Adenoviral Delivery of Angiotensin-(1-7) or Angiotensin-(1-9) Inhibits Cardiomyocyte Hypertrophy via the Mas or Angiotensin Type 2 Receptor

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Abstract

The counter-regulatory axis of the renin angiotensin system peptide angiotensin-(1-7) [Ang-(1-7)] has been identified as a potential therapeutic target in cardiac remodelling, acting via the mas receptor. Furthermore, we recently reported that an alternative peptide, Ang-(1-9) also counteracts cardiac remodelling via the angiotensin type 2 receptor (AT2R). Here, we have engineered adenoviral vectors expressing fusion proteins which release Ang-(1-7) [RAdAng-(1-7)] or Ang-(1-9) [RAdAng-(1-9)] and compared their effects on cardiomyocyte hypertrophy in rat H9c2 cardiomyocytes or primary adult rabbit cardiomyocytes, stimulated with angiotensin II, isoproterenol or arg-vasopressin. RAdAng-(1-7) and RAdAng-(1-9) efficiently transduced cardiomyocytes, expressed fusion proteins and secreted peptides, as demonstrated by western immunoblotting and conditioned media assays. Furthermore, secreted Ang-(1-7) and Ang-(1-9) inhibited cardiomyocyte hypertrophy (Control = 168.7 ± 8.4 μm; AngII = 232.1 ± 10.7 μm; AngII+RAdAng-(1-7) = 186 ± 9.1 μm, RAdAng-(1-9) = 180.5 ± 9.4 μm; P < 0.05) and these effects were selectively reversed by inhibitors of their cognate receptors, the mas antagonist A779 for RAdAng-(1-7) and the AT2R antagonist PD123,319 for RAdAng-(1-9). Thus gene transfer of Ang-(1-7) and Ang-(1-9) produces receptor-specific effects equivalent to those observed with addition of exogenous peptides. These data highlight that Ang-(1-7) and Ang-(1-9) can be expressed via gene transfer and inhibit cardiomyocyte hypertrophy via their respective receptors. This supports applications for this approach for sustained peptide delivery to study molecular effects and potential gene therapeutic actions.

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Introduction

The renin-angiotensin system (RAS) is recognized for its systemic actions, however the presence of RAS components in specific tissues (e.g. heart, brain, kidney), suggests the presence of a local RAS. Furthermore, a counter-regulatory axis of the RAS exists, which functions mainly via angiotensin converting enzyme 2 (ACE2)/angiotensin (Ang)-(1-7)/mas and inhibits many detrimental cardiovascular disease phenotypes [1,2]. Ang-(1-7) has been shown to antagonise pathological actions such as cardiac hypertrophy and fibrosis through the receptor mas [1,3]. Most recently we reported that Ang-(1-9), a poorly characterised peptide not previously reported as a receptor agonist, also had anti-hypertrophic effects on angiotensin II-induced cardiomyocyte hypertrophy, as a functional ligand at the angiotensin type 2 receptor (AT2R) [4]. Moreover, we also demonstrated that Ang-(1-9) reduced cardiac fibrosis in stroke prone spontaneously hypertensive rats through the AT2R [5]. These studies highlight the potential for therapeutic application of Ang-(1-7) and Ang-(1-9) in cardiovascular disease applications. Though active angiotensin peptides are generated extracellularly in the plasma via renin mediated cleavage of angiotensinogen to angiotensin I, followed by ACE-mediated cleavage to AngII, methods which enable their expression through gene transfer approaches are available. Transgenic expression of AngII and Ang-(1-7) can be mediated through the use of synthetic fusion protein expression cassettes which are expressed intracellularly and result in cleavage and secretion of active peptides. Such approaches have been utilised to demonstrate organ-specific effects of individual angiotensin peptides in the heart, kidney and brain [6–14]. Gene therapy approaches have also been reported for Ang-(1-7) in models of both myocardial infarction and diabetic retinopathy using viral vector-mediated gene transfer, highlighting their potential in this setting [15,16]. Here, we have generated adenoviral (Ad) vectors encoding fusion proteins expressing Ang-(1-7) or Ang-(1-9) and compared their effects in models of cardiomyocyte hypertrophy. We report that adenoviral gene transfer can be used to express different angiotensin peptides and it can be shown that these peptides are secreted from cells and maintain the receptor-specific interactions that have been reported for the endogenous peptides. This highlights the general applicability of this approach and importantly for the first time demonstrates that Ang-(1-9) can be
expressed via adenoviral gene transfer and mediate functional
effects at the AT_{2}R.

