Angiotensin II DNA Vaccine

Long-Term Reduction of High Blood Pressure by Angiotensin II DNA Vaccine in Spontaneously Hypertensive Rats

Hiroshi Koriyama, Hironori Nakagami, Futoshi Nakagami, Mariana Kiomy Osako, Mariko Kyutoku, Munehisa Shimamura, Hitomi Kurinami, Tomohiro Katsuya, Hiromi Rakugi, Ryuichi Morishita

Abstract—Recent research on vaccination has extended its scope from infectious diseases to chronic diseases, including Alzheimer disease, dyslipidemia, and hypertension. The aim of this study was to design DNA vaccines for high blood pressure and eventually develop human vaccine therapy to treat hypertension. Plasmid vector encoding hepatitis B core-angiotensin II (Ang II) fusion protein was injected into spontaneously hypertensive rats using needleless injection system. Anti-Ang II antibody was successfully produced in hepatitis B core-Ang II group, and antibody response against Ang II was sustained for at least 6 months. Systolic blood pressure was consistently lower in hepatitis B core-Ang II group after immunization, whereas blood pressure reduction was continued for at least 6 months. Perivascular fibrosis in heart tissue was also significantly decreased in hepatitis B core-Ang II group. Survival rate was significantly improved in hepatitis B core-Ang II group. This study demonstrated that Ang II DNA vaccine to spontaneously hypertensive rats significantly lowered high blood pressure for at least 6 months. In addition, Ang II DNA vaccines induced an adequate humoral immune response while avoiding the activation of self-reactive T cells, assessed by ELISPOT assay. Future development of DNA vaccine to treat hypertension may provide a new therapeutic option to treat hypertension. (Hypertension. 2015;66:167-174. DOI: 10.1161/HYPERTENSIONAHA.114.04534.) • Online Data Supplement

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Antihypertensive drugs, such as calcium channel blockers and angiotensin II (Ang II) receptor blockers are widely used in the treatment of hypertension. These medications are known to effectively lower high blood pressure (BP) with relatively minor side effects. Although current antihypertensive drugs seem to fulfill the unmet medical needs of hypertensive patients, there are still serious issues (eg, daily dosage of the medication and often for life) that may be a significant financial burden, particularly in developing countries. Therefore, next generation of the treatments for hypertension that can reduce or halt the use of antihypertensive drugs and reduce long-term medical expenses would be ideal. Vaccines against viral and bacterial pathogens have been established, and are widely used. Recent progress in therapeutic B-cell vaccines toward producing neutralizing auto-reactive antibodies against important mediators in common adult diseases, such as Alzheimer disease, obesity, diabetes mellitus, hypertension, and cancer. This study focuses on establishing an effective vaccine to treat hypertension.

Research has targeted the components of the renin–angiotensin system (RAS), such as renin, Ang I, Ang II, and Ang II type I receptor (AT1R), for the treatment of hypertension. Unexpectedly, the initial studies encountered the difficulties in hypertension vaccines against these targets: renin vaccine caused autoimmune side effects, whereas Ang I vaccine did not affect BP in a clinical trial. Vaccination against Ang II and AT1R successfully reduced BP, but some problems remained that inhibited their translation to clinical use, such as insufficient reduction in BP to warrant clinical usage.

Recently, clinical trials of DNA vaccines in humans have been widely conducted. These trials demonstrated DNA vaccines are able to increase antibody titers and mediate therapeutic effects in several diseases. In phase I clinical trial, neutralizing antibody responses to West Nile virus were elicited by DNA vaccination. A therapeutic cytomegalovirus DNA vaccine significantly reduced the occurrence and recurrence of cytomegalovirus viremia and improved the time-to-event for viremia episodes when compared with placebo in...
phase II clinical trial. In this study, we used DNA vaccine technology to treat hypertension because we hypothesized that it would be superior to peptide vaccine in the maintenance of target antigen expression and long-lasting immunologic effects. Here, we demonstrated the development of DNA vaccine that reduced BP for ≤6 months by targeting Ang II fusion with hepatitis B core (HBc) protein administered via the needleless jet injector in a rat model of hypertension.

Methods
Methods are available in the online-only Data Supplement.

Results

Construction of DNA Vectors
We selected Ang II as a target antigen to reduce BP in spontaneously hypertensive rats (SHRs), expecting that immunization against Ang II would minimize the inflammatory action of immune complexes because of the extremely low concentration of circulating Ang II. In addition, as Ang II receptor blockers are known to cause few severe adverse effects in clinical practice, we hypothesized that targeting a peptide in this pathway would have the similar safety profile with little side effects. To break the immunologic tolerance to Ang II, we constructed an HBc-Ang II fusion protein plasmid vector; the immunization of the fusion protein of HBc with any target peptide can produce high titers of antibody against the target peptide because of the highly immunogenic nature of the HBc protein.

Plasmids pcDNA3.1-HBc (control) and pcDNA3.1-HBc-Ang II containing an immunostimulatory sequence were both constructed (Figure S1A and S1B in the online-only Data Supplement), and transfected into HeLa cells. We analyzed the HBc-Ang II recombinant protein (Figure S1C) synthesized in cells by performing Western blots on whole cell protein lysates (Figure S2). One band ≈24 kDa in size was detected in the cell lysate of pcDNA3.1-HBc-Ang II-transfected HeLa cells by probing the immunoblot with anti-Ang II antibody; this band was also visible in the cell lysates of pcDNA3.1-HBc-Ang II- and pcDNA3.1-HBc-transfected HeLa cells when probing the immunoblot with anti-HBc antibody. These results indicated that the transcription and translation of the pcDNA3.1-HBc-Ang II plasmid in HeLa cells produced the desired target protein.

