research implicating digitalis led many scientists to extrapolate that endogenous digoxin-like substances were existent at supranormal levels. In the late 60s and early 70s, Bricker proposed the idea that hormonal adaptations may work to decrease renal function and participate in the pathogenesis of uremic cardiomyopathy. Bricker proposed a “trade-off” hypothesis that an inhibitor (digitalis) was part of a homeostatic feedback mechanism that functioned to maintain extracellular fluid volume and blood pressure, via electrolyte homeostasis. However, maintaining sodium homeostasis would come at a cost, due to other effects the hormone may carry.
Recently, cardiotonic steroids have been implicated in the pathogenesis of cardiomyopathy in the setting of renal failure. Kennedy, et al, produced a partial nephrectomy model, which served as an exemplar for the study of uremic cardiomyopathy.\textsuperscript{(12)} Through using these models, cardiotonic steroids are currently believed to play a central role in this process, yielding many of the features associated with uremic cardiomyopathy, e.g. diastolic dysfunction, oxidative stress, and fibrosis. Kennedy illustrates that treatment of cardiac cells with cardiotonic steroids induces structural and functional changes including down regulation of SERCA, increases of reactive oxygen species and thus oxidative stress, as well as marked increases in fibrosis.\textsuperscript{(7)} The proposed pathway in which cardiotonic steroids produce these effects is depicted in figure 1 below. Decreasing SERCA levels and increasing fibrosis in the heart results in diastolic dysfunction via attenuating both the active and passive stages of relaxation, respectively. The production of reactive oxygen species characterizes the systemic oxidant stress and chronic inflammation associated with uremic cardiomyopathy, thus building the foundation for future studies in an attempt to produce a potentially therapeutic clinical application.\textsuperscript{(7)}
Figure 1. “Schematic depicting sodium pump signaling in cardiac myocytes. In the presence of a cardiotonic steroid, Na/K-ATPase is converted to a signal transducer, which complexes with Src and the epidermal growth factor receptor. A signal cascade is initiated, which depends on Ras and results in the generation of reactive oxygen species (ROS) and activation of ERK. This, in turn, leads to altered gene expression, including decreases in SERCA expression, as well as alterations in calcium cycling.”

More recently, Elkareh et al, have studied the effects of cardiotonic steroids on fibrosis. In experimental renal failure, conditions and infusion of cardiotonic steroids (MBG), animals develop marked cardiac hypertrophy and fibrosis. Further investigations employing cardiac fibroblasts demonstrate clear increases in collagen and procollagen synthesis among other steroids such as digoxin and ouabain. The authors note, however, that MBG induction of collagen synthesis requires low levels of TGF-β to be present in the serum, thus illustrating that TGF-β is necessary, but not sufficient for this effect. The MBG induction pathway was found to be independent of TGF-β/Smad system, and likely undergoing a pathway similar to that described by Kennedy.
III. Na/K ATPase and Cardiotonic Steroids

The Na/K-ATPase (figure 2) is a ubiquitously expressed enzyme found in the plasma membrane of vertebrates, which maintains cellular resting potential and volume via maintaining a specific electrolyte balance.\(^\text{(14)}\) Crystallogical analysis of purified membranes from pig kidneys were used to determine the three dimensional structure of the Na/K-ATPase, facilitating the deciphering of its functions possible.\(^\text{(15)}\) Analysis of structure and function led to the categorization of the pump as a P-Type ATPase, which has subgroups that contain not only the Na/K-ATPase, but also H/K-ATPase, SERCA (sarcoplasmic endoplasmic reticulum calcium ATPase) and PMCA (plasma membrane calcium ATPase). The Na/K-ATPase is the only member of this family that permits binding of cardiotonic steroid binding.\(^\text{(16)}\)

Structurally, several isozymes of Na/K-ATPase have been identified, and are a molecularly heterogeneous protein formed from the association of two major peptides, the catalytic \(\alpha\) subunit, and a supporting \(\beta\) subunit. Ion movement across the membrane is coupled with hydrolysis of ATP, which induces a cation dependent conformational change from \(E_1\) to \(E_2\). Four isoforms for the \(\alpha\) subunit exist, all of which are transmembrane spanning proteins that provide the binding sites for cations, ATP, and cardiotonic steroids. The \(\beta\) subunit facilitates the binding of \(Na^+\) and \(K^+\) to the \(\alpha\) portion, as well as being crucial for the delivery of the \(\alpha\) polypeptide to the membrane. The \(\alpha\beta\) isozymes are highly conserved amongst species, and play a critical role in maintaining homeostasis.\(^\text{(16)}\)

The Na/K-ATPase functions to catalyze the active exchange of cytoplasmic \(Na^+\) for extracellular \(K^+\) across the plasma membrane via trading \(3Na^+\) ions for \(2K^+\) ions by
hydrolysis of one molecule ATP.\textsuperscript{17} Recent findings illustrate that this enzyme may also act as a signal transducer, modulating the effects of multiple gene-regulatory secondary messengers and pathways, including activation of Src, EGFR, Ras, PKC, MAPKs, and intracellular ROS production.\textsuperscript{(7,18)} Because the pump can act as a signal transducer, recent research has focused on decoding the underlying molecular mechanisms in which it signals, and determining the effects it may have. MBG and other cardiotonic steroids are thought to induce a signal transduction cascade through the plasmalemmal Na/K-ATPase reside in the caveolae, which results in the activation of Src, transactivation of the EGFR, generation of reactive oxygen species and, eventually, activation of p42/44 mitogen activated protein kinases, thus eliciting a myriad of effects that work to modulate cellular activity.\textsuperscript{(7)}

Signal transduction through the pump appears to significantly depend on the endocytosis of the Na/K-ATPase. Historically, dopamine or parathyroid hormone stimulation have been shown to induce the endocytosis of the plasmalemmal Na/K-ATPase, however it wasn’t until recently that other inducers have been implicated.\textsuperscript{(19,20)} Protein Kinase C (PKC) and Protein Kinase A (PKA) are understood to alter the activation of various proteins by means of phosphorylating specific amino acids found in the alpha chain of the Na/K-ATPase.\textsuperscript{(21)} Several pieces of work have established that cardiotonic steroids could stimulate internalization of the Na/K-ATPase in a clathrin dependent mechanism, when porcine proximal tubular (LLC-PK1) cells are treated with cardiotonic steroids in vitro, as well as in proximal tubular cells in vivo.\textsuperscript{(22,23,24,25)} Accumulation of cardiotonic steroids along with Na/K-ATPase has also been shown as a
result of Na/K-ATPase internalization. Thus, cardiotonic steroids are closely linked with the internalization and signal transduction properties of the Na/K-ATPase.\textsuperscript{(22)}

However, several studies have illustrated that caveolins play an important functional role in cellular modulation of signal transduction via compartmentalization of proteins, and internalization of proteins. Caveolins are believed to set off a signaling cascade when stimulated, either resulting in the activation of $G_s$ subunits, leading to the activation of adnylyl cyclase, thus increasing intracellular cAMP concentrations, and finally activating protein kinase A, or by having an activated EGFR receptor dock, resulting in activation of the proliferative pathway involving several caveolae associated proteins (Ras/Raf/MEK/ERK). Thus, signal transduction and internalization relies heavily on an intact caveolar architecture. Three types of caveolins act as scaffolding proteins that maintain the integrity of the caveolae, and lack of one or more than one type of caveolin is implicated in human disease.\textsuperscript{(26)} Liu and others demonstrated that cardiotonic steroids will stimulate a clathrin-dependent endocytosis pathway that lead to the translocation of the Na/K-ATPase into intracellular compartments via Src activation.\textsuperscript{(25)}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{sodium_channel_diagram.png}
\caption{Schematic diagram of the sodium channel $\alpha$ and $\beta$ subunits.\textsuperscript{(27)}}
\end{figure}
Cardiotonic Steroids:

For 50 years now, the mechanisms of action of digitalis on the Na/K-ATPase has been under extensive exploration, yielding much needed insight on its signaling capabilities. Digitalis’s ability to bind and inhibit the pumping action of the Na/K-ATPase is well established, it is however, the increases in intracellular calcium concentrations that provide for both the positive inotropic actions of the drug and its toxicity.\(^{(28)}\) Since its discovery, digitalis has been used extensively for therapeutic treatment of congestive heart failure, due mainly its positive inotropic actions on the heart.\(^{(29)}\) Prior to 1953, digitalis was thought to be xenobiotic to mammals. However, researchers soon discovered digitalis endogenously.\(^{(30)}\)

The 80s brought about a period in which doctors identified increased serum concentrations of digoxin in chronic renal failure medications. Puzzled as to the origin and motive behind these serum concentrations, doctors coined the term “false positive” to define the digoxin anomaly.\(^{(31)}\) These findings further fueled interest and research in the areas of cardiotonic steroid origin, action, and synthesis.