Results

Generation of RAdAng-(1-7) and RAdAng-(1-9)

The fusion protein expression cassette consists of a signal
peptide, an IgG molecule linked to Ang-(1-7) or Ang-(1-9) and a
cleavage site for furin protease enabling active peptides to be
secreted (Figure 1A). Western immunoblotting of Ad transduced
H9c2 cardiomyocytes demonstrated expression of each fusion
protein with a size of 32 kDa as expected (Figure 1B).

Assessment of the Effects of RAdAng-(1-7) and RAdAng-(1-9) Delivery on Cardiomyocyte Hypertrophy

First we assessed the efficiency of Ad transduction into H9c2 and
rabbit primary cardiomyocytes using a reporter gene (β-galactosi-
dase) expressing Ad vector. Doses of 500 and 1000 pfu/cell in H9c2
cells and 50 and 100 pfu/cell in rabbit cardiomyocytes produced
approximately 50 and a 100% transduction, respectively (data not
shown). To assess the effects of RAdAng-(1-7) and RAdAng-(1-9) on
cardiomyocyte hypertrophy we used in vitro stimulation with AngII as
described previously [4,17,18]. AngII induced cardiomyocyte
hypertrophy as expected (Figure 1C). Adenoviral gene delivery per se
did not affect cell size as RAd60 transduced cells were not
significantly different to AngII stimulated cells. However, at 500 and
1000 pfu/cell both RAdAng-(1-7) and RAdAng-(1-9) inhibited
AngII-induced hypertrophy (AngII = 232.1 ± 10.7 μm; RAdAng-(1-
7) 300 μm = 209.2 ± 10.4 μm; RAdAng-(1-9) 500 μm = 186.9 ± 9.1 μm; RAdAng-(1-7) 1000 μm = 189.5 ± 7.5 μm; RAdAng-(1-9)
500 μm = 180.5 ± 9.0 μm; RAdAng-(1-9) 1000 μm = 178.5 ± 9.1 μm; p<0.05), indicating that each Ad was functional and antagonized AngII-induced hypertrophy. Next,
RAdAng-(1-7) and RAdAng-(1-9) were assessed in adult rabbit left
ventricular primary cardiomyocytes. Similarly to what was observed
previously with exogenous peptides [4], RAdAng-(1-7) and RAdAng-
(1-9) were able to inhibit AngII-induced hypertrophy by preventing
the AngII-stimulated increase in cell width which is indicative of
concentric hypertrophic growth (Figure 1D).

Assessment of Peptide Secretion and Function from RAdAng-(1-7) and RAdAng-(1-9) Transduced Cells

To demonstrate that Ang-(1-7) and Ang-(1-9) were secreted
from the cells in an active form a conditioned media assay was
utilised. HeLa cells were transduced with 100 pfu/cell of either
RAdAng-(1-7), RAdAng-(1-9) or RAd60 and incubated in serum
free media for 48 hours. Conditioned media was then transferred
to AngII-stimulated H9c2 cardiomyocytes (Figure 1E). Transfer of
conditioned media from RAd60 transduced HeLa cells to AngII-
stimulated H9c2 cardiomyocytes had no effect on hypertrophy.
However, transfer of conditioned media from RAdAng-(1-7) or
RAdAng-(1-9) transduced HeLa cells to AngII-stimulated H9c2
cardiomyocytes had no effect on hypertrophy. However, transfer of conditioned media from RAdAng-(1-7) or
RAdAng-(1-9) transduced HeLa cells to AngII-stimulated cardio-
myocytes inhibited hypertrophy (p<0.01), thus confirming each
Ad expressed the fusion protein and secreted the active peptide
and produced effects equivalent to those observed previously with
direct peptide incubation [4]. To demonstrate that the peptides
expressed and secreted via gene transfer functioned similarly to
exogenous peptides, antagonists of mas and AT_{2}R function were
assessed. In similarity to observations with exogenous peptides
[3,4], addition of A779 abolished the anti-hypertrophic effect of
RAdAng-(1-7), while RAdAng-(1-9) was able to inhibit AngII-
induced hypertrophy in the presence of A779 (Figure 2A). Addition of PD123,319 did not block the anti-hypertrophic effect
of Ang-(1-7), but, completely eliminated those of RAdAng-(1-9)
(Figure 2B).