Evaluation of Ang II DNA Vaccine in SHR
To examine antibody production by plasmacytes, we measured the amounts of anti-Ang II antibodies produced in response to vaccination with pcDNA3.1-HBc-Ang II or pcDNA3.1-HBc. Because Ang II is an 8-amino-acid peptide and too small to be detected by Western blot, a BSA-Ang II conjugate was constructed with several (an average of 5) Ang II molecules conjugated to 1 BSA molecule at its N terminus. As shown in Figure 1A, sera from pcDNA3.1-HBc-Ang II–immunized rats bound the BSA-Ang II conjugate, but not BSA. Preimmune sera did not bind to either the BSA-Ang II conjugate or BSA. These results indicated that anti-Ang II antibody was successfully produced by immunization.

We subsequently immunized SHR with either pcDNA3.1-HBc-Ang II, pcDNA3.1-HBc or saline by intradermal administration 3× at 2-week intervals. Anti-Ang II antibodies were detected only in the pcDNA3.1-HBc-Ang II–immunized group when compared with inoculation with pcDNA3.1-HBc or saline. ELISA detected antibodies as early as 2 weeks after the first administration that lasted for at least 24 weeks until the last sampling of blood (Figure 1B). The production of anti-Ang II and anti-Ang I antibodies significantly increased after immunization, whereas the production of anti-Angiotensinogen (Ang-N) and anti-Ang1–7 antibodies did not significantly increase (Figure 1C). These results indicated that the immunization was specific to Ang I and Ang II.

To confirm the efficacy of vaccination, BP levels were measured in SHRs by the tail-cuff method. Systolic blood pressure (SBP) in pcDNA3.1-HBc-Ang II–immunized rats was significantly lower when compared with the pcDNA3.1-HBc- and saline-immunized groups at 8, 12, 16, 20, and 24
Systolic blood pressure (SBP) and plasma angiotensin II (Ang II) concentration were both reduced by hepatitis B core (HBc)-Ang II vaccination. A. The effect of vaccination on SBP in spontaneously hypertensive rats. Data are presented as the average of each group; error bars indicate the SEM. *P < 0.05 vs HBc and saline groups. **P < 0.05 vs saline group. ●, HBc-Ang II group (n=5); ▲, HBc-group (n=5); and ▲, saline group (n=4). B, Correlation between SBP (mmHg) and antibody response against Ang II (optical density [OD] at 450 nm) in HBc-Ang II-immunized rats. C, Ang II levels in the plasma of vaccinated rats. Ang II was extracted from plasma sampled 12 weeks after the first immunization of vaccinated rats. Horizontal bars indicate the averages of the groups; the symbols indicate individual values.

Figure 2. Systolic blood pressure (SBP) and plasma angiotensin II (Ang II) concentration were both reduced by hepatitis B core (HBc)-Ang II vaccination. A. The effect of vaccination on SBP in spontaneously hypertensive rats. Data are presented as the average of each group; error bars indicate the SEM. *P < 0.05 vs HBc and saline groups. **P < 0.05 vs saline group. ●, HBc-Ang II group (n=5); ▲, HBc-group (n=5); and ▲, saline group (n=4). B, Correlation between SBP (mmHg) and antibody response against Ang II (optical density [OD] at 450 nm) in HBc-Ang II-immunized rats. C, Ang II levels in the plasma of vaccinated rats. Ang II was extracted from plasma sampled 12 weeks after the first immunization of vaccinated rats. Horizontal bars indicate the averages of the groups; the symbols indicate individual values.

weeks after the first immunization (Figure 2A). As shown in Figure 2B, a significant inverse correlation was observed between anti-Ang II antibody response and BP (r = -0.704; P < 0.01). No significant differences in heart rate were detected between any treatment groups (data not shown). It is noteworthy that a significant decrease in plasma Ang II concentration was observed in the pcDNA3.1-HBc-Ang II group (−30 pg/mL, P < 0.001) when compared with the pcDNA3.1-HBc and saline groups (Figure 2C). Then, we measured plasma renin activity, plasma Ang I concentration, and daily urinary excretion of aldosterone. As a result, plasma renin activity tends to rise in the HBc-Ang II group (Figure S3A). These results indicate that blockade of Ang II signal by Ang II vaccine may induce the feedback system to increase the renin/angiotensin production. Interestingly, plasma Ang I did not change by Ang II vaccine (Figure S3B). These results also support that Ang II vaccine mainly neutralizes Ang II in our system. Daily urinary excretion of aldosterone was significantly decreased in the HBc-Ang II group (Figure S3C). These results suggest that the downstream pathway of Ang II in RAS is repressed by Ang II vaccine.