Ouabain (figure 3A), a potent cardiotonic steroid that originates from the African ouabaio tree and the *Stophantus gratus* plant, has been used extensively and in laboratory research.\(^{(5)}\) Serum ouabain levels have been revealed to be elevated in patients with renal failure and congestive heart failure, and have been established to be located in the hypothalamus, functioning as a neurosteroid.\(^{(29)}\) Since its detection as an endogenous compound, research studies have extensively focused on the site and synthesis of this hormone. Ouabain is currently believed to be synthesized in the zona fasciculata of the adrenal cortex, and synthesis likely mediated by ACTH, alpha adrenergic and
dopaminergic stimulation, Angiotensin II, vasopressin, as well as systemic hypoxia and physical exercise. Ouabain induced hypertension can be inhibited via ouabain antibodies in experimental models.\(^{(5)}\) Ouabain has been demonstrated to have a profoundly high affinity to \(\alpha\) subunits of the Na/K-ATPase, specifically the \(\alpha_2\) and \(\alpha_3\) isoforms, hence cells expressing other isoforms prove to be insensitive to ouabain administration. Ouabain (figure 3) exhibits a high affinity for the \(\alpha_2/\alpha_3\) Na/K-ATPase isoforms.\(^{32}\) It is important to note that ouabain is believed to signal through the pathway described by Kennedy, et al.

Marinobufagenin (figure 3B), an endogenous digitalis-like substance produced in toads, has been identified and isolated from an amphibian venom. Toads from the \textit{Bufo} family produce venoms from bufadienolide group of steroids in attempt to protect themselves from predators, and some even suggest the compound has antimicrobial properties.\(^{(33)}\) Unlike ouabain which only has a high affinity to the \(\alpha_2\) and \(\alpha_3\) isoforms, MBG exhibits a prominent affinity to the \(\alpha_1\) isoform of the Na/K-ATPase, and can induce natriuresis in attempt to overcome an impairment of sodium excretion. Bufodienolides are markedly different from cardenolides because of a doubly unsaturated six-membered lactone ring. Patients with renal failure have been documented to have high plasma concentrations of various endogenous digitalis, telocinobufagenin, and MBG. MBG has been shown to be elevated after myocardial infarction, and may act as a marker of severe congestive heart failure.\(^{(5)}\) Marinobufagenin has recently been found to exhibit potent vasoconstrictive effects in its endogenous form.\(^{(34,35)}\) The inhibitory impact of MBG and ouabain on the Na/K-ATPase although analogous, the resultant fibrosis varies depending on dosage and duration.\(^{(13)}\)
Digoxin (figure 3C), a cardiac glycoside extracted and purified from the foxglove plant, *Digitalis lanata*, is a common clinical pharmaceutical employed as a therapeutic in the treatment of various heart conditions including atrial flutter, heart failure, and most frequently for atrial fibrillation.\(^{36}\) The structural characteristics of digoxin that confer to its functionality are the lactone ring and three digitoxose sugars attached to a steroid nucleus. Digoxin may be metabolized through deglycosylation forming digoxin like immunoreactive factors (DLIF), e.g. digoxigenin bis-digitoxoside, digoxigenin monodigitoxoside, and digoxigenin. DLIF is found endogenously and is thought to be synthesized in the adrenal glands of humans.\(^{37}\) Digoxin works like all digitalis-like compounds by binding to the \(\alpha\)-subunit of the Na/K-ATPase, inhibiting the pumping action, causing an accumulation of intracellular sodium, which slows the extrusion of calcium via the Ca/Na exchanger, and opening voltage gated calcium channels, thus causing increases in intracellular calcium concentrations. The increases in calcium concentrations lead to increased contraction of myocytes via activation of SERCA and increased vagal activity, leading to increased contractility of the heart, and decreased conduction of electrical impulses through the AV node, respectively. Digitalis also elicits a signal transduction pathway that may initiate Src-EGFR mediated signal transduction, MAPK, phospholipase C, and PKC cascades. Several authors have reported a possible alleged role for digitalis on RyR functioning. Studies linking direct intracellular action of glycosides on SR calcium release channels implicate the localization occurs in near the ryanodine receptors (RyRs), suggesting that an association with RyR protein or function is critical to the positive inotropic effects of digitalis. A summary of the positive and negative inotropic of digitalis is illustrated in figure 4.\(^{28}\)
Figure 3: Chemical structures of (A) Ouabain, (B) Marinobufagenin, and (C) Digoxin.
Figure 4: “Summary of positive inotropic and intracellular signaling effects of cardiotonic steroids. Left: summary of interactions between Na/K-ATPase (NKA) inhibition and a putative effect of ryanodine receptor (RyR) activation to produce positive inotropic effects of digitalis. Glycoside binding to NKA inhibits its Na/K pumping function. Result is increased internal $[\text{Na}^+]$ and therefore $[\text{Ca}^{2+}]$ as a result of a net influx of $\text{Ca}^{2+}$ via the Na/Ca exchange (NCX) when the Na$^+$ gradient is reduced. In addition, glycoside may bind to and increase activity of RyRs when sarcoplasmic reticulum (SR) luminal $[\text{Ca}^{2+}]$ is elevated, thus amplifying SR $\text{Ca}^{2+}$ release at higher SR $\text{Ca}^{2+}$ load. Right: cell signaling pathways induced by digitalis binding to and inhibition of NKA. Binding of glycoside to and possibly pump inhibition may also initiate sarcoma kinase-epidermal growth factor receptor (SRC-EGFR)-mediated signal transduction-MAPK, and/or phospholipase C- (PLC)-protein kinase C (PKC) cascades. NKA is then depicted as residing both on the external sarcolemma and on the T-tubular membrane, possibly located in but probably not restricted to, the caveolae. AP-1, transcription activator protein 1; MAPK (ERK1/2), mitogen-activated protein kinase (extracellular-signal regulated kinases 1 and 2); NF-B, nuclear factor-B; RAF, renin-angiotensin factor kinase; MEK, mitogen activated ERK-activating kinase; RAS, renin-angiotensin system (GTPase); SHC, Src homology collagen-like protein; SOS, Son of Sevenless protein; SRC, sarcoma kinase; VGCC, voltage-gated $\text{Ca}^{2+}$ channel; SERCA, sarco(endo)plasmic reticulum $\text{Ca}^{2+}$-ATPase; ROS, reactive oxygen species.” (28)
IV. Fli-1 and Collagen Synthesis

Homeostasis in human physiology is a key aspect to normal functioning. In terms of normal healing and adaptation to stress, a balance between pro-fibrotic and anti-fibrotic factors is needed to allow for proper compensation or ordinary healing. ECM production and remodeling during stages of healing is tightly controlled and any shift in factors will either lead to an exaggerated or understated effect, causing many diseases. One gene, called Friend of Leukemia Integration-1 (Fli-1) has been implicated in fibrosis and collagen-1 production. Fli-1 generally competes with Ets-1, a family of transcription factors that bind an Ets promoter domain, in an Sp-1 dependent manner, shifting the balance of collagen production via stimulating or repressing the Col-1a2 promoter. Three-dimensional analysis of the Fli-proteins illustrated and helix-loop-helix secondary structure which it uses to read the 5’ and 3’ ends of the Ets domain.

