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Effects of cardiotonic steroids on dermal collagen synthesis and wound healing

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El-Okdi N, Smaili S, Raju V, Shidyak A, Gupta S, Fedorova L, Elkareh J, Periyasamy S, Shapiro AP, Kahaleh MB, Malhotra D, Xie Z, Chin KV, Shapiro JI. Effects of cardiotonic steroids on dermal collagen synthesis and wound healing. *J Appl Physiol* 105: 30–36, 2008. First published May 15, 2008; doi:10.1152/japplphysiol.00119.2008.—We previously reported that cardiotonic steroids stimulate collagen synthesis by cardiac fibroblasts in a process that involves signaling through the Na-K-ATPase pathway (Elkareh et al. *Hypertension* 49: 215–224, 2007). 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 h ($P < 0.01$). Similar increases in proline incorporation were seen following treatment with digoxin, ouabain, and marinobufagenin (10 nM × 24 h, all results $P < 0.01$ vs. control). The coadministration of the Src inhibitor PP2 or *N*-acetylcysteine 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 induce increases in collagen synthesis by dermal fibroblasts, as could potentially be exploited to accelerate wound healing.

digitalis-like substances; sodium-potassium-adenosinetriphosphatase; signaling; fibrosis

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 level observed in experimental renal failure induced cardiac fibrosis to a similar degree, but immunization against MBG before 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-reactive oxygen species cascade (8).

As the rate of collagen formation plays a major role in wound healing (18), and because low concentrations of cardiotonic steroids, some of which are commonly employed in clinical medicine, have dramatic effects on collagen production, we examined the effect of these compounds on dermal collagen synthesis and wound healing.

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 h). 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 Bioanalyzer (Agilent, Santa Clara, CA). Gene expression profiling was conducted using the Human OneArray (Phalanx Biotechnology) 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-in. × 3-in. glass slides.

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 mRNA 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 mRNA 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

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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 no. 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; http://rsb.info.nih.gov/ij/).

In vivo wound healing. All of the animal experimentation described in this study 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.

Male Sprague-Dawley rats weighing 300–350 g were subjected to full-thickness skin biopsy performed with a 8.0-mm punch biopsy (item no. 501912, Harris Uni-core, World Precision Instruments, 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 (no. 25 mm Chamber System, Hill Top Research, Miamiville, OH). Digital photographs were taken immediately after and 1, 2, 3, 4, 7, and 9 days following wounding. Photographs were analyzed with ImageJ as described in the vitro wound healing model. At 9 days, animals were euthanized, and the healing wound was 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, deparrafinized sections were preincubated in the dark (covered with aluminum foil) with a solution of 0.1% fast green in picric acid for 15 min 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 min 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 0.1 N NaOH and absolute methanol. One milliliter of elution solution was added to each section. The eluted stain was then read at 605 nm and 540 nm using a spectrophotometer with quantification as described previously (14). Collagen and total protein are expressed as a fraction of control samples.

Statistical analysis. Data presented are means ± SE. 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 ANOVA was performed before 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