To examine the effect of HBc-Ang II vaccine on normoten- sive rats, WKY rats were vaccinated 3× with pcDNA3.1-HBc- Ang II at 2-week intervals, and SBP was measured at 6 weeks after the first immunization. As shown in Figure S4, SBP of WKY rats was not significantly affected by vaccination. We think that this is because homeostatic control of BP is regulated by not only RAS but also catecholamine, vasopressin, or the autonomic nervous system.

To investigate whether the decrease in BP is the primary result of increase Ang-(1-7)-mas axis activation with simultaneous Ang II-AT1R suppression, we conducted the additional experiments to examine whether the administration of A779 or Olmesartan would affect SBP in the immunized rats. As shown in Figure S5, administration of A779 (0.8 mg/kg per day IP) for 7 days did not significantly change SBP in HBc-Ang II–immunized rats, and oral administration of Olmesartan (3 mg/kg per day) for 7 days did not decrease SBP in HBc-Ang II–immunized rats. Both HBc-Ang II group and Olmesartan group decreased SBP to the same degree in SHR rats.

Because SHRs exhibited severe organ damage such as fibrosis in various organs, including the kidney and heart because of high BP and Ang II concentration, we examined hearts and aortas from SHRs at 24 weeks after immunization for pathological changes. Severe perivascular fibrosis was detected in hearts from SHRs treated with saline or pcDNA3.1-HBc using Masson trichrome staining, whereas fibrosis was significantly reduced in SHRs treated with saline or pcDNA3.1-HBc using Masson trichrome staining, whereas fibrosis was significantly reduced in SHRs treated with pcDNA3.1-HBc-Ang II (Figure 3A and 3B). At 24 weeks after the first immunization, the area of aortic media in the HBc-Ang II group was significantly reduced than that in the HBc and saline groups (Figure 3C and 3D). In contrast, we confirmed the safety of the Ang II DNA vaccine by histochemical analysis of the kidney, heart, liver, and aorta, all of which showed no evidence of pathological changes and T cell or macrophage infiltrations at 24 weeks after immunization (Figures S6 and S7).

To evaluate long-term safety and efficacy of this vaccine, we compared survival rates between HBc-Ang II group and control groups. Because it is reported that disruption of the Ang II type1 receptor promotes longevity in mice,9 we reimmunized SHR with either pcDNA3.1-HBc-Ang II, pcDNA3.1-HBc, or saline at 24 weeks after first immunization, as a booster effect (Figure 4A). As shown in Figure 4B, the survival rate was significantly improved in the HBc-Ang II group (log rank P < 0.05). The serum from the rats that lived longest in HBc-Ang II group showed the highest anti-Ang II antibody response measured by ELISA at 48 weeks after first immunization (data not shown). We also sampled pleural effusion from the rat that lived longest on the date of death. The sampled effusion showed high anti-Ang II antibody response by ELISA (data not shown). These results demonstrated that
anti-Ang II antibody production could be sustained >1 year after the last vaccination.

**Evaluation of T-cell Activation After Immunization**

Our hypothesized mechanism of action for Ang II–targeted vaccine therapy is illustrated conceptually in Figure S8. We immunized mice by introducing HBc-Ang II as an antigen. During immunization, antigen-presenting cells phagocytose the HBc-Ang II fusion protein and present T-cell epitopes of this antigen on their major histocompatibility complexes. T cells bind their cognate epitopes through their T-cell receptors, activating and differentiating into effector T cells (step 1, shown as 1 in Figure S8). B cells that have a B-cell receptor specific for Ang II also phagocytose HBc-Ang II and present corresponding T-cell epitopes of HBc-Ang II on major histocompatibility complex toward T cells. These B cells then differentiate to plasmacytes and produce antibodies with the help of activated effector T cells (step 2, shown as 2 in Figure S8). These steps are required for the efficient production of antibodies.

To examine the initial immunization step, the activation of T cells was evaluated by measuring their cytokine production in rats immunized by pcDNA3.1-HBc or pcDNA3.1-HBc-Ang II. Splenocytes from rats immunized with either pcDNA3.1-HBc-Ang II or pcDNA3.1-HBc produced interferons (IFN)-γ and interleukin (IL)-2 (T-helper type 1 cytokines) when stimulated in vitro with recombinant HBc protein (Figure 5A and 5B), but did not produce IL-10 or IL-4 (Th2 cytokines) after exposure to the same stimuli (Figure 5C and 5D). These data indicated that HBc-Ang II and HBc contain sufficiently immunogenic T-cell epitopes to activate T cells. None of the pcDNA3.1-HBc-Ang II–immunized rats produced cytokines.
Transient, influenza-like symptoms. In the preclinical study, a sustained to 1 year, and (2) potential side effects, such as tions, including (1) relatively long BP reduction that was not peptide immunization also seemed to exhibit several limita-
72 patients with mild-to-moderate hypertension. However, blindness randomized, placebo-controlled phase IIa trial enrolling day time BP from baseline at week 14 in a multicenter, double-
II exhibited \(-9.0/-4.0\) mmHg reduction in mean ambulatory BP, compared with saline (n=2) at 0, 2, 4, and 24 weeks after first immunization. Vaccination was started at 6 weeks of age in rats. The Kaplan–Meier curve of time to survival rate, log rank \(P<0.05\) between HBC-Ang II group and nonimmunization group (HBC and saline).