Experimentation on dermal fibroblasts yielded results supporting the competitive antagonistic effects of Ets-1/Fli-1 on Col-1a2 promoter gene. Because scleroderma, systemic sclerosis, and nephrogenic sclerosing dermopathy are due to marked increases of collagen deposition ensuing fibrotic scar formation, research has focused on elucidating the mechanism in which this occurs. Interestingly, there is data that suggest collagen transcription is suppressed in scleroderma fibroblasts. Epigenetic mechanisms, such as DNA methylation and histone deacetylation, are believed to play an important role in Fli-1 gene repression, particularly in patients with scleroderma. However TGF-β stimulation has been shown to cause reversible acetylation of Fl-1 in human dermal fibroblasts, implicating that the TGF-β/Smad pathway causes dissociation
of FlI-1 from the Col-1a2 promoter gene, hence de-repressing collagen-1 synthesis and producing systemic fibrosis.\(^{(40)}\)

V. Wound Healing

Wound healing is a highly complex, orchestrated, and dynamic sequence of events that takes place in order to repair and regenerate a damaged tissue, a function crucial to survival. The mechanisms in which healing is thought to take place, the cross-talk that occurs between cells, and the molecules implicated in ECM production and remodeling (Table 1) will be briefly discussed. Wound healing is composed of three overlapping phases, inflammation, tissue formation, and tissue remodeling, each of which contains a myriad of events that move the process forward.\(^{(43)}\)

After the production of injury, a release of inflammatory mediators will initiate a clotting cascade containing anti-fibrinolytic agents. This first stage is termed the inflammatory phase and consists of several events. When platelets become exposed to components of the ECM – chiefly released intracellular stores of calcium and collagen – aggregation will ensue, resulting in hematostasis and clot formation.\(^{(44,45,46)}\) The clotting cascade facilitates the formation of blood clots and lays down the provisional extracellular matrix.\(^{(45)}\) Vasodilation will take place approximately 20 minutes post-injury as result of platelet degranulation, attempting to increase the blood flow to the wound area. Myofibroblasts, epithelial, and endothelial cells are believed to be stimulated by platelet degranulation, thus producing matrix metalloproteinases (MMPs), which disrupt the basement membrane, ergo facilitating the passage of inflammatory and immune cells to the wound area.\(^{(46)}\) Additionally, epithelial and endothelial cells will produce and secrete growth factors (PDGF, TGF-β, etc.)\(^{(45)}\), cytokines, and chemokines
to stimulate the proliferation and migration of leukocytes across the provisional ECM. The influx of inflammatory cells, chiefly neutrophils and macrophages, defines the inflammatory phase. As neutrophils become more abundant at the wound site due to the increased blood flow and signaling of other cells, they begin to degranulate and die, thus allowing for the additional recruitment of macrophages via their degranulation products.\(^{(46)}\) The arrival of macrophages plays a vital role in normal wound healing.\(^{(45)}\) As activated macrophages and neutrophils accumulate, they begin to eliminate tissue, debris, and microorganisms, producing cytokines and chemokines in the process, that serve as a feed-forward mechanism, further amplifying the inflammatory response. Finally, as T-cells become activated and begin to arrive at the wound milieu, they produce pro-fibrotic cytokines such as IL-3 and TGF-β that work to activate cells of the proliferative stage.\(^{(46)}\)

The close of the inflammatory phase unlocks the process of repair, which consists of two dichotomous processes: the regeneration of injured tissue by parenchymal cells of the affected tissue and the replacement of connective tissue in a fibrotic process.\(^{(4)}\) The proliferative stage is marked by the arrival of non-inflammatory cells to the wound site. Mitogens and chemotactic factors produced by macrophages and neutrophils in the inflammatory phase have paved the way for endothelial and fibroblast cell migration to the place of injury.\(^{(46)}\)

Angiogenesis (neovascularization) is initiated by the advent of endothelial cells, and is mediated by growth factors released by macrophages, e.g. low oxygen tension, elevated lactic acid levels, bFGF, VEGF, angiogenenin, angiotropin, angiopoeitin 1, thrombospondin, and TNFα.\(^{(43)}\) Angiogenesis generally precedes ECM secretion, as fibroblasts require significant quantities of oxygen in order support their growth and
proliferation. Endothelial cells originate from uninjured areas of the blood vessels develop pseudopodia and migrate towards the wound site following angiogenic stimulus enter and wound site via the dissolved basement membrane of the vessels. As endothelial cells mature, they cause the growth and remodeling of the capillary tubes.\(^{(47)}\)

Because VEGF also increases vascular permeability, in addition to its angionenic effects, plasma proteins will be exuded and deposited providing the provisional ECM needed for fibroblast migration. Fibroblast migration and proliferation are triggered by multiple growth factors that are secreted by platelets, activated epithelium, and inflammatory cells (chiefly macrophages), that include TGF-\(\beta\), PDGF, EGF, FGF, and cytokines such as IL-1 and TNF. TGF-\(\beta\) appears to have the most prominent role in inflammatory fibrosis, as it causes fibroblast migration and proliferation, increased synthesis of collagen and fibronectin, and decreased degradation of e ECM by metalloproteinases\(^{(47)}\)

Fibroblasts are responsible for the formation of granulation tissue via deposition of ground substances and collagen. Fibrillar collagens (primarily type I and type III in dermal wounds) form the major portion of the connective tissue, providing the tensile strength needed for the healing wound.\(^{(45)}\) This process typically occurs between 3-5 days and may last for several weeks. Collagen synthesis is known to be enhanced by growth factors (PDGF, FGF, TGF-\(\beta\)) and cytokines (IL-1, IL-13) secreted by leukocytes and fibroblasts themselves in healing wounds.\(^{(47)}\) Net collagen deposition relies heavily on shifting the balance of collagen formation via increasing collagen synthesis, downregulating MMPs, and upregulating TIMPs.\(^{(48)}\) The granulation tissue formed consists of fibroblasts, new blood vessels, inflammatory cells, endothelial cells and ECM.
A scar will be produced from the granulation tissue, becoming an assortment of spindle-shaped fibroblasts, elastic tissue, dense collagen, and other ECM components. As the scar matures, vascularization will decrease, and the scar will become pale and avascularized. It is important to note that a process of epitheliazation occurs in some tissues, in which epithelial cells (keratinocytes in skin) migrate across the granulation tissue to form a new epithelial layer. In the skin, basal keratinocytes will form the wound and dermal appendages (hair follicles, sweat glands, and sebaceous glands), and the various layers of the skin through differentiating into five stratum. \(^{(47)}\)

The final and longest phase of wound healing is tissue remodeling and maturation. The composition of the ECM must be altered to produce a scar from the weak and dispersed granulation tissue, and this balance between ECM synthesis and degradation provides for the remodeling of the connective tissue framework. Collagen synthesis and collagen catabolism, as well as matrix composition must be modulated via activation/inhibition of metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), thus resulting in the modification of the ECM. This family of proteins is zinc-dependent endopeptidases, and any shift in their activity could result in diseases states ranging from arthritis to fibrosis and keloid development. Table 2 provides a summary of MMPs actions. \(^{(48)}\) The angiogenesis produced during the proliferative phase begins to recede and the loose provisional matrix will gradually be replaced by collagen fibers, which continue to be reorganized via synthesis and proteolysis, consequently providing increased tensile strength and producing the scar. Although type III collagen dominates the provisional matrix, type I collagen begins to dominate the mature scar during and after remodeling, yielding a mature scar. Scar maturation and
resolution, in humans, is typically complete 6-12 months post-injury.\(^{(45)}\) It is important to note that the organization of fibrillar collagens in the wound area never return to normal, thus the scar tissue only gains a maximum of 70% the tensile strength of normal skin tissue.\(^{(43)}\)

