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Adenovirus-mediated delivery of relaxin reverses cardiac fibrosis

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Abstract

We have evaluated the effectiveness of systemic adenovirally delivered mouse relaxin on reversing fibrosis in a transgenic murine model of fibrotic cardiomyopathy due to β_2-adrenergic receptor (β_2AR) overexpression. Recombinant adenoviruses expressing green fluorescent protein (Ad-GFP), rat relaxin (Ad-rRLN) and mouse relaxin (Ad-mRLN) were generated and Ad-rRLN and Ad-mRLN were demonstrated to direct the expression of bioactive relaxin peptides *in vitro*. A single systemic injection of Ad-mRLN resulted in transgene expression in the liver and bioactive relaxin peptide in the plasma. Ad-mRLN, but not Ad-GFP, treatment reversed the increased left ventricular collagen content in β_2AR mice to control levels without affecting collagen levels in other heart chambers or in the lung and kidney. Hence a single systemic injection of adenovirus producing mouse relaxin reverses cardiac fibrosis without adversely affecting normal collagen levels in other organs and establishes the potential for the use of relaxin gene therapy for the treatment of cardiac fibrosis.

Keywords: Relaxin; RXFP1; Fibrosis; Extracellular matrix; Adenovirus; Gene therapy

1. Introduction

Cardiac fibrosis is a process of myocardial remodeling where there is abnormal expansion of the cardiac extracellular matrix (ECM) due to fibroblast activation and excessive accumulation of collagen and other components (Weber, 2000; Tyagi et al., 1996). Fibrosis is a common hallmark of cardiovascular diseases and interferes with normal myocardial architecture and function leading to a variety of pathophysiological problems such as diastolic and systolic dysfunction, increased risk of myocardial ischemia, arrhythmias, sudden cardiac death, and worsening of heart failure (Weber, 2000). Current therapies such as angiotensin-converting enzyme inhibitors and aldosterone inhibitors (Lijnen and Petrov, 2003; Mandarim-de-Lacerda and Pereira, 2003) are effective but slow-acting with undesirable side-effects and thus novel specific and effective therapies, with shorter treatment periods, to abolish or attenuate the development of fibrosis are highly desired.

In recent years, the polypeptide hormone relaxin has surfaced as a potent regulator of collagen in the ECM of multiple organs including reproductive organs, skin, kidney, lung, liver, and heart (Samuel et al., 2003, 2004a,b; Garber et al., 2001; Williams et al., 2001; Unemori et al., 1996; Unemori and Amento, 1990). Investigations into the cardiac phenotype of relaxin gene-knockout mice (RLX−/−) have revealed that there is an age-related development of cardiac fibrosis, leading to altered diastolic function (Du et al., 2003). More importantly, several studies have demonstrated that relaxin delivery via osmotic mini-pumps reverses fibrotic cardiomyopathy in three rodent models of established cardiac fibrosis: (i) in mice lacking the relaxin gene (Samuel et al., 2004a); (ii) in transgenic (TG) mice with cardiac-restricted overexpression of β_2-adrenergic receptors (β_2AR) (Samuel et al., 2004a); and (iii) in spontaneously hypertensive rats (Lekgabe et al., 2005). Together, these studies have established that relaxin is an innate regulator of collagen deposition.

Recombinant adenovirus-mediated gene delivery has been utilized for experimental gene therapy in a variety of heart disorders (Hajjar et al., 2000; Hammond and McKirnan, 2001; Jones

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and Koch, 2005) and pilot clinical trials have also been initiated (Relph et al., 2005). In almost all studies, intra-coronary or intra-myocardial injection of adenovirus was adopted. Furthermore, few studies have tested interventions that specifically target myocardial fibrosis. In this study, we have directly investigated the feasibility of systemic adenoviral-mediated relaxin delivery. We have characterized adenoviruses producing bioactive rat and mouse relaxin peptides and evaluated the effectiveness of intravenous injection of these viruses in a transgenic murine model of cardiac fibrosis. The results demonstrate that systemic relaxin gene delivery can address the complicating factor of cardiac fibrosis associated with cardiovascular diseases, without any notable side-effects.

2. Materials and methods

2.1. Adenovirus construction

The pAd-Easy-1 virus and shuttle plasmid, pAd-TrackCMV, were obtained from Dr. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD (He et al., 1998)). Rat and mouse relaxin full length cDNAs were generated from pregnant ovary cDNA using RT-PCR and ligated into pBS-KS using KpnI and NotI linkers. The constructs were sequenced on both strands to verify the sequence and then subcloned into the shuttle vector, pAdTrack-CMV to yield pAdTrack-rRLN (rat relaxin) and pAdTrack-mRLN (mouse relaxin). The pAdTrack-CMV vector also expresses the green fluorescent protein (GFP) under the control of a separate CMV promoter. Recombinant adenovirus, Ad-GFP, Ad-rRLN and Ad-mRLN were generated by bacterial homologous recombination between pAdTrack-CMV, pAdTrack-rRLN and pAdTrack-mRLN and pAdEasy-1. Large-scale amplification and purification of Ad-GFP (Empty vector), Ad-rRLN and Ad-mRLN viruses was performed as described (He et al., 1998; Brandenburger et al., 2001).