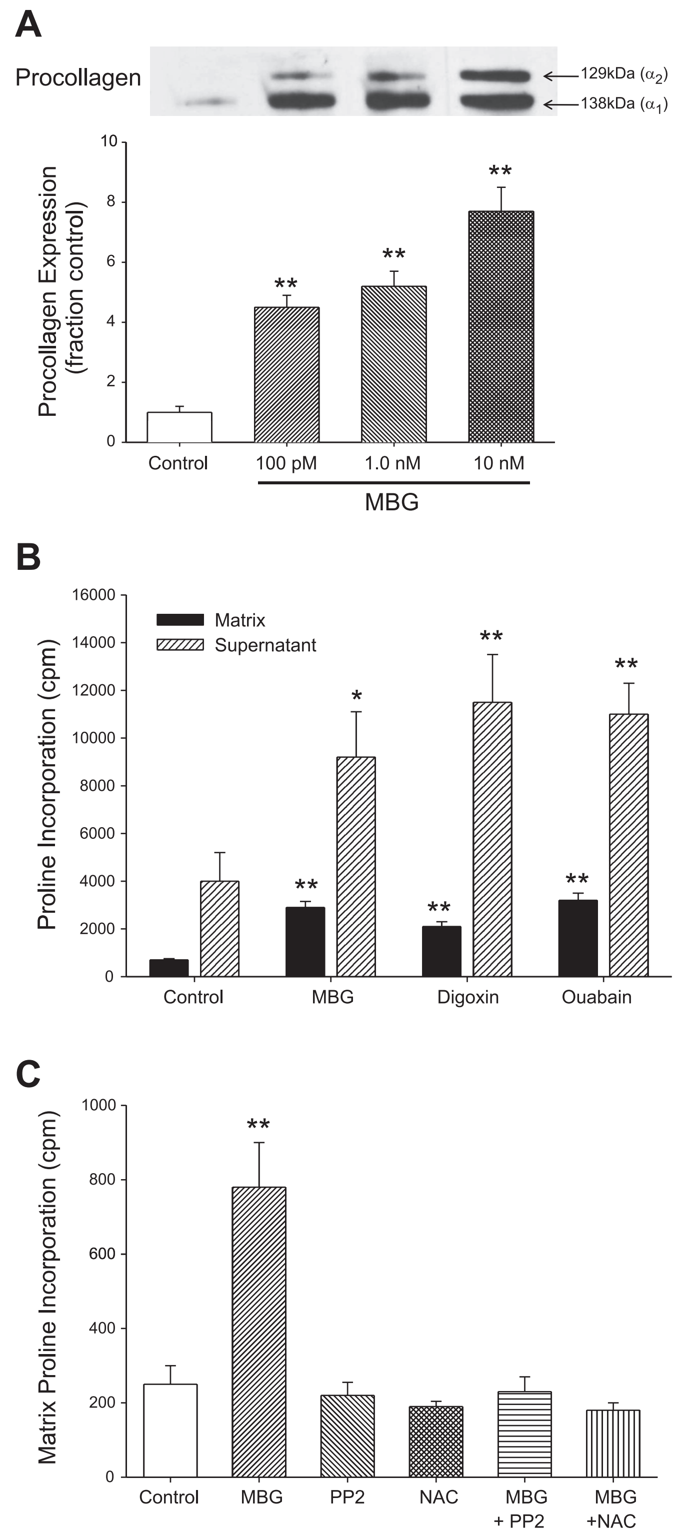

Fig. 1. Production of collagen by dermal fibroblasts in response to cardiotonic steroids. *A*: Western blot analysis of procollagen synthesis by human dermal fibroblasts exposed to 3 different concentrations of marinobufagenin (MBG). Cells were grown to confluence and serum-starved before study. *B*: radiolabeled proline incorporation in human dermal fibroblasts grown to confluence. Hatched bars refer to the supernatant, and black bars refer to matrix (residual after removing supernatant and washing with buffered saline). *C*: radiolabeled proline incorporation induced by MBG alone or in combination with the Src inhibitor PP2 (1 μM) or the antioxidant *N*-acetylcysteine (NAC, 5 mM). All data are shown as means ± SE of 6 determinations in each group. *$P < 0.05$, **$P < 0.01$ vs. control.