### Table 1: Growth Factors and Cytokines Involved in Regeneration and Wound Healing

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Symbol</th>
<th>Source</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal growth factor</td>
<td>EGF</td>
<td>Platelets, macrophages, saliva, urine milk, and plasma</td>
<td>Mitogenic for keratinocytes and fibroblasts; stimulates keratinocyte migration and granulation tissue formation</td>
</tr>
<tr>
<td>Transforming growth factor alpha</td>
<td>TGF-α</td>
<td>Macrophages, T lymphocytes, keratinocytes, and many other tissues</td>
<td>Similar to EGF; stimulates replication of hepatocytes and certain epithelial cells</td>
</tr>
<tr>
<td>Hepatocyte growth factor/scatter factor</td>
<td>HGF</td>
<td>Mesenchymal cells</td>
<td>Enhances proliferation of epithelial and endothelial cells, and of hepatocytes; increases cell motility</td>
</tr>
<tr>
<td>Vascular endothelial cell growth factor (isoforms A,B,C,D)</td>
<td>VEGF</td>
<td>Mesenchymal cells</td>
<td>Increases vascular permeability; mitogenic for endothelial cells</td>
</tr>
<tr>
<td>Platelet-derived growth factor (isoforms A,B,C,D)</td>
<td>PDGF</td>
<td>Platelets, macrophages, endothelial cells, keratinocytes, smooth muscle cells</td>
<td>Chemotactic for PMNs, macrophages, fibroblasts, and smooth muscle cells; activates PMNs, macrophages, and fibroblasts; mitogenic for fibroblasts, endothelial cells and smooth muscle cells; stimulates production of MMPs, fibroectin, and HA; stimulates angiogenesis and wound contraction; remodeling; inhibits platelet aggregation; regulates integrin expression</td>
</tr>
<tr>
<td>Fibroblast growth factor-1 (acidic) -2 (basic) and family</td>
<td>FGF</td>
<td>Macrophages, mast cells, T lymphocytes, endothelial cells, keratinocytes, fibroblasts, and many tissues</td>
<td>Chemotactic for fibroblasts; mitogenic for fibroblasts and keratinocytes; stimulates keratinocyte migration, angiogenesis, fascia wound contraction and matrix deposition</td>
</tr>
<tr>
<td>Transforming growth factor beta (isoforms 1,2,3); other members of the family are BMP and activin</td>
<td>TGF-β</td>
<td>Platelets, T lymphocytes, keratinocytes, smooth muscle cells, fibroblasts</td>
<td>Chemotactic for PMNs, macrophages, lymphocytes, fibroblasts, and smooth muscle cells; stimulates TIMP synthesis, keratinocyte migration, angiogenesis, and fibroplasia; inhibits production of MMPs and keratinocyte proliferation; regulates integrin expression and other cytokines; induced TGF-β production</td>
</tr>
<tr>
<td>Keratinocyte growth factor (also called FGF-7)</td>
<td>KGF</td>
<td>Fibroblasts</td>
<td>Stimulates keratinocyte migration, proliferation, and differentiation.</td>
</tr>
<tr>
<td>Insulin-like growth factor-1</td>
<td>IGF-1</td>
<td>Macrophages, fibroblasts and other cells</td>
<td>Stimulates synthesis of sulfated proteoglycans collagen keratinocyte migration, and fibroblast proliferation; endocrine effects similar to growth hormone</td>
</tr>
<tr>
<td>Tumor necrosis factor</td>
<td>TNF</td>
<td>Macrophages, mast cells, T lymphocytes</td>
<td>Activates macrophages; regulates other cytokines; multiple functions</td>
</tr>
<tr>
<td>Interleukins</td>
<td>IL-1,</td>
<td>Macrophages, mast cells, keratinocytes, lymphocytes, and many tissues</td>
<td>Many functions. Some examples: chemotactic for PMNs (IL-1) and fibroblasts (IL-4), stimulation of MMP-1 synthesis (IL-1), TIMP synthesis (IL-6); regulation of other cytokines</td>
</tr>
<tr>
<td>Interferons</td>
<td>IFN-α,</td>
<td>Lymphocytes and fibroblasts</td>
<td>Activates macrophages; inhibits fibroblast proliferation and synthesis of MMPs; regulates other cytokines</td>
</tr>
</tbody>
</table>

BMP, bone morphogenetic proteins; PMNs, polymorphonuclear leukocytes; MMPs, matrix metalloproteinases; HA, hyaluronic acid; TIMP, tissue inhibitor of matrix metalloproteinase

From Robbins and Cotran *Pathological Basis of Disease*\(^{(47)}\)
<table>
<thead>
<tr>
<th>MMP Subtypes</th>
<th>Substrates</th>
<th>Activating Capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>Collagen I, II, III (MMP-1), IV, VII, X, gelatin, aggrecan, versican, pro-collagen III (MMP-1), tenascin, decorin, lumican, semaphorin 3A, TIMP-3</td>
<td>MMP-2, 4, 6, plasmin, kallikrein, thrombin, matrix</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Collagen I, II, III, IV, VII, X, N, gelatin, aggrecan, versican, fibromodulin, lumican, semaphorin 3A</td>
<td>MMP-2, 4, 6, plasmin</td>
</tr>
<tr>
<td>MMP-4</td>
<td>Gelatin, fibronectin I, V, VII, X, X, N, aggrecan, versican, pro-collagen III, tenascin, decorin, lumican, semaphorin 3A, TIMP-1, 2</td>
<td>MMP-2, 4, 6, plasmin, -13, -14, -15, -24</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatin, fibronectin I, V, VII, X, X, N, aggrecan, versican, pro-collagen III, tenascin, decorin, lumican, semaphorin 3A, TIMP-1, 2</td>
<td>MMP-2, 4, 6, plasmin, -13</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Collagen III, IV, V, IX, X, gelatin, aggrecan, versican, pro-collagen I, tenascin, decorin, lumican, semaphorin 3A, TIMP-1, 2, TIMP-3, kallikrein, MMP, angiostatin</td>
<td>MMP-2, 4, 6, plasmin, -13, -14, -15, -24</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Collagen III, IV, V, IX, X, gelatin, aggrecan, versican, pro-collagen I, tenascin, decorin, lumican, semaphorin 3A, TIMP-1, 2, TIMP-3, kallikrein, MMP, angiostatin</td>
<td>MMP-2, 4, 6, plasmin, -13, -14, -15, -24</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Collagen I, II, III, IV, V, IX, X, X, N, gelatin, aggrecan, versican, pro-collagen I, tenascin, decorin, lumican, semaphorin 3A, TIMP-1, 2, TIMP-3, kallikrein, MMP, angiostatin</td>
<td>MMP-2, 4, 6, plasmin, -13, -14, -15, -24</td>
</tr>
<tr>
<td>MMP-14</td>
<td>Collagen I, II, III, IV, V, IX, X, X, N, gelatin, aggrecan, versican, pro-collagen I, tenascin, decorin, lumican, semaphorin 3A, TIMP-1, 2, TIMP-3, kallikrein, MMP, angiostatin</td>
<td>MMP-2, 4, 6, plasmin, -13, -14, -15, -24</td>
</tr>
<tr>
<td>MMP-16</td>
<td>Collagen I, II, III, IV, V, IX, X, X, N, gelatin, aggrecan, versican, pro-collagen I, tenascin, decorin, lumican, semaphorin 3A, TIMP-1, 2, TIMP-3, kallikrein, MMP, angiostatin</td>
<td>MMP-2, 4, 6, plasmin, -13, -14, -15, -24</td>
</tr>
<tr>
<td>MMP-17</td>
<td>Collagen I, II, III, IV, V, IX, X, X, N, gelatin, aggrecan, versican, pro-collagen I, tenascin, decorin, lumican, semaphorin 3A, TIMP-1, 2, TIMP-3, kallikrein, MMP, angiostatin</td>
<td>MMP-2, 4, 6, plasmin, -13, -14, -15, -24</td>
</tr>
<tr>
<td>MMP-18</td>
<td>Collagen I, II, III, IV, V, IX, X, X, N, gelatin, aggrecan, versican, pro-collagen I, tenascin, decorin, lumican, semaphorin 3A, TIMP-1, 2, TIMP-3, kallikrein, MMP, angiostatin</td>
<td>MMP-2, 4, 6, plasmin, -13, -14, -15, -24</td>
</tr>
<tr>
<td>MMP-19</td>
<td>Collagen I, II, III, IV, V, IX, X, X, N, gelatin, aggrecan, versican, pro-collagen I, tenascin, decorin, lumican, semaphorin 3A, TIMP-1, 2, TIMP-3, kallikrein, MMP, angiostatin</td>
<td>MMP-2, 4, 6, plasmin, -13, -14, -15, -24</td>
</tr>
<tr>
<td>MMP-20</td>
<td>Collagen I, II, III, IV, V, IX, X, X, N, gelatin, aggrecan, versican, pro-collagen I, tenascin, decorin, lumican, semaphorin 3A, TIMP-1, 2, TIMP-3, kallikrein, MMP, angiostatin</td>
<td>MMP-2, 4, 6, plasmin, -13, -14, -15, -24</td>
</tr>
</tbody>
</table>