2.2. Adenovirus characterization

The ability of Ad-mRLN and Ad-rRLN viruses to produce bioactive relaxin protein from infected mammalian cells was first tested *in vitro* using HEK-293T cells. Exponentially growing HEK-293T cells were infected with purified adenoviruses at a multiplicity of infection (MOI) of 5. This dose of virus was sufficient to give 100% infectivity as determined by GFP expression using fluorescence microscopy. Approximately 24 h after infection, media was collected and immediately stored at −20 °C and the cells resuspended in lysis buffer (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.1% SDS, 2 μg/ml Aprotinin, 5 μg/ml Leupeptin, 0.7 μg/ml Pepstatin). Cell extracts were then centrifuged at 10,000 × *g* and the supernatant frozen at −20 °C for further analysis. Proteins from the cell media were further purified using a well characterized C-18 mini-column protocol which has been used successfully previously to purify relaxin peptides (Bathgate et al., 2002). All eluted fractions were collected, freeze dried and then resuspended in 100 μl of bi-distilled water and frozen at −20 °C for further analysis.

Western blotting was performed on cell lysates, media and C-18 fractionated proteins from Ad-GFP and Ad-rRLN infected cells as described previously (Bathgate et al., 2002). An antibody (AS#2) which was raised against the native rat relaxin peptide (Gunnersen et al., 1995) was used to visualize immunoreactive proteins. Native rat relaxin extracted from pregnant rat ovaries (Wade et al., 1996) was used as a positive control and Ad-GFP cell extracts and media as negative control. Western blotting on Ad-mRLN infected cells was not performed as an antibody to mouse relaxin is not available and the rat relaxin antisera does not recognize mouse relaxin (data not shown).

The presence of bioactive relaxin peptide in the Ad-GFP, Ad-rRLN and Ad-mRLN infected cell media was tested using HEK-293T cells stably expressing the human relaxin receptor (RXFP1) (Halls et al., 2005). Various amounts of cell media were diluted to a total volume of 200 μl with control media and isobutylmethylxanthine (IBMX) added to a final concentration of 50 μM before being added to RXFP1 stably expressing HEK-293T cells followed by incubation at 37 °C for 30 min. cAMP levels were measured in cell lysates using the cAMP Biotrak EIA system as per the manufacturers protocol (Amersham Corp., NSW, Australia). Recombinant human gene-2 (H2) relaxin (a gift of BAS Medical, San Mateo, CA) and native rat relaxin at various concentrations diluted in media with IBMX were used as positive controls. Relaxin bioactivity in the plasma of Ad-GFP and Ad-mRLN infected mice was tested using HEK-293T cells stably transfected with LGR7 and a pCRE-β-galactosidase reporter gene as previously described (Scott et al., 2006). For individual cell media experiments data are expressed as fmol cAMP accumulated in the cell lysate from a representative experiment. For the dose response and media dilution curves as well as mouse plasma experiments, data is expressed as mean ± S.E.M. of percent maximum H2 relaxin (10 nM) response. All experiments were performed in triplicate. Curves were plotted using the sigmoidal dose response curves in the Graphpad PRISM program (Graphpad Software Inc., San Diego, CA, USA).

2.3. Animals and adenoviral gene delivery

The TG mice used in this study were generated at the Howard Hughes Medical Institute, Duke University Medical Centre (Milano et al., 1994) and subsequently bred at the Baker Heart Research Institute. These TG mice overexpress the β_2AR in the heart by 200-fold and have a mixed genetic background of C57BL and SJL strains. Genotype was determined individually (Milano et al., 1994). In this study, both male and female TG and wild-type (WT; negative littermates) mice of 7 months of age were used. It has been previously well documented that these TG mice develop a fibrotic cardiomyopathy phenotype and have a significant sex-dimorphism, with females having less severe cardiac pathology than males (Gao et al., 2003; Du et al., 2000c). Thus, male and female mice were separated for likely gender-dependent changes. Animals were housed at a constant room temperature with a 12 h light–dark cycle and had free access to water and standard rodent chow. All experiments in this study were performed with the approval of a local animal ethics committee, which adheres to the Australian code of practice for the care and use of laboratory animals for scientific purposes. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

The adenovirus was injected intravenously via the tail vein at a dose of 5×10^{10} pfu/mouse diluted to 200 μl with PBS. This dose is sufficient to give robust expression of transgenes predominantly in the liver but also other organs including the heart and lung (Hannan and Du, unpublished data). Adenoviral gene delivery is transient due to the epichromosomal nature of the virus and the mild immune responses. Following adenovirus gene delivery, expression of the recombinant proteins usually peaks at 3–4 days and disappears 7–10 days later (Munch et al., 2005; Burcin et al., 1999). Accordingly, mice were sacrificed at different time-points; 3 days ($n = 6$), 7 days ($n = 4$), 10 days ($n = 3$) and 14 day ($n = 22$) post-infection to assess the efficiency of the *in vivo* gene therapy. At all time-points, tissue and plasma was collected. Relaxin bioactivity assay was performed on the plasma collected, whereas the tissue collected was used for assessment of GFP expression. To assess collagen content, histology and hydroxyproline assay was carried out on the tissue collected at 14 days post-infection.

2.4. Assessment of GFP transgene expression by confocal microscopy

We isolated the LV, kidney, lung, liver and spleen of mice at 3 days, 7 days, 10 days and 14 days post-infection. Tissues were subsequently fixed overnight in 10% neutral buffered formaldehyde before being processed and embedded in paraffin. Transverse sections (6 μm) were cut and mounted on Super Frost plus charged slides (Menzel-Glaser). Prior to microscopy, sections were deembedded with xylene and a graded series of alcohols, brought to water and cover-slipped using Vectashield mounting solution (H-1000, Vector Labs, Burlingame, CA). To view GFP expression, confocal microscopy was performed using a Zeiss LSM510 META. Excitation was carried out using the 488 nm line on an argon-ion laser. Fluorescent light was collected in the green region of the spectrum (500–550 nm). Digital images were obtained using the Zeiss LSM510 software. All images were collected with an Apochromat 20 × 0.6 Ph2 objective.