**Figure 4.** The survival time was significantly prolonged in the hepatitis B core (HBC)-angiotensin II (Ang II) group. A, Time course of DNA vaccination. Arrows indicate vaccination with either pcDNA3.1-HBC-Ang II (n=6), pcDNA3.1-HBC (n=6), or saline (n=2) at 0, 2, 4, and 24 weeks after first immunization. Vaccination was started at 6 weeks of age in rats. B, Kaplan–Meier curve of time to survival rate, log rank \(P<0.05\) between HBC-Ang II group and nonimmunization group (HBC and saline).

when stimulated in vitro with either Ang II or Ang-N, indicating that Ang II does not contain the immunogenic T-cell epitope. ELISPOT assays for IFN-\(\gamma\) and IL-2 were also performed (Figure 5E and 5F), and responses against recombinant HBc protein were detected in pcDNA3.1-HBc-Ang II–immunized rats and pcDNA3.1-HBc–immunized rats; in contrast, responses against Ang II or Ang-N were not detected.

**Discussion**

In this study, we demonstrated that DNA vaccine against Ang II successfully reduced high BP in SHRs, suggesting that it might be a novel therapy to treat hypertension. BP was continuously decreased for at least 6 months after 3-dose immunization regimen. The long-term BP reduction observed in this study might be because of the prolonged high-level production of antibodies against Ang II and Ang I by DNA vaccine. Previous reports using the peptide vaccine against Ang II exhibited \(-9.0/-4.0\) mmHg reduction in mean ambulatory daytime BP from baseline at week 14 in a multicenter, double-blind, randomized, placebo-controlled phase IIa trial enrolling 72 patients with mild-to-moderate hypertension. However, peptide immunization also seemed to exhibit several limitations, including (1) relatively long BP reduction that was not sustained to 1 year, and (2) potential side effects, such as transient, influenza-like symptoms. In the preclinical study, a transient reduction in BP by the peptide vaccine against Ang II was observed, but this reduction was not sustained ≤6 months different from this study using DNA vaccine. Therefore, DNA vaccine might be superior in sustaining the effects of vaccination.

The contrasting maintenance of efficacy begets the question: Why is Ang II DNA vaccine superior to the peptide vaccine to reduce BP? One potential reason might be that both anti-Ang II and anti-Ang I antibodies were produced by Ang II DNA vaccine. The production of both anti-Ang I and anti-Ang II antibodies by DNA vaccine would synergistically act to decrease BP in SHRs. Because tissue Ang II is known to accelerate fibrosis, this study successfully demonstrated a significant decrease in cardiac perivascular fibrosis in the HBc-Ang II–treated group. Considering that aldosterone breakthrough is known to accelerate fibrosis, a significant decrease in circulating Ang II might be even more beneficial for the prevention of fibrosis.

In contrast, anti-Ang1–7 antibody was not produced for an unidentified reason. Presumably, the phenylalanine residue in Ang II that is absent in Ang1–7 may be important as a B-cell epitope (Figure 1C). The axis formed by angiotensin-converting enzyme 2/Ang-(1–7)/Mas represents an endogenous counter-regulatory pathway within the RAS whose actions oppose the vasoconstrictor/proliferative arm of the RAS that is composed of angiotensin-converting enzyme/Ang II/AT1R. The HBc-Ang II DNA vaccine used here is expected to restrain the RAS effectively without inhibiting the action of Ang1–7 because of the lack of anti-Ang1–7 antibody. In fact, administration of A779 did not significantly change SBP in HBc-Ang II–immunized rats (Figure S5). These results indicate that the decrease in BP is the primary result of Ang II-AT1R suppression.

To enhance the therapeutic efficacy of DNA vaccines for the treatment of hypertension, we used the HBc-Ang II fusion antigen to produce antibody for a more sustained period of time. Because HBc self-aggregates into a sphere, Ang II sequence inserted into the B-cell epitope (aa80-81) of HBc is presented on the surface of the sphere, and is recognized by the immune system as a repetitive epitope. In addition to the capacity of the HBc carrier moiety to provide T cell help to inserted sequences, the HBc capsid mediates T-cell–independent humoral response to inserted epitopes because of the high degree of repetitiveness of the epitopes and their spacing. The anti-HBc antibody is also known to last longer than the anti-HBs antibody in humans after infection by HB virus. These characteristics of DNA vaccine using the HBc system would contribute to the maintenance of antibody production.

Virus-like particles are highly organized spheres that self-assemble from virus-derived structural antigens. These stable, versatile subviral particles possess excellent adjuvant properties and are capable of inducing both innate and cognate immune responses. The structural components of some virus-like particles have proven amenable to the insertion or fusion of foreign antigenic sequences, allowing for the production of chimeric virus-like particles that expose the foreign antigen on their surface. Among virus-like particles, those based on the HBc protein have been studied intensively and used in previous clinical trials. It is widely accepted that the HBc carrier is capable of eliciting high levels of B- and T-cell immunogenicity to foreign epitopes. HBc particles can present on their spherical surface any peptide inserted between A80-S81, which are located within the major immunodominant region. The injection system used for delivery is also important in vaccine efficacy. Because the chosen method and route of administration could play key roles in the magnitude and quality of the resultant immune response, DNA vaccines require an appropriate delivery technology. Dermal delivery will elicit...
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a humoral response with the production of IgA and IgG, whereas intramuscular injection will prime a cellular response with the activation of cytotoxic T lymphocytes and the production of IgG2a. Traditionally, percutaneous injection has been a popular approach for vaccination owing to its accessibility, size, and cell population consisting of Langerhans cells, antigen-presenting cells, and migrating lymphocytes. Although there are several methods to deliver DNA vaccines, such as gene gun, electroporation, and liposomes, we chose the needleless jet injection system. This method was reported to be highly effective at gene transfection of naked plasmid DNA to the skin in rats at levels similar to those in viral vector systems. Indeed, our previous study demonstrated that local gene expression was ≈100× higher by the spring-powered jet injector, Shima Jet, when compared with delivery by needle alone.