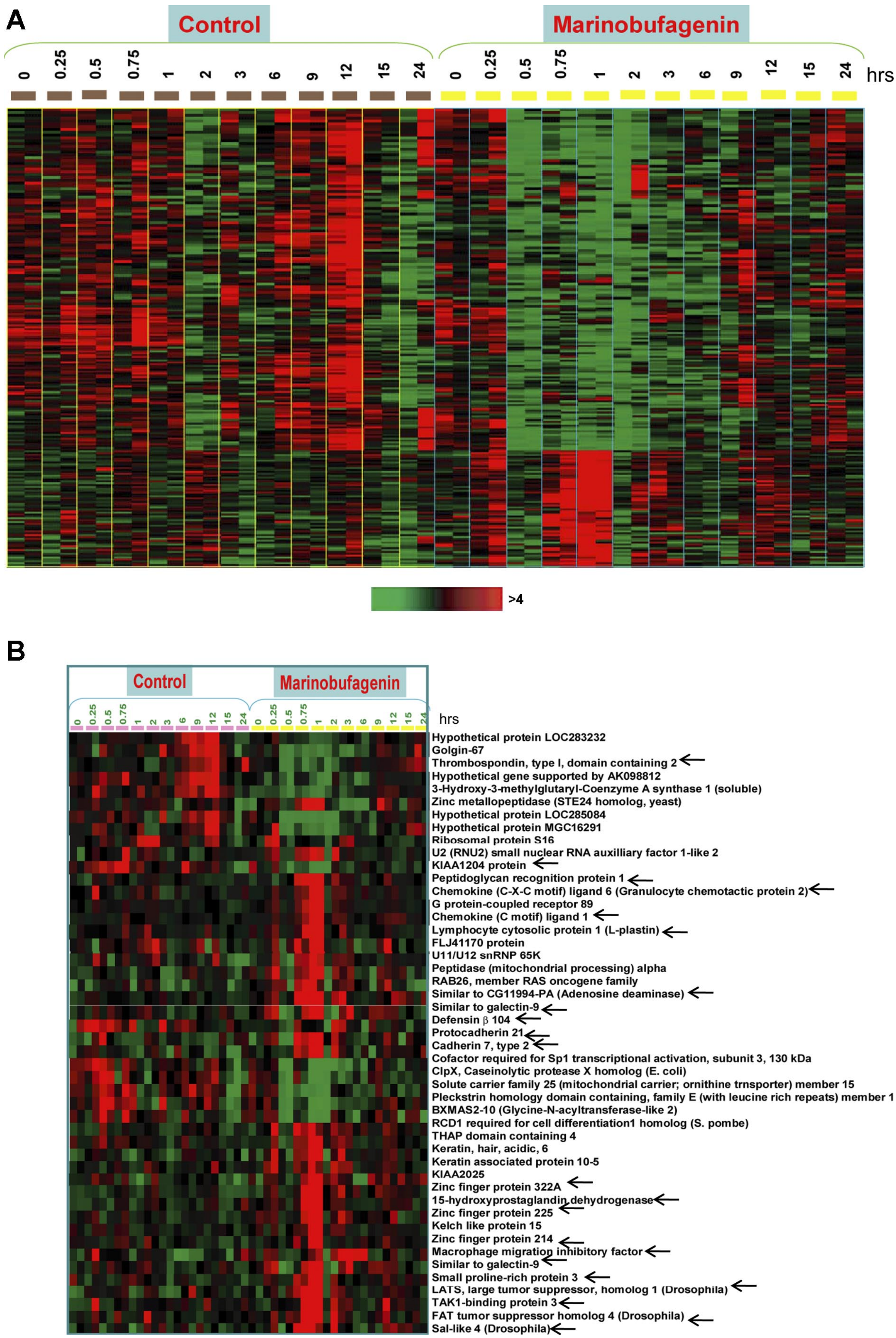
A
Control
Marinobufagenin
0 0.25 0.5 0.75 1 2 3 6 9 12 15 24
0 0.25 0.5 0.75 1 2 3 6 9 12 15 24 hrs
>4
B
Control
Marinobufagenin
0 0.25 0.5 0.75 1 2 3 6 9 12 15 24
0 0.25 0.5 0.75 1 2 3 6 9 12 15 24
hrs
Hypothetical protein LOC283232
Golgin-67
Thrombospondin, type I, domain containing 2
Hypothetical gene supported by AK098812
3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)
Zinc metallopeptidase (STE24 homolog, yeast)
Hypothetical protein LOC285084
Hypothetical protein MGC16291
Ribosomal protein S16
U2 (RNU2) small nuclear RNA auxilliary 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-plastin)
FLJ41170 protein
U11/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
ClpX, Caseinolytic protease X homolog (E. coli)
Solute carrier family 25 (mitochondrial carrier; ornithine trnsporter) member 15
Pleckstrin homology domain containing, family E (with leucine rich repeats) member 1
BXMAS2-10 (Glycine-N-acyltransferase-like 2)
RCD1 required for cell differentiation1 homolog (S. pombe)
THAP domain containing 4
Keratin, hair, acidic, 6
Keratin associated protein 10-5
KIAA2025
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
LATS, large tumor suppressor, homolog 1 (Drosophila)
TAK1-binding protein 3
FAT tumor suppressor homolog 4 (Drosophila)
Sal-like 4 (Drosophila)