2.5. Collagen content quantification: histology and hydroxyproline assays

The heart, kidney and lung were assessed for collagen content after completion of the 14-day treatment period. Left ventricle (LV), right ventricle (RV), and atria of the heart were separated. The apical portion of the LV was fixed, embedded in paraffin, sectioned and stained with Masson's trichrome for quantitative histology. The remainder of the LV and kidneys were snap frozen in liquid nitrogen and stored at −80 °C for hydroxyproline assay. Images of the LV sections were gathered with a CCD video camera (Optimas, Bioscan, Edmonds, WA, USA), digitized, and quantified using the Optimas Live 6.5 program. Approximately 20–25 fields of each LV were analyzed. Interstitial collagen content was determined by measuring the blue stained area per field and the results were averaged and expressed as a percentage of a field. Hydroxyproline content of the LV, RV, atria, and kidney were determined as described previously (Samuel et al., 2004b; Du et al., 2003) and then converted to collagen content by multiplying by a factor of 6.94 (Samuel et al., 1996). Results are expressed as collagen concentration per tissue dry weight.

2.6. Statistical analysis

All results were analyzed using one-way ANOVA and the student Newman–Kuels tests for multiple comparisons between the groups. All data are expressed as the mean ± S.E.M. with statistical significance accepted at the level of $P < 0.05$.

3. Results

3.1. In vitro characterization of Ad-rRLN and Ad-mRLN

Full-length cDNAs for mouse and rat relaxin were cloned from pregnant ovaries and inserted into the pAd-TrackCMV vector and sequenced. Before Ad-rRLN and Ad-mRLN were used in *in vivo* studies they were first tested for their ability to produce immunoreactive relaxin peptide in HEK-293T cells. Ad-GFP infected cells were used as a negative control.

The presence of relaxin peptide was first tested in Ad-rRLN infected cells and media using a rat relaxin antibody and Western blotting. As an antibody to mouse relaxin is not available and the rat relaxin antisera does not recognize synthetic mouse relaxin (data not shown) Western blotting on Ad-mRLN infected cells was not performed. Initial experiments indicated the presence of an immunoreactive band at ∼18 kDa in cell media from Ad-rRLN infected cells, but not Ad-GFP infected cells (data not shown). The immunoreactive band was concentrated over a C-18 minicolumn, where it eluted with 50% and 80% acetonitrile (ACN), but not in the 10% ACN washes. Additionally, there were no immunoreactive bands present in the Ad-GFP infected cell extracts. Hence, the C-18 columns effectively concentrated the immunoreactive band. Samples of cell lysate, cell media, 50% and 80% ACN fractions from both Ad-GFP and Ad-rRLN infected cells were then analyzed by Western blot (Fig. 1). The 18 kDa immunoreactive band is clearly present in cell media and ACN extracts from Ad-rRLN infected cells. A weak band can also be seen in the cell lysate. No immunoreactive bands are seen in the Ad-GFP infected cells. Hence the Ad-rRLN is directing the production of an 18 kDa immunoreactive band which is mostly secreted from the cell. The predicted size of rat prorelaxin based on the known signal peptide cleavage site is 17927.3 Da, suggesting that the 18 kDa immunoreactive band most likely represents a rat prorelaxin peptide. No bands are present at the position of native rat relaxin (∼6 kDa) in either cell lysate or media indicating that no cleaved rat relaxin peptide is produced by the HEK-293T cells. Importantly, previous studies have demonstrated that transfection of mammalian cells with relaxin transgenes results in the production of prorelaxin only and these prorelaxins are equally bioactive to the processed forms (Bathgate et al., 2006).

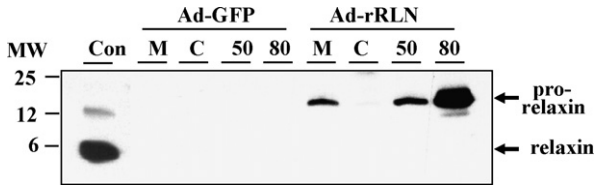

Fig. 1. Analysis of relaxin peptide production by Ad-rRLN infected HEK-293T cells using Western blotting with an anti-rat relaxin antibody. Rat relaxin (500 ng) was used as a positive control (Con). Samples are cell media (M), cell lysate (C) and 50% (50) and 80% (80) ACN fractions of C-18 mini-column purified cell media (see text for details) from HEK-293T cells infected with either Ad-GFP or Ad-rRLN. MW indicates molecular weight in kDa. The positions of the 6 kDa relaxin control band and approximately 18 kDa rat prorelaxin band are indicated.