Assessment of RAdAng-(1-7) and RAdAng-(1-9) in Cardiomyocyte Hypertrophy Induced with Different Stimuli

To provide evidence that the anti hypertrophic effects of
RAdAng-(1-7) or RAdAng-(1-9) delivery were not confined to
AngII stimulation, effects in cardiomyocyte hypertrophy induced
by isoproterenol and arg-vasopressin were measured (Figure 2C–
D). Both RAdAng-(1-7) and RAdAng-(1-9) were able to inhibit
isoproterenol and arg-vasopressin induced hypertrophy. This
verified that gene delivery of Ang-(1-7), or Ang-(1-9) was able to
inhibit cardiomyocyte hypertrophy induced with different stimuli
relevant to cardiovascular disease.

Discussion

We have generated adenoviral vectors which selectively over-
express Ang-(1-7) or Ang-(1-9). RAdAng-(1-7) and RAdAng-(1-9)
and transduce neonatal rat cardiomyocyte cell lines and adult
primary rabbit cardiomyocytes and secrete RAS peptides.
Transducing each respective cell type with viral vector doses
which achieved 50 or 100% transduction efficiency resulted in
secreted peptides inhibiting cardiomyocyte hypertrophy in the
same manner as described for exogenous peptides, via mas and
AT_{2}R respectively [3,4], confirming over-expression and release of
active peptides via adenoviral transduction.

Currently, there is great interest in studying the counter-
regulatory ACE2/Ang-(1-7)/mas axis and our own recent
publications have also highlighted the importance of studying
other peptides such as Ang-(1-9), which may have, as yet, relatively
unexplored functions [4,5]. Most studies of angiotensin peptide
function utilise systemic delivery of peptides via osmotic mini-
pumps, however, transgenic models directing tissue-specific
production of angiotensin peptides to avoid confounding systemic
effects have also been developed [6-14]. These studies have
proved invaluable in our understanding of endogenous tissue-
specific RAS. However, the possibility of developmental abnor-
malities caused by embryonic over-expression following transgenic
model generation cannot be discounted. Such effects may explain
the discrepancies between transgenic studies and gene over-
expression studies. For example, ACE2 null mice have been
reported to have increased blood pressure with no cardiac changes
[2] while ACE2 overexpression by adeno-associated virus (AAV)
6-mediated gene delivery leads to severe cardiac fibrosis [19].
Furthermore, Ang-(1-7) has previously been investigated as a gene
therapeutic approach utilising viral vector mediated delivery. In a
rat model of myocardial ischaemia lentiviral gene transfer of Ang-
(1-7) 5 weeks before induction of myocardial infarction prevented
decreased myocardial performance as indicated by improvements
in fractional shortening and decreased myocardial wall thinning
[15]. In a model of diabetic retinopathy local ocular delivery of
adeno-associated virus (AAV) expressing Ang-(1-7) prevented
diabetes-induced retinal vascular damage and inflammation
[16]. Furthermore, adenoviral gene transfer of AngII to vascular
smooth muscle cells in vitro resulted in secretion of AngII and
functional effects on smooth muscle cell hypertrophy [20]. These
studies highlight the potential for gene transfer of angiotensin
peptides for molecular investigations and therapeutic approaches
and our study builds on these findings.
Figure 1. Detection of fusion protein expression and functional assessment of RAdAng-(1-7) and RAdAng-(1-9).

(A) Schematic of fusion protein, consisting of a renin signal peptide to ensure secretion, murine IgG to provide mass for efficient production of the protein, a furin protease cleavage domain (to invoke peptide release), and each peptide [6].

(B) H9c2 cardiomyocytes were transduced with 500 or 1000 pfu/cell of RAdAng-(1-7), or RAdAng-(1-9), or RAd60 lysed after 48 h and subjected to electrophoresis. Fusion protein expression was detected by western immunoblotting using a α-IgG2b antibody. kDa = kilodaltons.

(C) H9c2 cardiomyocytes were transduced with RAdAng-(1-7), RAdAng-(1-9) or RAd60 at 500 and 1000 pfu/cell 24 h before AngII addition. Following 96 h incubation cells were fixed, stained with crystal violet and cell size measured. *p<0.01 vs. unstimulated cells; #p<0.05 vs. AngII stimulated cells.