When considering the clinical application of immunotherapy for hypertension, the safety of the vaccine is extremely important. For example, a clinical trial of an amyloid β vaccine for Alzheimer disease was halted because of a severe adverse event. Therefore, a vaccine against self-antigen requires an adequate and reversible humoral immune response from B cells while avoiding the activation of self-reactive T cells. In this study, the splenocytes in the HBc-Ang II group stimulated with HBc produced T-helper type 1 cytokines, such as IFN-γ and IL-2. In the ELISPOT assay, the production of IFN-γ and IL-2 by splenocytes stimulated with HBc was also observed. However, Th2 cytokines, such as IL-10 and IL-4 were scarcely produced.

These outcomes indicate that these immunization conditions established a T-helper type 1–dominant immune response, a result that is in agreement with other studies on DNA vaccines. However, the stimulation of splenocytes in the HBc-Ang II group with Ang II or Ang-N did not lead to the secretion of any cytokines (IFN-γ, IL-2, IL-10, or IL-4) detected by ELISA or ELISPOT assay. These results demonstrated that immunization by pcDNA3.1-HBc-Ang II did not establish T-cell immune responses against Ang II or Ang-N. Generally, antibody production requires helper T-cell activation to assist the expansion of B cells; therefore, antigens generally contain B- and T-cell epitopes. However, the derivation of the sequence to activate T cells (the T cell-epitope) and to activate B cells (the B-cell epitope) may be different, similar to the relationship between hapten and carrier, where the former has the only B-cell epitope and the latter possesses the T-cell epitope. In this scenario, the antigen composed of self–B-cell epitope and non-self T-cell epitope could potentially induce antiself antibody without inducing antiself T-cell activation.

Figure 5. Splenocytes from hepatitis B core (HBc)-angiotensin II (Ang II)–immunized rats exhibit a T-helper type 1 response against HBc, but not against Ang II or angiotensinogen. A–D, Cytokine levels produced by splenocytes cultured after stimulation with 50 μg/mL phytohemagglutinin (PHA), 3 μg/mL recombinant HBc, 10 μg/mL Ang II or 10 μg/mL angiotensinogen (Ang-N). (-), no stimulation. Levels of interferons (IFN)-γ (A), interleukin (IL)-2 (B), IL-10 (C), and IL-4 (D) were determined by ELISA on cell culture supernatants. E and F, Cytokine (IFN-γ [E] and IL-2 [F])-producing cells were quantified by ELISPOT assays on splenocytes stimulated ex vivo with 50 μg/mL PHA, 3 μg/mL HBc, 10 μg/mL Ang II, or 10 μg/mL Ang-N. The results are expressed as the means±SEM of the numbers of cytokine (IFN-γ [E] and IL-2 [F])-producing cells/10⁶ splenocytes. HBc-Ang II group, n=3; HBc group, n=3; and Saline group, n=3.
In this study, we used HBc as a carrier with several non-self helper T-cell epitopes and succeeded in producing antibody against Ang II without T-cell activation toward Ang II or Ang-N. Indeed, no cytokine was secreted in ELISA or ELISPOT assay, and any pathological symptoms were discovered in the kidney, liver, or heart. Ang II is assumed not to be a highly responsive cytotoxic T lymphocyte epitope in SHRs. Overall, our results revealed that (1) Ang II did not act as a T-cell epitope, (2) T cells were activated by a T-cell epitope in HBc but not Ang II, and (3) helper T-cell activation by HBC-Ang II was predominantly skewed toward a T-helper type 1 phenotype.

Theoretically, DNA vaccines raise fewer safety concerns because they can induce both long-lasting cellular and humoral immune responses, but do not revert to virulence. Many clinical trials with DNA vaccines have observed lower incidences of systemic adverse effects, such as redness, transient pain, swelling, fever, and headache in >3000 patients.26 The fact that survival time was extended in the HBC-Ang II group may also be considered as a strong support for high safety of this vaccine.

Perspectives
Here, we reported on the first DNA vaccine against hypertension using HBc. DNA vaccination against Ang II in SHRs produced anti-Ang II antibodies and lowered SBP for 6 months postvaccination without any apparent side effects. Although there are no US Food and Drug Administration-approved DNA vaccines for use in humans, this study presents the newest vaccine platform currently under development. Further research on this DNA vaccine platform, including increase the longevity of BP reduction, may eventually provide a new therapeutic option to treat hypertensive patients.