The ability of Ad-rRLN and Ad-mRLN to produce secreted bioactive relaxin peptide *in vitro* was tested by treatment of RXFP1 stably transfected HEK-293T cells with the media from adenovirus infected cells. The results from one representative batch of Ad-GFP, Ad-rRLN and Ad-mRLN infected HEK-293T cell media are shown in Fig. 2a. Fifty microlitres of media was tested in parallel with a maximal dose (10 nM) of H2 relaxin, the native ligand of human RXFP1. Clearly both Ad-rRLN and Ad-mRLN media stimulates cAMP production from RXFP1 stably transfected cells, whereas Ad-GFP media treated cAMP levels are not significantly different from levels in unstimulated cells (Control). Other batches of Ad-rRLN and Ad-mRLN infected cells demonstrated similar results (data not shown). The Ad-rRLN and Ad-mRLN media was then tested for its ability to elicit a dose response in comparison to H2 relaxin and rat relaxin. Fig. 2b demonstrates that media dilutions were able to parallel the dose response curves of both forms of relaxin. Thus, both Ad-rRLN and Ad-mRLN viruses are able to direct the production and secretion of bioactive relaxin peptide *in vitro*. Additionally, comparison of dose response curves for Ad-rRLN media with that of the rat relaxin peptide allows a rough calculation of the levels of bioactive rat relaxin equivalent peptide in the media. This comparison results in an estimated level of relaxin peptide of 50 ng rat relaxin equivalents/ml of Ad-rRLN infected cell media.

3.2. Assessment of in vivo expression and activity of transgenes after systemic delivery

The ability of Ad-mRLN to direct the secretion of bioactive relaxin peptide into the plasma after systemic injection of virus was tested in conjunction with the localization of GFP transgene expression in tissues. The plasma samples from infected mice were also tested for their ability to activate RXFP1 sta-

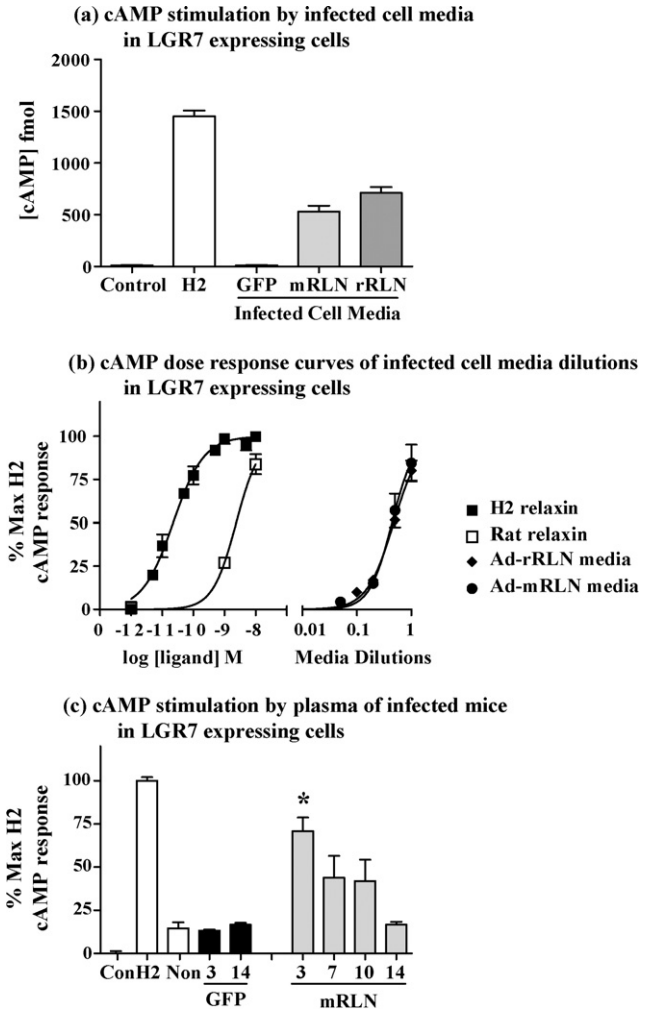

Fig. 2. (a) Analysis of the presence of bioactive relaxin peptide in the cell media from Ad-GFP (GFP), Ad-mRLN (mRLN) and Ad-rRLN (rRLN) infected HEK-293T cells. Relaxin activity was measured as cAMP stimulation in HEK-293T cells stably expressing RXFP1. H2 relaxin (H2 at 10 nM) was used as a positive control. Data are from one representative experiment performed in triplicate. (b) Dose response relationship of different dilutions of Ad-rRLN and Ad-mRLN infected cell media in comparison to H2 relaxin and rat relaxin dose response curves. Data are the mean ± S.E.M. of triplicate determinations and are expressed as a percentage of the maximum H2 relaxin response. (c) Analysis of the presence of bioactive relaxin peptide in plasma of mice infected with Ad-GFP (GFP; 3 and 14 days post-infection) and Ad-mRLN (mRLN; 3, 7, 10 and 14 days post-infection) compared to non-infected animals (Non). Relaxin activity was measured as cAMP stimulation in HEK-293T cells stably expressing RXFP1. H2 relaxin (H2 at 10 nM) has been used as a positive control and non-treated cells have been used as a negative control (Con). Data are the mean ± S.E.M. of triplicate determinations. *$P < 0.05$ vs. GFP control.

bly transfected HEK-293T cells in parallel to a H2 relaxin positive control and plasma from Ad-GFP infected and non-infected mouse controls. As can be seen in Fig. 2c, plasma from Ad-mRLN infected mice 3-day post-infection demonstrated a significant increase in activity compared to Ad-GFP infected and non-infected animals ($P < 0.05$). One of three animals at both 7-day and 10-day post-infection also showed increased relaxin bioactivity, however the other two animals in each group did not demonstrate activity above control levels. No relaxin bioactivity was observed in any animal 14-day post-infection. This data demonstrates that Ad-mRLN is able to produce bioactive relaxin peptide *in vivo* which is secreted into the plasma of infected mice for up to 7-day post-infection. A crude estimate of relaxin activity in the plasma calculated as above, suggests that the plasma levels in Ad-mRLN infected mice is 100 ng rat relaxin equivalent/ml.