Table 1. *Effect of cardiotonic steroids on in vitro wound healing*

	Control	100 pM	1.0 nM	10 nM
MBG				
0 h	100±3	100±3	100±3	100±3
3 h	88±3	78±3*	61±2†	64±3†
7 h	57±6	50±3	34±2†	37±2†
12 h	27±3	15±3†	10±2†	7±2†
Ouabain				
0 h	100±3	100±8	100±3	100±3
3 h	84±2	81±4	75±3†	72±2†
7 h	56±4	43±3†	35±3†	39±3†
12 h	22±3	10±2†	11±3†	12±2†
Digoxin				
0 h	100±3	100±5	100±3	100±3
3 h	84±2	83±3	66±4†	66±2
7 h	57±4	49±4	32±3†	33±3†
12 h	24±2	16±5	9±3†	9±3†

Data are expressed as means ± SE of $n = 6$ samples (with each sample read digitally in triplicate) with each sample read as % of average initial value. MBG, marinobufagenin. *$P < 0.05$, †$P < 0.01$ versus control.

(Fig. 1*A*) and radiolabeled proline incorporation (Fig. 1, *B* and *C*). 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 (Fig. 1*B*). Procollagen expression (or acceleration of in vitro wound closure; see below) was not further increased by simultaneous addition of MBG and ouabain or digoxin and ouabain. Coadministration of *N*-acetylcysteine or inhibition of Src with PP2 blocked the stimulation of collagen synthesis by MBG (Fig. 1*C*). 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 genomewide 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 h) was conducted to examine the temporal changes in gene expression in response to MBG (Fig. 2). RNA was 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 are displayed by TreeView software (7) (Fig. 2*B*).

A distinct pattern of gene expression was observed that was recapitulated in duplicate microarrays (Fig. 2*A*). Unfortunately, many genes involved in fibrosis (e.g., col1a) 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 (Fig. 2*B*). 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 β 104 within 1 h 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 that 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

Fig. 2. Dermal fibroblast gene expression patterns following exposure to MBG. *A*: dendrogram of MBG-induced temporal gene expression changes compared with controls. Primary cultures of human dermal fibroblasts were treated with MBG as described in MATERIALS AND METHODS for 0, 0.25, 0.5, 0.75, 1, 2, 3, 6, 9, 12, 15, and 24 h compared with 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. *B*: selected subset of genes that were specifically up- or downregulated in response to MBG. Arrows indicate up- or downregulated genes (see RESULTS for further description).