From: http://ethesis.helsinki.fi/julkaisut/laa/klin/vk/vaalamo/table2.gif
VI. Nephrogenic Sclerosing Dermopathy

Patients with renal failure have become increasingly prone to developing a fibrosing disorder similar to scleroderma called nephrogenic sclerosing dermopathy. Although previously believed to afflict only hemodialysis patients, later studies world-wide found the condition to exist in patients prior to dialysis. The disease presents itself as acute, lumpy, plaque-like indurations, mostly affecting the lower extremities, but seldom the upper appendages and torso. The lesions produced by this disease can result in functional disability, as they are often painful, chronic, and unremitting in most patients. Histological examination of patients with this disease reveals similarities with scleroderma, however nephrogenic sclerosing dermopathy presents itself additionally with increased depositions of dermal mucin. Myofibroblasts have also been discovered to actively participate in this disease process, providing important implications, as they are central to ECM production and the development of fibrosis. Ergo, nephrogenic sclerosing dermopathy may be an unusual and prolonged response to tissue injury and thus a form of wound healing. Because the existence of subtle inflammatory cell populations of macrophages are present, as well as a population of proliferating fibroblasts which differentiate into myofibroblasts exist, it becomes likely that these populations may be responsible for the increases in fibrosis. It has been proposed that nephrogenic sclerosing dermopathy is the result of an unidentified toxin creating an exaggerated reparative response.\(^{(42)}\) While the etiology and pathogenesis of this disease remain poorly understood, there has recently been a high correlation of its development with gadolinium exposure.\(^{(49)}\) One should keep in mind that further work is needed to identify other toxins that may be producing this disease.
The Effects of Cardiotonic Steroids on
Dermal Collagen Synthesis and Wound Healing

By

Nasser El-Okdi, Sleiman Smaili MD, Vanamala Raju MD, Amjad Shidyak MD, Shalini
Gupta, Larisa Fedorova PhD, Jihad Elkareh PharmD,
Sankaridrug Periyasamy PhD, Anna P Shapiro, M Bashar Kahaleh MD,
Deepak Malhotra MD PhD, Zijian Xie PhD, Khew Voon Chin PhD,
and Joseph I Shapiro MD.

From

Departments of Medicine and Physiology/Pharmacology
University of Toledo College of Medicine
Running Title: Cardiotonic steroids stimulate dermal collagen production

Corresponding Author: Joseph I. Shapiro, M.D.

Chairman, Department of Medicine

University of Toledo College of Medicine

3000 Arlington Avenue, Mail Stop 1186,

Toledo, Ohio 43614-2598

Phone: (419) 383-6030 FAX: (419) 383-6244

Email: joseph.shapiro@utoledo.edu

Pages of Text: 20 (including title pages and references)

Tables: 1

Figures: 8

Abstract: 241 words

Introduction: 203 words

Discussion: 566 words

References: 24

Nonstandard abbreviations: None.

Key Words: Digitalis-Like Substances, Na/K-ATPase, Signaling, Fibrosis, Wound Healing, Collagen
Abstract:

We previously reported that cardiotonic steroids stimulate collagen synthesis by cardiac fibroblasts in a process that involves signaling through the Na/K-ATPase pathway. In this study, we examined the effect of cardiotonic steroids on dermal fibroblasts, collagen synthesis, and on wound healing. Increased collagen expression by human dermal fibroblasts was noted in response to the cardiotonic steroid, marinobufagenin, in a dose- and time-dependent fashion. An eightfold increase in collagen synthesis was noted when cells were exposed to 10 nM marinobufagenin for 24 hours (p<0.01). Similar increases in proline incorporation were seen following treatment with digoxin, ouabain and marinobufagenin (10 nM X 24 hours, all results p< 0.01 vs. control). The co-administration of the Src inhibitor, PP2, or N-Acetyl Cysteine completely prevented collagen stimulation by marinobufagenin. Next, we examined the effect of digoxin, ouabain, and marinobufagenin on the rate of wound closure in an in vitro model where human dermal fibroblasts cultures were wounded with a pipette tip and monitored by digital microscopy. Finally, we administered digoxin in an in vivo wound healing model. Olive oil was chosen as the digoxin carrier because of a favorable partition coefficient observed for labeled digoxin with saline. This application significantly accelerated in vivo wound healing in rats wounded with an 8 mm biopsy cut. Increased collagen accumulation was noted 9 days after wounding (both p< 0.01). The data suggest that cardiotonic steroids can induce increases in collagen synthesis by dermal fibroblasts, as well as accelerate recovery.
Introduction:

We have previously noted that the cardiotonic steroid, marinobufagenin (MBG), mediates cardiac fibrosis in an experimental renal failure model (8, 10). (Specifically, we observed that infusion of MBG to normal animals to achieve a plasma level similar to the levels observed in experimental renal failure models induced cardiac fibrosis similar to the one seen in renal failure models.) Moreover, immunization against MBG prior to induction of experimental renal failure markedly attenuated the development of cardiac fibrosis (10). We also observed that relatively low concentrations of MBG and other cardiotonic steroids such as ouabain and digoxin induced cardiac fibroblasts to produce greater amounts of collagen as assessed by radiolabeled proline incorporation and Western blot analysis. Furthermore, we noted that the induction of collagen production by cardiotonic steroids depended on the integrity of signaling through the Na/K-ATPase-Src-ROS cascade (8).

Because low concentrations of cardiotonic steroids had dramatic effects on collagen production and since systemic cardiotonic steroids are extensively used clinically, we examined the effect of these compounds on collagen synthesis and wound healing. The rate of collagen formation plays a major role in wound healing (18). Thus, we tested the effect of cardiotonic steroids on an in vitro and an in vivo wound healing models.
Methods:

Cell culture: Human skin fibroblasts, obtained from Cambrex, were maintained in culture as described (2). Collagen production was assessed by Western blot and by radiolabeled proline incorporation as previously described (8).

DNA Microarray: Human dermal fibroblasts were treated with MBG for variable times (0, 0.25, 0.5, 0.75, 1, 2, 3, 6, 9, 12, 15, and 24 hrs). Cells were harvested and RNA was isolated. RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Integrity of isolated RNA was examined using Agilent’s RNA LabChip kits on the 2100 Bioanlyzer (Agilent, Santa Clara, CA). Gene expression profiling was conducted using the Human OneArray (Phalanx Biotechnology, Taiwan,) containing over 30,000 sixty-mer polynucleotide probes with each probe mapped to the latest draft of the human genome (GoldenPath) printed on standard 1” x 3” glass slide.