Successful viral delivery and the tissue sites of transgene expression were determined by detecting GFP expression using confocal microscopy. The liver was the predominate site of transgene expression in all animals with only very weak GFP expression being seen in other organs in some animals (data not shown). Fig. 3 demonstrates representative images of the liver from an uninfected control mouse (Fig. 3a), 3-day post Ad-GFP infection (Fig. 3b), 3-day post Ad-mRLN infection (Fig. 3c), 7-day post Ad-mRLN infection (Fig. 3d), 10-day post Ad-mRLN infection (Fig. 3e) and 14-day post Ad-mRLN infection (Fig. 3f). GFP expression was highest 3-day post-infection and was detectable in the liver of all animals studied. Expression was concentrated around major blood vessels with robust GFP expression clearly present in hepatic vascular endothelial cells with moderate to extensive invasion of the adjacent hepatocytes. Levels were reduced by 7- and 10-days post-infection and were lower in those animals which had low plasma bioactivity levels as shown in Fig. 2c.

3.3. Effect of adenoviral relaxin gene transfer

The TG mouse model with cardiac-restricted overexpression of β_2AR used in this study developed an age-dependent increase in LV collagen concentration. Hydroxyproline assay of the LV demonstrates a significant increase in collagen concentration as early as 3 months (Fig. 4a). Hence, all further *in vivo* experiments to investigate the antifibrotic effect of relaxin in the heart were carried out using 7-month TG mice with well established cardiac fibrosis. High levels of collagen concentration (male $P < 0.05$ and female $P < 0.01$, Fig. 4b) and content (male $P < 0.05$ and female $P < 0.01$; Fig. 5) were consistently detected in the LV of β_2AR mice compared to WT mice, using both hydroxyproline assay and quantitative histology, thereby confirming the presence of cardiac fibrosis. The rise in collagen observed in the LV was similar in both males and females. In contrast to the findings in the LV, at this age studied, there was no significant increase in collagen concentration in the other chambers of the heart (RV and atria) and in other organs such as the kidney (Fig. 4c) and lung (results not shown) from TG animals. Adenoviral gene transfer of mouse relaxin led to a significant reduction in the elevated extracellular collagen in the LV of the TG mice as detected by histology (male $P < 0.05$ and females $P < 0.01$; Fig. 5) and hydroxyproline assay (male $P < 0.05$ and female $P < 0.01$; Fig. 4b). This therapy had similar efficiency in males and females. Importantly, collagen levels in the other chambers of the heart (Fig. 4c), kidney (Fig. 4c) and lung (results not shown) remained unaffected with relaxin gene therapy, demonstrating that relaxin specifically attenuates the accumulation of collagen in diseased tissues only.

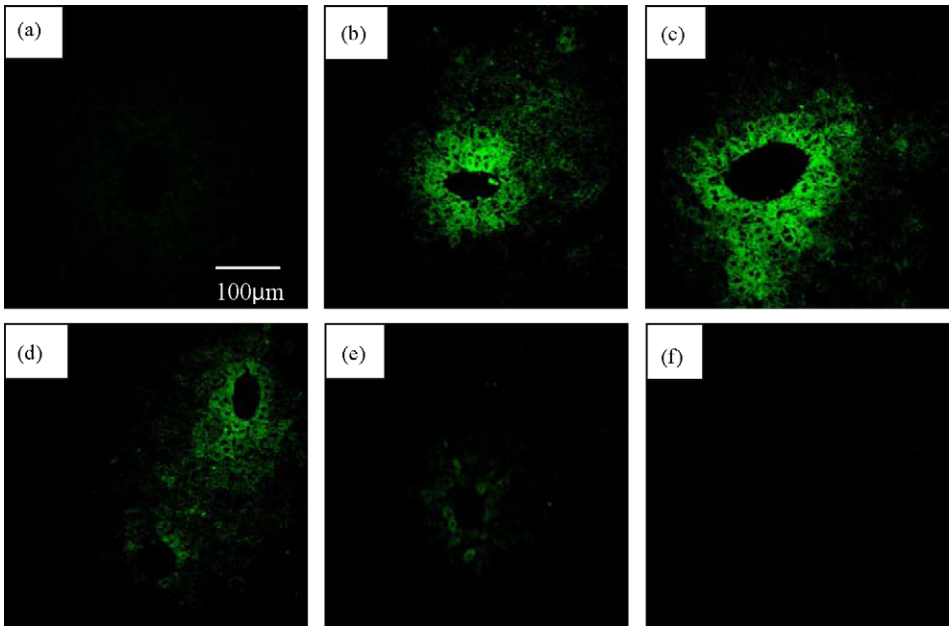

Fig. 3. Representative immunofluorescence images of the liver obtained by fluorescence microscopy from mice: (a) uninfected control; (b) 3-day post-infection with Ad-GFP; (c) 3-day post-infection with Ad-mRLN; (d) 7-day post-infection with Ad-mRLN; (e) 10-day post-infection with Ad-mRLN; and (f) 14-day post-infection with Ad-mRLN. (images are 20× magnification).