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Disclosures
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What Is New?
• Antihypertensive drugs are well known to effectively lower high blood pressure, however, next-generation treatment for hypertension might be ideal, leading to the reduction in medical expenses. Vaccine has been recently developed to different pathologies to target self-antigen, such as Alzheimer disease. In this study, we succeeded to evaluate the efficiency and safety of the angiotensin II (Ang II) DNA vaccine for hypertensive rat.

What Is Relevant?
• Plasmid vector encoding hepatitis B core-Ang II fusion protein was injected to hypertensive rat by needle less injection system. As a result, anti-Ang II antibody was successfully produced in hepatitis B core-Ang II group and sustained at least ≤6 months. Consistently, SBP was lower in hepatitis B core-Ang II group after the immunization, and BP reduction was continued at least ≤6 months.

Summary
Our findings proposed that future development of DNA vaccine to hypertension might provide new therapeutic option to treat hypertensive population.
Online Supplement for

Long-Term Reduction of High Blood Pressure

by Angiotensin II DNA Vaccine in Spontaneously Hypertensive Rats

Hiroshi Koriyama M.D., Ph.D a, Hironori Nakagami M.D., Ph.D a,1, Futoshi Nakagami, M.D., Ph.D b,c, Mariana Kiomy Osako, Ph.D a, Mariko Kyutoku, Ph.D b, Munehisa Shimamura M.D., Ph.D a, Hitomi Kurinami M.D., Ph.D a, Tomohiro Katsuya M.D., Ph.D b, Hiromi Rakugi M.D., Ph.D c, and Ryuichi Morishita M.D., Ph.D c,1

aDivision of Vascular Medicine and Epigenetics, Osaka University United Graduate School of Child Development. 2-1 Yamada-oka, Suita, Osaka, 565-0871, Japan

bDepartment of Clinical Gene Therapy, Osaka University Graduate School of Medicine. 2-2 Yamada-oka, Suita, Osaka, 565-0871, Japan

cDepartment of Geriatric Medicine and Nephrology, Osaka University Graduate School of Medicine. 2-2 Yamada-oka, Suita, Osaka, 565-0871, Japan

1Address correspondence to:
Ryuichi Morishita, M.D., Ph.D. Professor, Division of Clinical Gene Therapy, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka, 565-0871, Japan. Tel: +81-6-6879-3406, Fax: +81-6-6879-3409, E-mail: morishita@cgt.med.osaka-u.ac.jp
and
Hironori Nakagami, M.D., Ph.D. Professor, Division of Vascular Medicine and Epigenetics, Osaka University United Graduate School of Child Development. 2-1 Yamada-oka, Suita, Osaka, 565-0871, Japan. Tel: +81-6-6879-4142, Fax: +81-6-6879-4142 E-mail: nakagami@gts.med.osaka-u.ac.jp
Methods

Reagents

Anti-Ang II antibody, anti-Ang I antibody, anti-Ang1-7 antibody, anti-Ang-N antibody, and anti-BSA antibody were purchased from Abcam. Anti-β actin antibody was purchased from Sigma-Aldrich. Olmesartan was purchased from Daiichi-Sankyo (Tokyo, Japan.).

Vaccine synthesis

The HBc gene was obtained by PCR from the plasmid pPLc3 (BCCMTM/LMBP, Plasmid Collection, University of Ghent, Belgium). To construct pcDNA3.1-HBc, the HBc sequence was amplified by PCR using HBcF (5’-gccatggatcagctctataaaaaagatcggagc-3’) and HBcR (5’-gggctctcataacatggagatcggagatcggagcagtttaa-3’) as forward and reverse primers, respectively. The amplified fragment was inserted into the pcDNA3.1/V5-His-TOPO vector (Invitrogen) by TA cloning according to the manufacturer’s protocol.

To construct pcDNA3.1-HBc-AngII, PCR was performed three times. First, the 5’ half of the HBc ORF was amplified by PCR using HBcF and H2 (5’-ggtctggtataacgccggtcagctctataaaaaagatcggagc-3’) primers. Second, the 3’ half of the HBc ORF was amplified by PCR using H3 (5’-cgctgtataaccccttacgctcagctctataaaaaagatcggagc-3’) and HBcR primers. Primer H2 contains the sequence of the spacer Ile-Thr dipeptide and the N-terminal half of Ang II. Primer H3 contains the sequence of the C-terminal half of Ang II and the tripeptide spacer Gly-Ala-Thr. Using these two amplified fragments as templates, a third PCR was performed using primers HBcF and HBcR. The final amplified fragment contained the Ang II sequence within the immunodominant region (aa 80-81) of HBc; this fragment was inserted into the pcDNA3.1/V5-His-TOPO vector by TA cloning.

ISS containing four CpG motifs (CpG-A D19, 5’-ggtctggtataacgccggtcagctctataaaaaagatcggagc-3’; CpG-B 1018, 5’-tgactgtgaacggtcagctctataaaaaagatcggagc-3’; CpG-C 274, 5’-tcgctggtataacgccggtcagctctataaaaaagatcggagc-3’ and CpG-C 695 5’-tcgctggtataacgccggtcagctctataaaaaagatcggagc-3’) was synthesized by PCR using the following primers: CpGF (5’ttcactgtgaacggtcagctctataaaaaagatcggagc-3’), CpGR (5’-gtctggtataacgccggtcagctctataaaaaagatcggagc-3’) and templates: CpG1 (5’-ggctgtggtataacgccggtcagctctataaaaaagatcggagc-3’) and CpG2 (5’-aacgttcagctctataaaaaagatcggagc-3’).
acc-3'). The ISS fragment (112 bp) was digested with the restriction endonuclease \textit{DraIII} and inserted into the backbone of the pcDNA3.1-HBc-AngII-ISS(-) and pcDNA3.1-HBc-ISS(-) plasmids.