Target Synthesis, Array Hybridization, Image Processing, and Data Analysis: cDNA were synthesized from the isolated RNA by reverse transcription followed by second strand synthesis using the Amino Allyl MessageAmp II aRNA Amplification kit (Ambion, Austin, TX) with oligo(dT) primer that contains a T7 RNA polymerase promoter sequence, according to the manufacturer’s specification. In vitro transcription was then performed with the purified cDNA and amino allyl UTP was incorporated during transcription to produce amino allyl modified aRNA that was subsequently coupled to cy dye label for hybridization to the microarray. Scanned images of microarray were analyzed using ImaGene (Biodiscovery, Danville, CA) and the output intensity data were further filtered and analyzed using custom statistical software. In brief, normalized expression data for each gene were analyzed by regression models to fit
polynomial functions of the logarithm of time up to the third degree. A subset of genes with >2-fold changes for at least two consecutive time points and with significant coefficients of the highest degree (i.e., linear, quadratic, or cubic) at the significance level of 2% (to control possible type I error rate from multiple comparisons) was included in the polynomial regression analysis. Local regression models were also used to fit quadratic polynomials of the logarithm of time. For the selected genes, the patterns of gene expression were log transformed, centered by median, and subjected to cluster analyses by centered correlation and average linkage as the similarity/distance metric using the hierarchical cluster algorithm in Cluster and TreeView software suite (3, 4, 22, 24). The entire microarray data set is available for searches at http://www.ncbi.nlm.nih.gov/projects/geo accession number GSE9806.

*In vitro* wound healing: Fibroblasts were grown to confluence and then wounded with a 10 μl pipette tip as was described (11, 16, 20). Digital photographs were taken to monitor the *in vitro* wound closure. Quantification of the wound closure was performed by taking the average of at least three measurements of the distance separating the intact cells at different times following the wound and expressing it as a fraction of the average distance immediately following wounding. These measurements were performed using ImageJ (version 1.32j) software (National Institutes of Health, USA http://rsb.info.nih.gov/ij/).

*In vivo* wound healing: Male Sprague Dawley rats weighing 300-350 gms were subjected to full thickness skin biopsy performed with a 8.0 mm punch biopsy (Item No. 501912, Harris Uni-core™, World Precision Instruments, Inc., Sarasota, Fl). Two symmetric lesions were made on the dorsum of the thorax and treated with either vehicle alone
(olive oil) or MBG in olive oil (Sigma, St. Louis, MO) at 30 nM concentration and covered with hill-top bandages (# 25 mm Chamber System, Hill Top Research, Miamiville, Ohio). Digital photographs were taken immediately after and 1, 2, 3, 4, 7 and 9 days following wounding. Photographs were analyzed with Image J as described in the vitro wound healing model. At 9 days, animals were euthanized and the healing wound excised for histological study and measurement of protein and collagen content. Paraffin sections were prepared and stained with trichrome stain as was reported (8, 10). Quantification of collagen on these sections was also performed using Sirius red as described by Lopez-De Leon and Rojkind (14). Briefly, deparaffinized sections were pre-incubated in the dark (covered with aluminum foil) with a solution of 0.1% Fast Green in picric acid for 15 minutes on a rotary shaker. The fast green solution was then replaced by a 0.1% Sirius red and 0.1% fast green mixture in picric acid and again incubated in the dark for 30 minutes on a rotary shaker. The staining fluids were withdrawn, and the sections were rinsed several times with distilled water to remove unbound stain. Samples were either fixed for photography, or eluted for collagen studies. The elution solution consisted of a 1:1 mixture of 0.1N NaOH and absolute methanol. One milliliter of elution solution was added to each section. The eluted stain was then read at 605nm and 540nm using a spectrophotometer with quantification as described previously (14). Collagen and total protein are expressed as a fraction of control samples. All of the animal experimentation described in the article was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals using protocols approved by the University of Toledo Health Science Campus Institutional Animal Use and Care Committee.
**Statistical analysis:** Data presented are mean± standard error of the mean. Data obtained were first tested for normality. If the data did not pass the normality test, the Tukey test (for multiple groups) or the Mann-Whitney Rank Sum test were used to compare data. If the data did pass the normality test, parametric comparisons were performed. If more than two groups were compared, one-way analysis of variance was performed prior to comparison of individual groups with the unpaired Student’s t-test with Bonferroni’s correction for multiple comparisons. If only two groups of normal data were compared, the Student’s t-test was used without correction (23). Statistical analysis was performed using SPSS™ software.
Results:

First, we examined whether MBG and other cardiotonic steroids stimulated dermal fibroblasts in a manner similar to that seen with cardiac fibroblasts (8). Exposure of human dermal fibroblasts to MBG resulted in dose-dependent increases in procollagen expression determined by Western blot (Figure 1a) and radiolabeled proline incorporation (Figures 1b and 1c). In fact, the magnitude of this response was considerably more than what was seen previously with primary cardiac fibroblasts (8). Administration of other cardiotonic steroids (ouabain and digoxin) resulted in similar increases in proline incorporation (Figure 1b). Co-administration of N-acetyl cysteine or inhibition of Src with PP2 blocked the stimulation of collagen synthesis by MBG (Figure 1c). Exposure of dermal fibroblasts to greater than 10 nM concentrations of MBG, ouabain or digoxin did not result in greater degrees of proline incorporation. Similarly, simultaneous exposure of dermal fibroblasts to 10 nM MBG and either ouabain or digoxin at 10 nM concentration did not result in more proline incorporation than seen with 10 nM MBG alone (data not shown).

To further understand the biological pathways activated by cardiotonic steroids, we investigated the genomic-wide effects of MBG on human skin fibroblasts by gene expression profiling. A time-course study (0, 0.25, 0.5, 0.75, 1, 2, 3, 6, 9, 12, 15, and 24 hrs) was conducted to examine the temporal changes in gene expression in response to MBG (Figure 2). RNA were isolated from fibroblasts at the indicated time and compared with untreated cells. Samples were reverse-transcribed and hybridized to the human whole genome array. All the samples were hybridized in duplicate arrays. Normalized data were analyzed by regression models and the expression levels for each gene were
fitted to polynomial functions relative to the logarithm of time up to the third degree as described previously (24). Statistically, significant alterations in gene expression were recorded. A subset of these gene changes was selected for cluster analysis and the results displayed by TreeView software (7), figure 2b).

A distinct pattern of gene expression that was recapitulated in duplicate microarrays was observed (Figure 2a). Unfortunately, many genes involved in fibrosis (e.g. collagen) were maximally expressed at baseline, and MBG induced increases in the expression of these genes was not possible with the loading conditions employed. However, we were able to identify a cluster of genes with common functions in inflammation, proliferation, and fibrosis as a target of the MBG effect (Figure 2b). For example, we found changes in the expression of various chemokines including chemokine (C-X-C motif) ligand 6, chemokine (C motif) ligand 1, macrophage migration inhibitory factor 1, and defensin β104 within an hour following exposure to MBG. Altered expression of other genes involved in inflammatory response, specifically a gene similar to CG11994-PA (adenine deaminase), a gene similar to galectin 9, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1, 15-hydroxyprostaglandin dehydrogenase, small proline rich protein 3, and lymphocyte cytosolic protein 1 (L-plastin) were also observed. Other immune regulatory genes, such as the peptidoglycan recognition protein 1 and β-defensin 104, were also targeted by MBG. Genes involved in cell growth regulation including two of the genes in the drosophila FAT signaling pathway (5), the FAT tumor suppressor homolog 4 and LATS, large tumor suppressor, homolog 1, as well as the DENN/MADD domain containing 4B (DENND4B or KIAA0476) gene, were also either up- or downregulated by MBG, respectively. We also found the induction of a small
cluster of zinc and ring finger transcription factors including the ring finger and CCCH-type zinc finger domains 1 (RC3H1 or KIAA2025), zinc finger proteins 214, 225, and 322A, and transcription factor 21, by MBG.

ADAMTS (A Disintegrin And Metalloproteinase domain, with Thrombospondin type-1 modules) is a recently described family of zinc-dependent proteases which play important roles in a variety of normal and pathological conditions, including arthritis and cancer (1). Some members of this family of proteins have recently been shown to be involved in tissue fibrosis (15). Therefore, altered expression of a cluster of genes including the thrombospondin, type I, domain containing 2, the TAK1-binding protein 3, and sal-like 4 (SALL4) by MBG may be important for the onset of fibrosis. Coordinated changes in the expression of cadherins (protocadherin 21 and cadherin 7, type 2), the actin-bundling protein L-plastin, kelch-like protein 2 (a synaptotagmin-like protein) and KIAA1204 (a GTPase-activating protein for Cdc42) were also observed.