4. Discussion

In this study, we employed a somatic gene therapy approach to explore the therapeutic potential of relaxin on cardiac fibrosis in mice. We have used a model of cardiac fibrosis, cardiac-restricted overexpression of β_2AR, in which we have previously reversed fibrosis using H2 relaxin peptide delivered via osmotic mini-pumps implanted subcutaneously (Samuel et al., 2004a). As relaxin cannot be delivered orally, and has a short plasma half-life, continuous infusion is the only viable form of delivery by conventional means. Viral mediated relaxin delivery offers an alternative means to have continuous treatment with relaxin. This study clearly demonstrates that a single intravenous injection of adenovirus producing mouse relaxin has a similar efficacy to H2 relaxin infusion in reversing cardiac fibrosis. Importantly, there are no consequences to collagen levels in disease-free organs. Hence, virally driven delivery of relaxin may represent a viable method for clinical applications with relaxin.

Adenoviral vectors were constructed to express the mouse and rat relaxin genes in tandem with GFP. The presence of GFP allowed the assessment of optimal infectivity of HEK-293T cells *in vitro* using fluorescence microscopy and also provided an Ad-GFP (control) vector for relevant *in vitro* and *in vivo* controls. The ability of the constructed Ad-rRLN and Ad-mRLN to produce bioactive relaxin peptide was first characterized *in vitro* via infection of HEK-293T cells. The production of relaxin peptide was confirmed by Western blotting with an anti-rat relaxin antibody (no mouse relaxin antibody was available). The results of these studies clearly demonstrated that the Ad-rRLN vector produced rat prorelaxin peptide that was secreted into the cell medium. No processed rat relaxin peptide was detected, which is consistent with previous results using adenovirally driven H2 relaxin expression whereby only prorelaxin was detected (Silvertown et al., 2003). Additionally, it has previously been shown in other mammalian cell expression systems that relaxin transgenes only produce prorelaxin peptides (Bathgate et al., 2006). The media from Ad-rRLN and Ad-mRLN infected HEK-293T cells was then tested for the presence of bioactive relaxin peptide. It has previously been demonstrated that prorelaxin peptides have similar bioactivity to the native processed relaxin peptides (Bathgate et al., 2006). Indeed, the media from both Ad-rRLN and Ad-mRLN infected cells demonstrated that bioactive relaxin peptide was being produced. Comparison of the relaxin activity of the media to that of purified rat relaxin peptide allowed the crude calculation of relative levels of relaxin peptide in the media. We estimate that Ad-rRLN and Ad-mRLN vectors directed the production of approximately 50 ng rat relaxin equivalents per ml of media. These levels are similar to those which have been demonstrated previously with adenoviral driven H2 relaxin expression in mammalian cells (Silvertown et al., 2003).

Groups of animals were treated with Ad-GFP and Ad-mRLN and plasma and tissues collected at 3 days, 7 days, 10 days and 14 days to assess the duration of transgene expression. Plasma from Ad-mRLN infected mice clearly demonstrated relaxin bioactivity whereas plasma from Ad-GFP infected mice showed identical activity to non-infected mouse plasma. The low levels of relaxin activity seen in plasma from control animals is likely due to some background cAMP stimulated activity of the plasma and is unlikely to represent plasma levels of the native relaxin peptide as levels in male mice are too low to be detected by this technique. Hence the Ad-mRLN was able to direct the expression of bioactive relaxin peptide, which was secreted into the plasma. The relative level of peptide was calculated, as above, suggesting that plasma levels were around 100 ng rat relaxin equivalents per ml for Ad-mRLN. These levels are only slightly lower than those seen during pregnancy