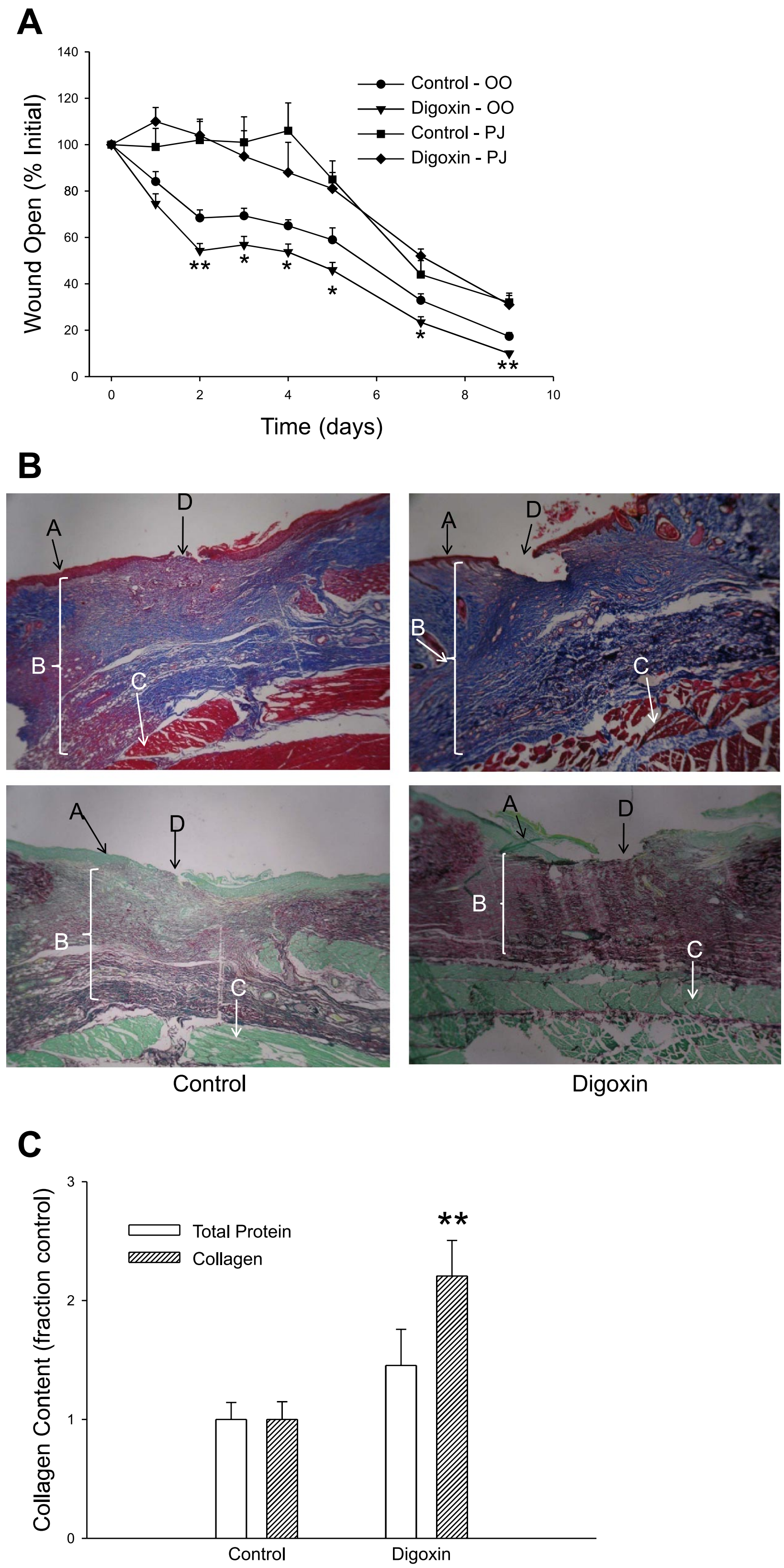

Fig. 3. Wound closure data obtained from in vivo experiment. *A*: quantitative wound closure data with digoxin administered in olive oil (OO) or in petroleum jelly (PJ). *B*: representative trichrome (*top* panels) and Sirius red (*bottom* panels) obtained from wounds treated with olive oil carrier and annotated as follows: epidermis (A), dermis (B), muscle (C), and residual wound (D). *C*: quantitative measurement of collagen eluted from Sirius red/fast green-stained slides obtained from rats treated with olive oil carrier. Data in *A* and C are shown as means ± SE of $n = 20$ determinations (olive oil) or $n = 10$ determinations (petroleum jelly). *$P < 0.05$, **$P < 0.01$ vs. control.

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. On this basis, we chose to treat wounds with 30 nM digoxin in olive oil or with olive oil alone. We observed that digoxin administered in this fashion led to a substantial acceleration of wound closure on the basis of digital photographs that were analyzed similarly to that described for the in vitro model (Fig. 3*A*). Histological analysis also revealed that a greater amount of dermal collagen appeared to be present in the wound area (Fig. 3*B*). Quantification of the Sirius red and fast green data confirmed this subjective assessment demonstrating larger amounts of collagen in the wound area (Fig. 3*C*).

DISCUSSION

In this study, we describe a potent effect for cardiotonic steroids on collagen production by human dermal fibroblasts that was more prominent than that which we previously reported in rat cardiac fibroblasts. 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 β 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 that corresponds closely to the circulating concentrations of MBG and other cardiotonic steroids seen in clinical scenarios, beg the question 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 that 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 sex-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 nonwounded skin where increased dermal collagen would also be desirable.

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GRANTS

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