Each of these constructed vectors was used to transform \textit{E. coli} TOP10 competent cells (Invitrogen), and resultant colonies were screened for ampicillin resistance and sequenced to confirm correct insertions.

\textit{Animals and DNA immunization}

The experiments were approved by the Ethical Committee for Animal Experiments of the Osaka University Graduate School of Medicine. Eight-week-old male spontaneously hypertensive rats (SHRs) were purchased from The Oriental Yeast (Osaka, Japan) and were housed in a temperature- and light cycle-controlled animal facility with free access to food and water. These experimental protocols were approved by the Ethical Committee for animal experiments of the Osaka University Graduate School of Medicine. Rats were randomly assigned into three groups; each group of rats was anesthetized, and received DNA immunizations by a needleless injector, the Shima Jet (Shimazu, Japan). The HBc-Ang II group was vaccinated three times with pcDNA3.1-HBc-AngII, while the HBc group received pcDNA3.1-HBc and the saline group received saline only, all at 2-week intervals. Each shot delivered 100 \( \mu \text{g} \) of DNA, and two shots were delivered to each rat on shaved back skin at each immunization. Blood samples before immunization and 2, 4, 8, 12, 16, 20 and 24 weeks after the first immunization were collected. Sera were isolated and stored at -70 \( \degree \text{C} \) until use.

\textit{Measurement of BP and Ang II concentration}

Arterial BP was measured at 4, 8, 12, 16, 20 and 24 weeks by the tail-cuff method (BP-98A, SOFTRON, Japan). During measurements, the animals were held in a restraining device. The SBP values are shown as the average of 10 readings for each animal at each time. Plasma immunoreactive Ang II concentrations, Ang I concentrations, plasma renin activity, and daily urinary excretion of aldosterone were measured by radioimmunoassay by an external organization (FALCO Biosystems, Kyoto, Japan). Briefly, plasma Ang II is measured by competitive ELISA. The protocol is as follows, 1) the ELISA plate is coated by anti-angiotensin II antibody, 2) add sample plasma(s) to the ELISA plate with defined radiolabelled angiotensin II, 3) plasma angiotensin II concentration is quantified by measuring radioactivity bound to plate.
**Enzyme-linked immunosorbent assay (ELISA)**

Ang II-specific antibody responses were measured by coating ELISA plates with BSA-Ang II conjugate. Ang II was conjugated with BSA at its N-terminus by suberic acid bis (PEPTIDE INSTITUTE Inc., Osaka, Japan). BSA-Ang II conjugate was coated onto ELISA plates at 10 µg/ml in carbonate buffer overnight at 4 °C. After blocking with 5% skim milk solution in phosphate-buffered saline (PBS)/Tween, serum samples from immunized animals were diluted 1:100 in PBS containing 5% skim milk and then incubated for 2 hours on the plate. Detection was performed by goat anti-rat IgG horseradish peroxidase conjugate (NA935, GE Healthcare, Tokyo, Japan). The specificity of the signal was confirmed by assaying preimmune serum, which gave the signal background. The absorbance was read by a microplate reader (Bio-Rad Inc., Japan). Anti-Ang I and anti-Ang1-7 antibody responses were measured by coating ELISA plates with BSA-Ang I conjugate and BSA-Ang1-7 conjugate (PEPTIDE INSTITUTE Inc., Osaka, Japan), respectively. IFN-γ, IL-2, IL-4, and IL-10 were measured by Quantikine ELISA kit (R&D systems).

**ELISPOT Assay**

IFN-γ- and IL-2-producing T cells were quantified in splenocytes by *ex vivo* ELISPOT assay after peptide stimulation for 24 hours. Briefly, sterile 96-well ELISPOT plates (Millipore, Tokyo, Japan) were coated at 4 °C overnight with 50 µl mouse IFN-γ or IL-2 mAb as recommended by the manufacturer (R&D Systems, Tokyo, Japan). After overnight incubation, wells were blocked with 1.5% BSA and 5% sucrose. Freshly isolated splenocytes from individual immunized rats were seeded in wells (10⁶ cells/well) and restimulated by 50 µg/ml Phytohemagglutinin (PHA), 3 µg/ml recombinant HBc, 10 µg/ml Ang II or 10 µg/ml Ang-N in RPMI1640 medium. Splenocytes from saline-injected rats and cells in culture medium alone were used as negative controls to determine background levels. Each cell population was titrated in triplicate. A positive response was defined as a median number of spot-forming cells (SFCs) in triplicate wells at least twice that observed in control wells containing medium and at least 50 SFCs per million splenocytes.