Next, we examined the effects of cardiac steroids on in vitro wound healing. Human dermal fibroblasts were grown to confluence and then injured with a 10 μl pipette tip as described above. These cells were either exposed to vehicle, or to MBG, ouabain or digoxin at concentrations ranging from 0.1 nM to 100 nM. We found that MBG, ouabain and digoxin significantly accelerated wound closure in this in vitro wound healing model at concentrations as low as 1 nM. Each of these cardiotonic steroids had similar effects on wound healing (Table 1).

Next we examined the effects of cardiotonic compounds on the in vivo model of wound closure in the rat. Rats were wounded with an 8 mm biopsy punch, creating a full thickness lesion on both flanks. We first treated the wounds with digoxin at 10 nM
concentration administered in petroleum jelly, but no effects were noted on wound closure (data not shown). Next, we examined the partition coefficient of radioactive digoxin between petroleum jelly and saline and found it to be nearly infinite. Thus, we sought a carrier with a more practical partition coefficient and a mixture of olive oil: saline was found to have a partition coefficient of 3:1. Based on this, we chose to treat wounds with digoxin 30 nM in olive oil and olive oil alone. We observed that digoxin administered in this fashion led to a substantial acceleration of wound closure based on digital photographs which were analyzed similarly to that described for the in vitro model (figure 3a). Histological analysis also revealed that a greater amount of dermal collagen appeared to be present in the wound area (figure 3b). Quantification of the Sirius Red and Fast Green data confirmed this subjective assessment demonstrating larger amounts of collagen in the wound area (figure 3c).
Discussion:

We describe in this study a potent effect for cardiotonic steroids on collagen production by human dermal fibroblasts that were more prominent than we previously reported using rat cardiac fibroblasts, at least as compared to basal production by those cells. More importantly, we observed that cardiotonic steroids promote and accelerate wound healing both in an *in vitro* and *in vivo* model of wound healing, raising the real possibility that cardiotonic steroids can help in facilitating wound healing in the clinical setting. The ability of the cardiotonic steroids to initiate wound healing was corroborated by gene expression profiling analysis of MBG added to human dermal fibroblasts. We observed distinct temporal changes in the expression of genes involved in inflammation and fibrosis, that are consistent with the onset of wound healing (6, 9), that is characterized by changes in the expression of various inflammatory chemokines (chemokine (C-X-C motif) ligand 6, chemokine (C motif) ligand 1, macrophage migration inhibitory factor 1, and defensin 104), and genes involved in the inflammatory response (protein similar to CG11994-PA (adenine deaminase), a gene similar to galectin 9, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1, 15-hydroxyprostaglandin dehydrogenase, small proline rich protein 3, and L-plastin), and genes that drive fibrogenesis (thrombospondin, type I, domain containing 2, the TAK1-binding protein 3, and sal-like 4).

Our data, specifically the relatively low threshold for the cardiotonic steroid induced dermal fibroblast stimulation which corresponds closely to the circulating concentrations of MBG and other cardiotonic steroids seen in clinical scenarios, begs the question of whether rapid wound healing and/or excessive fibrosis is observed in the skin
of such afflicted patients. Unfortunately, the answer at this point is only a very qualified “yes.” Patients with end stage renal disease, the clinical condition which is associated with some of the very highest-circulating concentrations of cardiotonic steroids have been reported to occasionally manifest a progressive dermal fibrotic condition called nephrogenic sclerosing dermopathy. This condition is quite similar to the disease, scleroderma, but appears to be unique to the setting of renal disease (21). Recently, this syndrome has been associated with exposure to gadolinium when magnetic resonance imaging studies are performed in patients with advanced renal failure, but the pathophysiology is still very poorly understood (12). Also, people of African extraction are known to have substantially higher prevalence of volume-sensitive, low renin, hypertension compared with age and gender matched Caucasians or Asians, as well as a higher incidence of excessive scar formation following surgical or incidental wounds (13, 19). Unfortunately, there has not been any substantial attempt to systematically explore a connection between circulating concentrations of cardiotonic steroids and these clinical skin conditions, at least to the best of our knowledge, and our comments on the possible association is highly speculative.

Our data in the in vivo wound healing experiments (using one cardiotonic steroid at a single concentration) suggest that cardiotonic steroids may be helpful in situations where stimulation of fibroblast collagen production is desirable, as in accelerating wound healing. We would also stress that many of these cardiotonic steroids are either found naturally or have been used clinically for many years at concentrations far beyond what would be achieved with topical administration (17). Further development of this class of agents as therapeutics administered in a topical manner may be of interest in either
accelerating wound repair or increasing dermal collagen production in settings involving non wounded skin where increased dermal collagen would also be desirable.

Acknowledgements:

Portions of this study were supported by the National Institutes of Health (HL67963).

The authors would like to thank Ms. Carol Woods for her excellent secretarial assistance.
Table 1: Effect of Cardiotonic Steroids on In Vitro Wound Healing

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>100 pM</th>
<th>1.0 nM</th>
<th>10 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>100+/−3</td>
<td>100+/−3</td>
<td>100+/−3</td>
<td>100+/−3</td>
</tr>
<tr>
<td>3 hr</td>
<td>88+/−3</td>
<td>78+/−3*</td>
<td>61+/−2**</td>
<td>64+/−3**</td>
</tr>
<tr>
<td>7 hr</td>
<td>57+/−6</td>
<td>50+/−3</td>
<td>34+/−2**</td>
<td>37+/−2**</td>
</tr>
<tr>
<td>12 hr</td>
<td>27+/−3</td>
<td>15+/−3**</td>
<td>10+/−2**</td>
<td>7+/−2**</td>
</tr>
<tr>
<td>Ouabain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>100+/−3</td>
<td>100+/−8</td>
<td>100+/−3</td>
<td>100+/−3</td>
</tr>
<tr>
<td>3 hr</td>
<td>84+/−2</td>
<td>81+/−4</td>
<td>75+/−3**</td>
<td>72+/−2**</td>
</tr>
<tr>
<td>7 hr</td>
<td>56+/−4</td>
<td>43+/−3**</td>
<td>35+/−3**</td>
<td>39+/−3**</td>
</tr>
<tr>
<td>12 hr</td>
<td>22+/−3</td>
<td>10+/−2**</td>
<td>11+/−3**</td>
<td>12+/−2**</td>
</tr>
<tr>
<td>Digoxin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hr</td>
<td>100+/−3</td>
<td>100+/−5</td>
<td>100+/−3</td>
<td>100+/−3</td>
</tr>
<tr>
<td>3 hr</td>
<td>84+/−2</td>
<td>83+/−3</td>
<td>66+/−4**</td>
<td>66+/−2</td>
</tr>
<tr>
<td>7 hr</td>
<td>57+/−4</td>
<td>49+/−4</td>
<td>32+/−3**</td>
<td>33+/−3**</td>
</tr>
<tr>
<td>12 hr</td>
<td>24+/−2</td>
<td>16+/−5</td>
<td>9+/−3**</td>
<td>9+/−3**</td>
</tr>
</tbody>
</table>

Data expressed as mean+/SEM of N=6 samples (with each sample read digitally in triplicate. * p< 0.05, ** p< 0.01 vs Control.)
Literature Cited:


Figure Legends:

Figure 1: Production of collagen by dermal fibroblasts in response to cardiotonic steroids. Panel a demonstrates Western blot analysis of procollagen synthesis by human dermal fibroblasts exposed to three different concentrations of MBG. Cells grown to confluence and serum starved prior to study. Panel b demonstrates radiolabeled proline incorporation in human dermal fibroblasts grown to confluence. Open bars refer to the supernatant and solid bars refer to matrix (residual after removing supernatant and washing with buffered saline). Panel c shows radiolabeled proline incorporation induced by MBG alone or in combination with the Src inhibitor, PP2 (1 M) or the antioxidant, N-Acetyl Cysteine (NAC, 5 mM). All data shown as mean +/- SEM of 6 determinations in each group. ** P< 0.01 vs. control.