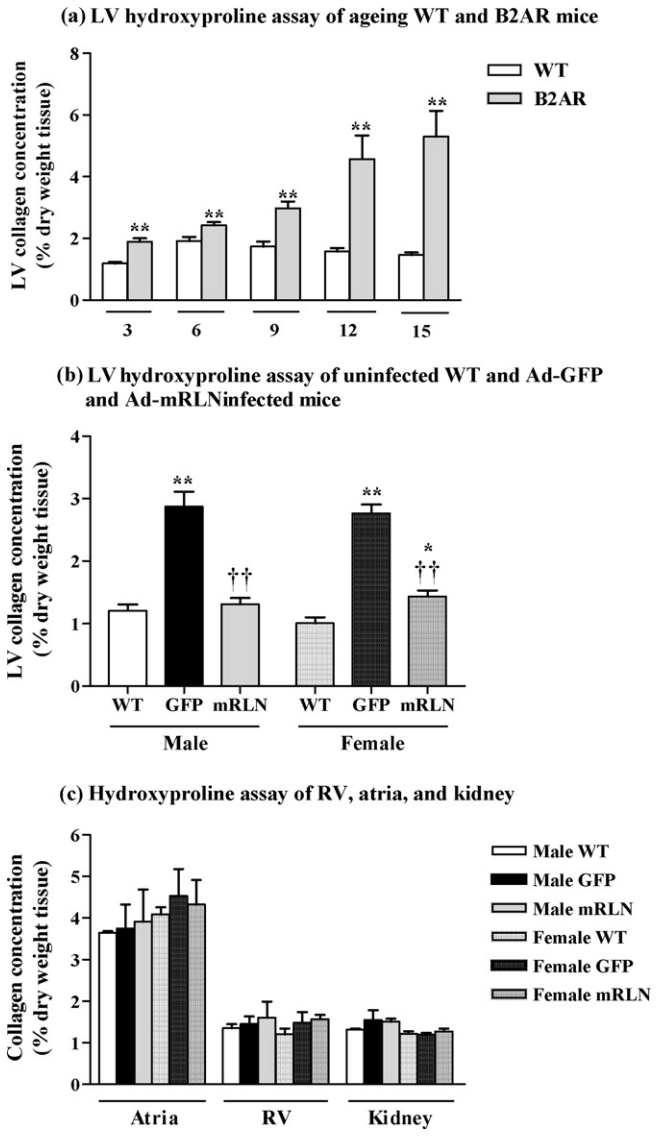

Fig. 4. Analysis of collagen concentration (μg/mg dry weight) using hydroxyproline assay was performed on: (a) left ventricle (LV) of wild-type and β_2-adrenergic receptor (β_2AR) overexpressing male mice aged 3–15 months ($n = 4$–6 mice per group); (b) LV of 7-month-old uninfected WT, Ad-GFP (GFP) and Ad-mRLN (mRLN) infected β_2AR mice ($n = 3$–4 mice per group) 14 days post-infection; and (c) atria, RV, and kidney of these same mice ($n = 3$–4 mice per group). $^{*}P < 0.05$ vs. male WT, $^{**}P < 0.01$ vs. WT, and $^{\dagger\dagger}P < 0.01$ vs. GFP.

in rats (Golos and Sherwood, 1982) and mice (O'Byrne and Steinetz, 1976) and are similar to levels of H2 relaxin produced with osmotic mini-pump infusion in mice (Samuel et al., 2003). Hence, adenoviral infection is able to drive the production of physiologically relevant levels of plasma relaxin. The levels of relaxin bioactivity in the plasma were highest at 3 days post-infection and decreased by 7 days post-infection. These levels correlated with the tissue expression of GFP which was localized predominantly in the liver associated with large blood vessels. Previous studies using intravenous injection of adenovirus have demonstrated that these viruses are highly hepatotrophic (Beck et al., 2004; van der Eb et al., 1998; Herrmann et al., 2004) which correlates with the results of our study and suggests that the liver is the major source of circulating relaxin in our study. The duration of transgene expression driven by the adenovirus (up to 7 days) is also consistent with previous studies (Stone et al., 2000).

Once the Ad-mRLN virus was demonstrated to be able to produce bioactive relaxin peptide *in vivo*, it was utilized to treat a mouse model of cardiac fibrosis (Liggett et al., 2000; Du et al., 2000c; Gao et al., 2003). We chose a TG model of cardiac fibrosis, caused by cardiac restricted overexpression of β_2AR and resultant tonic β-adrenergic drive to the heart, because the fibrosis is reversed by a 2-week period of H2 relaxin treatment (Samuel et al., 2004a). Accumulatively our studies presented here demonstrate that Ad-mRLN infection reverses fibrosis in both genders of this murine model with an efficacy that matches the H2 relaxin peptide delivered using osmotic mini-pumps implanted subcutaneously (Samuel et al., 2004a). This is consistent with the plasma bioactivity data above and further highlights that adenoviral infection is able to induce significant relaxin levels and that these levels, and the short duration of expression, are enough to elicit a therapeutic effect in this murine model of cardiac fibrosis. Quantitative histology and hydroxyproline assays confirmed a significant decrease in collagen content following Ad-mRLN infection. These findings are consistent with our recent reports that relaxin peptide infusion reverses cardiac fibrosis in two other models of cardiac fibrosis; spontaneously hypertensive rats and relaxin knockout mice (Samuel et al., 2004a; Lekgabe et al., 2005). This effect was restricted to the LV, where there was abnormal collagen, whereas other organs with normal collagen levels were unaffected—a feature also consistent with *in vitro* studies (Samuel et al., 2004a). These results illustrate that systemic delivery of Ad-mRLN is an efficient and specific approach in reversing cardiac fibrosis in this murine model.

Our recent study on primary rat ventricular fibroblasts demonstrated that relaxin potentially acts through various mechanisms to induce its anti-fibrotic actions in the heart (Samuel et al., 2004a); by (i) inhibiting the pro-fibrotic influence of angiotensin II and transforming growth factor-β1 (TGF-β1) in promoting cardiac fibroblast proliferation and differentiation into myofibroblasts; (ii) inhibiting cardiac myofibroblast-induced *de novo* collagen synthesis; and (iii) promoting matrix metalloproteinase (MMP)-induced collagen degradation. Consistent with the findings of the current study, relaxin did not affect basal collagen expression in other organs, promoting its status as a safe anti-fibrotic. Several other groups have utilized fibroblast cultures from other organs (Samuel et al., 2004a; Unemori and Amento, 1990; Unemori et al., 1996; Williams et al., 2001; Masterson et al., 2004) to demonstrate that relaxin consistently abrogates TGF-β1-stimulated collagen production, while increasing the expression and activity of MMPs, suggesting that its collagen-remodeling effects are independent of the source of fibroblasts it is applied to. We have since demonstrated that H2 relaxin treatment of spontaneously hypertensive rats *in vivo* (Lekgabe et al., 2005) over a 2-week treatment period also resulted in decreased cardiac fibroblast proliferation and differentiation, decreased collagen accumulation and increased MMP production, further suggesting that the collagen remodeling effects of relaxin are consistently observed, regardless of the