(D) Freshly isolated left ventricular adult rabbit primary cardiomyocytes were transduced with RAdAng-(1-7), RAdAng-(1-9) or RAd60 (50, 100 and 300 pfu/cell) 1 h before AngII (500 nM) addition. After 24 h cell width was measured.
measured. *p<0.01 vs. unstimulated cells; **p<0.01 vs. AngII stimulation. (E) HeLa cells were transduced with RAdAng-(1-7), RAdAng-(1-9) or RAd60 (100 pfu/cell) and incubated for 48 hours. Culture media from HeLa transduced cells (C.M; conditioned media) was transferred to H9c2 cardiomyocytes and incubated for 30 minutes before AngII (100 nM) addition. 96 hours later cells were fixed, stained with crystal violet and cell size measured. *p<0.01 vs. unstimulated cells; **p<0.01 vs. AngII stimulated cells.

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Figure 2. Effect of Mas and AT1R antagonism on AngII-stimulated H9c2 cardiomyocyte hypertrophy following RAdAng-(1-7), or RAdAng-(1-9) transduction. H9c2 cardiomyocytes were transduced with RAdAng-(1-7), RAdAng-(1-9) or RAd60 (negative control) +/- (A) the Mas antagonist A779 (10 μM) or (B) the AT1R antagonist PD123,319 (500 nM) 24 h before AngII addition. Cells were incubated for 96 h before fixing, staining and measurement of cell size. *p<0.01 vs. unstimulated cells, **p<0.001 vs. AngII stimulated cells. H9c2 cardiomyocytes were transduced with RAdAng-(1-7), RAdAng-(1-9) or RAd60 at 500 pfu/cell 24 h before addition of (C) 1 μM isoproterenol (Iso) or (D) 1 μM arg-vasopressin (vaso). Cell size was measured at 96 h. *p<0.05 vs. unstimulated cells, **p<0.05 vs. isoproterenol or arg-vasopressin stimulated cells.

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myocardial infarction (MI) and furthermore, their tropism is efficient for other cell types in other organs such as kidney, brain and skeletal muscle, which are also important to study tissue-specific RAS effects. Moreover, for long term expression of peptides in the heart AAV, particularly serotypes such as 1, 6 and 9 [21], can produce stable expression in myocardium following non-invasive intravenous delivery which may be beneficial to modulate long term detrimental remodelling in the heart following an MI, in order to counter progression to heart failure. Therefore, there are a range of delivery vectors which will enable assessment of Ang-(1-7) and Ang-(1-9) in gene therapy approaches.

In summary, our data demonstrates that Ang-(1-7) and Ang-(1-9) can be expressed via adenovalien gene transfer and inhibit cardiomyocyte hypertrophy via their respective receptors. Future work with these vectors is required in vivo to assess potential therapeutic effects induced following cardiac-selective delivery.

Materials and Methods

Production of Recombinant Adenoviruses (RAd)

RAds expressing Ang-(1-7) [RAdAng-(1-7)] or Ang-(1-9) [RAdAng-(1-9)] were generated by modifying pBluescript-pre-fc-proAngII [22] (Figure 1A). The furin cleavage site and AngII encoding nucleotides were excised via BglII and EcoRI restriction and the vector backbone gel purified. Oligonucleotides encoding a furin cleavage site, Ang-(1-7) or Ang-(1-9) flanked by BglII and EcoRI restriction sites were commercially synthesised: Ang-(1-7) Forward 5'-GATCTCGGCTACGCACTAAACGCGACCGGGTGTAACA-TACACCCCTGAG-3', Reverse 3'-AGCGGATGCGT-GATTTGGCTGGCCACCAGTGTTATGTTGGGACTCTTA-5'; Ang-(1-9) Forward 5'-GATCTCGGCTACGCACTAAACGCGGACCGTTGACA-TACACCCCTGGAG-3', Reverse 3'-AGCGCATGCGT-GATTTGGCTGGCCACCAGTGTTATGTTGGGACTCTTA-5'. Annealed oligonucleotides encoding Ang-(1-7) or Ang-(1-9) were ligated into pBluescript-pre-fc-pro and transformed into competent E.coli JM109 (Promega, Southampton, UK). Sequencing confirmed their correct insertion. Next, Ang-(1-7) or Ang-(1-9) fusion protein encoding expression cassettes were excised from pBluescript-pre-fc-pro, purified and cloned into pVQ-CMV-KNp (pBluescript-pre-fc-proAn-

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