Figure 2: Dermal fibroblast gene expression patterns following exposure to MBG.

Panel a illustrates a dendrogram of MBG induced temporal gene expression changes compared with controls. Primary cultures of human dermal fibroblasts were treated with 10nM MBG as described in Materials and Methods for 0, 0.25, 0.5, 0.75, 1, 2, 3, 6, 9, 12, 15, and 24 hrs, compared to untreated cells sampled at the same time points. Microarray was conducted in duplicate and expression data were subjected to cluster analysis using hierarchical cluster algorithm and then displayed using the TreeView software suite. Gene expression signal intensities are depicted using a log2 pseudocolor scale. Panel b illustrates selected subset of genes that were specifically up- or downregulated in respond to MBG.
Figure 3: Wound closure data obtained from in vivo experiment. Panel a shows quantitative wound closure data with digoxin administered in olive oil (OO) or in petroleum jelly (PJ). Panel b shows representative trichrome (top figures) and Sirius red (bottom figures) obtained from wounds treated with olive oil carrier and annotated as follows: A- Epidermis, B- Dermis, C, Muscle, D-Residual wound. Panel c shows quantitative measurement of collagen eluted from Sirius Red/Fast Green stained slides obtained from rats treated with olive oil carrier. Data in panel a and panel c shown as mean +/- SEM of N=20 determinations (olive oil) or N=10 determinations (petroleum jelly). * p< 0.05 and ** p<0.01 vs control.
Procollagen Expression (fraction control)

Control      100 pM      1.0 nM      10 nM

MBG

138kDa ($\alpha_2$)  
129kDa ($\alpha_1$)

Figure 1a

129kDa ($\alpha_2$)  
138kDa ($\alpha_1$)
Figure 1b
Figure 1c
Figure 2a
Figure 2b

- Hypothetical protein LOC283232
- Golgin-87
- Thrombospondin, type I, domain containing 2
- Hypothetical gene supported by AK098812
- 3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)
- Zinc metalloprotease (STE24 homolog, yeast)
- Hypothetical protein LOC289084
- Hypothetical protein MGC16291
- Ribosomal protein S16
- U2 (RN2U) small nuclear RNA auxiliary factor 1-like 2
- KIAA1204 protein
- Peptidoglycan recognition protein 1
- Chemokine (C-X-C motif) ligand 6 (Granulocyte chemotactic protein 2)
- G protein-coupled receptor 89
- Chemokine (C motif) ligand 1
- Lymphocyte cytosolic protein 1 (L-plasmin)
- FLJ41170 protein
- U117/U12 snRNP 65K
- Peptidase (mitochondrial processing) alpha
- RAB26, member RAS oncogene family
- Similar to CG11994-PA (Adenosine deaminase)
- Similar to galectin-9
- Defensin β 104
- Protocadherin 21
- Cadherin 7, type 2
- Cofactor required for Sp1 transcriptional activation, subunit 3, 130 kDa
- CipX, Caseinolytic protease X homolog (E. coli)
- Solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 15
- Pleckstrin homology domain containing, family E (with leucine rich repeats) member 1
- BXMAB2-16 (Glycine-N-acetyltransferase-like 2)
- RCD1 required for cell differentiation1 homolog (S. pombe)
- THAP domain containing 4
- Keratin, hair, acidic, 6
- Keratin associated protein 10-5
- KIAA2825
- Zinc finger protein 322A
- 15-Hydroxyprostaglandin dehydrogenase
- Zinc finger protein 225
- Kelch like protein 15
- Zinc finger protein 214
- Macrophage migration inhibitory factor
- Similar to galectin-9
- Small proline-rich protein 3
- LAT5, large tumor suppressor, homolog 1 (Drosophila)
- TAK1-binding protein 1
- FAT tumor suppressor homolog 4 (Drosophila)
- Sal-like 4 (Drosophila)
Figure 3a
Collagen Content (fraction control)

Total Protein
Collagen

Figure 3c

Control
Digoxin

Collagen Content (fraction control)

**
SUMMARY

Aberrant wound healing currently places a physical and financial burden on patients and society. Current interventions attempting to attenuate wound healing have had little success, and the etiology of several fibrosing diseases are still poorly understood and seldom controlled. Here we attempt to employ a novel mechanism of increased collagen production and fibrosis from a setting of renal failure to one of dermal wound healing.

Results indicated increased collagen synthesis in human dermal fibroblasts post-treatment with MBG to have an eight-fold increase in production. Using a wound healing scratch assay and digital imaging, wound closure was found to notably increase in cells treated with cardiotonic steroids. Genetic analysis via microarray yielded increased gene expression of inflammatory cytokines, pro-fibrotic genes, and genes involved with increased proliferation. Verification of the accelerated wound healing effect was also illustrated in an in vivo model using Sprague-Dawley rats and an olive vector for treatment with digitalis. Thus, treatment with cardiotonic steroids elicits an effect similar to what is seen in renal failure models, serving as potential technique to modulate wound healing processes in fibrotic diseases, such as uremic cardiomyopathy and nephrogenic sclerosing dermopathy, as well as hasten wound closure rates in individuals post-surgically.

Future work should consist of studying gene profiling, histology, and in vivo trials at more time points. It is especially important to focus on the inflammatory phase of wound healing, as gene profiling reveals several inflammatory mediators upregulated in this wound healing process. Cell migration studies may yield robust information on the
exact mechanisms and cross-talk cardiotonic steroids provide. A thymidine incorporation
assay may also be useful in determining the rate of proliferation MBG induces. Taken
together, these data show promise in attenuating the wound healing process, and can
potentially direct the production of future therapeutics.
CONCLUSIONS

The following preliminary conclusions can be drawn from the present study:

1) Treatment of human dermal fibroblasts with cardiotonic steroids, especially MBG, produces increased collagen synthesis in a dose and time-dependent manner.

2) Using an *in vitro* wound healing model, the wound closure rate of skin fibroblasts wounded with a pipette tip was significantly increased with treatment of digoxin, ouabain and marinobufagenin, as monitored by digital microscopy.

3) Microarray analysis of the genetic profile of dermal skin fibroblasts treated with MBG reveals increased gene expression of inflammatory cytokines, proliferative, and profibrotic genes.

4) Administration of digoxin in an *in vivo* wound healing model, using olive oil as a vector, notably accelerated *in vivo* wound healing in surgically wounded rats. Because significant increases in collagen accumulation were noted 9 days after wounding, the accelerated wound closure appears to be dependent on increased matrix deposition.
ABSTRACT

We previously reported that cardiotonic steroids stimulate collagen synthesis by cardiac fibroblasts in a process that involves signaling through the Na/K-ATPase pathway. In this study, we examined the effect of cardiotonic steroids on dermal fibroblasts collagen synthesis and on wound healing. Increased collagen expression by human dermal fibroblasts was noted in response to the cardiotonic steroid, marinobufagenin, in a dose and time dependent-fashion. Next, we examined the effect of digoxin, ouabain and marinobufagenin on the rate of wound closure in an in vitro model where human dermal fibroblasts cultures were wounded with a pipette tip and monitored by digital microscopy. Finally, we administered digoxin in an in vivo wound healing model, using olive oil as a vector. This application significantly accelerated in vivo wound healing in rats wounded with an 8 mm biopsy cut. Increased collagen accumulation was noted 9 days after wounding (both p< 0.01). These data suggest that cardiotonic steroids induce increases in collagen synthesis by dermal fibroblasts, and accelerate wound healing.
REFERENCES


57


34 Flier, J, Edwards, MW, Daly, JW, and Myers, CW. (1980). Widespread occurrence in frogs and toads of skin compounds interacting with the ouabain site of Na+, K+-ATPase. Science 208:503-505