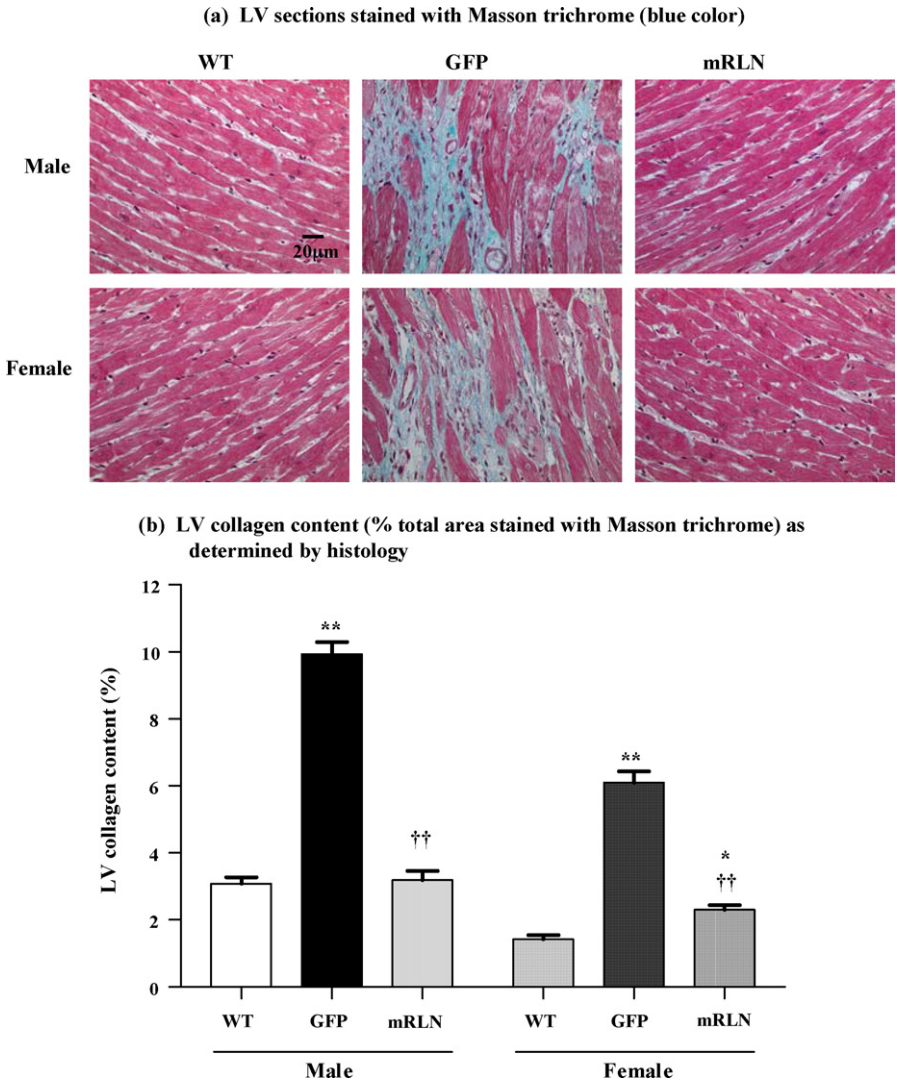

Fig. 5. Analysis of interstitial collagen in the LV myocardium of 7-month-old wild type (WT) compared to Ad-infected mice 14 days post-infection using Masson trichrome stain. (a) Representative LV sections illustrate increased interstitial collagen (blue color) in both male and female β_2-adrenergic receptor (β_2AR) over-expressing mice infected with Ad-GFP (GFP) compared to both WT counterparts and β_2AR mice infected with Ad-mRLN (mRLN). (b) The average percentage of total area of collagen stained by Masson trichrome in a field was measured. An average of 20–25 fields per section per animal was analyzed. ($n = 3$–10 mice per group; images are 20× magnification). $^{**}P < 0.05$ vs. male WT, $^{\dagger\dagger}P < 0.01$ vs. male GFP, $^{\ddagger}P < 0.05$ vs. female WT, $^{\ddagger\ddagger}P < 0.01$ vs. female WT, and $^{\P\P}P < 0.01$ vs. female GFP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

experimental model it is applied to. Based on these combined findings, we propose that Ad-mRLN would similarly be inducing its anti-fibrotic effects via the same mechanisms outlined above.

One limitation of the present study is the lack of functional assessment in the mice to evaluate physiological consequence of such efficacy on cardiac fibrosis. Unfortunately, the extremely high heart rate seen in the β_2AR TG mice makes assessment by echocardiography of LV diastolic function impossible (Liggett et al., 2000; Du et al., 2000c; Gao et al., 2003). Also, our previous studies on the β_2AR TG mice of a similar age revealed no significant change, compared with wild-type littermates, in catheter or echocardiography derived functional parameters at baseline, except an enhanced systolic function (Du et al., 2000a,b). Hence the potential effects of adenoviral deliver of relaxin on heart functional parameters remains to be explored in future studies.

To our understanding, this is the first study to: (1) describe the characterization of adenoviral vectors that can drive the production of bioactive mouse and rat relaxin peptides both *in vitro* and *in vivo*; and (2) demonstrate that intravenous delivery of these adenoviral vectors is effective in reversing cardiac fibrosis in an established model of cardiac fibrosis. The combined findings of this study support the accumulating evidence that relaxin is a potent antifibrotic agent with therapeutic potential for fibrotic cardiomyopathic disorders and these disorders are treatable with virally produced relaxin. The conclusion that emerges from this proof-of-concept study suggests that relaxin is an effective collagen-degrading peptide with virtually no consequence to collagen levels in disease-free organs. This study thereby provides a rational basis for this technology in achieving gene therapy of organ fibrosis using relaxin. Future studies should utilize the prolonged transgene expression and minimal

immune response provided by adeno-associated vectors and/or lentiviruses (Rivera et al., 1999; Blomer et al., 1997) in order to determine the long-term efficacy of reversing fibrosis in chronic models of fibrotic heart disease.

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