**Western Blot Analysis.**
BSA and the BSA-AngII conjugate were separated electrophoretically by SDS/PAGE and blotted onto poly(vinylidene difluoride) membranes (Millipore). The blots were incubated with sera from rats immunized with the HBc-AngII vaccine, preimmune, anti-BSA antibody, or commercially available anti-Ang II antibody. For evaluation of the expression of plasmid DNA in the transfected Hela cell, the cell lysate was collected in RIPA lysis buffer (Millipore) and then subjected to SDS/PAGE. After electrophoresis, the membrane was incubated with anti-Ang II antibody, anti-HBc antibody, or anti-β actin antibody, respectively. After incubation with HRP-conjugated antibodies specific for rat IgG (GE Healthcare), chemiluminescence signal was detected with a FujiFilm LAS 1000 camera and analyzed with MultiGauge version 3.2 software.

Statistics

All values are expressed as the mean ± standard errors. Data were compared using two-way analysis of variance (ANOVA) followed by Dunnett’s test for pair-wise comparisons against the control group and Tukey’s test for multiple comparisons. P values less than 0.05 were considered statistically significant. All statistical analyses were performed using JMP8 software (SAS Institute, Inc., Cary, NC, USA).
**Fig. S-1. Construction of DNA vaccines.** A, Plasmid map of pcDNA3.1-HBc. HBc gene was cloned downstream of CMV promoter. ISS sequence containing CpG-A D19, CpG-B 1018, CpG-C C274 and CpG-C C695 was also inserted. B, Plasmid map of pcDNA3.1-HBc-AngII. DNA fragment encoding angiotensin II and its N- and C-terminal linkers were inserted at the position corresponding to amino acid 80-81 of HBc. C, Schematic presentation of fusion protein HBc-angiotensin II expressed by plasmid pcDNA3.1-HBc-AngII. Angiotensin II was inserted in HBc protein at aa 80-81 and the N-terminal I-T dipeptide linker and a C-terminal G-A-T tripeptide were designed in frame to angiotensin II to allow flexibility in the conformation of angiotensin II when surface-exposed on HBc particle. The angiotensin II and the linkers were represented by single-letter codes.
**Fig. S-2. Expression of plasmid DNA.** Hela cells were transfected with plasmids, and expression of HBc-AngII protein (Allowhead) was only detected in the cell lysate transfected with pcDNA3.1-HBc-AngII by immunoblot using anti-Angiotensin II antibodies. Expression of HBc-AngII protein and HBc protein was detected in the immunoblot with anti-HBc antibody. Lane 1, the untreated cell lysate; lane 2, the cell lysate transfected with pcDNA3.1-HBc-AngII; and lane 3, the cell lysate transfected with pcDNA3.1-HBc. *, HBc makes dimers.
**Fig. S-3. Plasma renin activity, Plasma Ang I concentration, and Daily urinary excretion of aldosterone.**

**A,** Plasma renin activity of vaccinated rats. Plasma samples were collected from rats 8 weeks after the first immunization. Saline group, n=5; HBc-AngII group, n=6. **B,** Plasma Ang I concentration of vaccinated rats. Plasma samples were collected from rats 8 weeks after the first immunization. Saline group, n=2; HBc-AngII group, n=3. **C,** Daily urinary excretion of aldosterone. Urine samples were collected from rats 24 weeks after the first immunization. HBc and Saline group, n=6; HBc-AngII group, n=5. Data are presented as the average of each group; error bars indicate the standard error of the mean. *P<0.01 vs. HBc-AngII group.*
Fig. S-4. The effect of vaccination on systolic BP in WKY rats. Eight-week-old male WKY rats (n=5) were vaccinated three times with pcDNA3.1-HBc-AngII at 2-week intervals. Systolic BP was measured at 0 and 6 weeks after the first immunization. Data are presented as the average of each group; error bars indicate the standard error of the mean.
Fig. S-5. The effect of A779 or ARB treatment in the HBc-AngII group. SHRs were vaccinated three times with pcDNA3.1-HBc-AngII at 2-week intervals (0, 2nd, 4th week). The administration of A779 (0.8 mg/kg/day, I.P., for 7 days) or olmesartan (3 mg/kg/day, P.O., for 7 days) was started at 5th week. Systolic BP was measured at 6th week. Data are presented as the average of each group; error bars indicate the standard error of the mean. *P<0.05 vs. saline group. n=4 per group.
Fig. S-6. Histological conditions of kidney and heart. A, Photomicrographs of sections of kidney from SHR of the saline group, the pcDNA3.1-HBc-AngII vaccinated group and the pcDNA3.1-HBc group. Scale bar, 100μm. B, Photomicrographs of sections of heart from SHR of the saline group, the pcDNA3.1-HBc-AngII vaccinated group and the pcDNA3.1-HBc group. Scale bar, 100μm.
**Fig. S-7. Histological conditions of liver and aorta.**  
**A,** Photomicrographs of sections of liver from SHR of the saline group, the pcDNA3.1-HBc vaccinated group and the pcDNA3.1-HBc-AngII group. Scale bar, 100µm.  
**B,** Photomicrographs of sections of aorta from SHR of the saline group, the pcDNA3.1-HBc vaccinated group and the pcDNA3.1-HBc-AngII group. Scale bar, 100µm.
Fig. S-8. Conceptual map of this experiment. Our working hypothesis of AngII vaccine therapy was shown as a conceptual figure. We immunized mice with HBc-AngII as an antigen. As an immunization phase, antigen presenting cells (APCs) phagocyte HBc-AngII and present T cell epitope of HBc-AngII to T cells through major histocompatibility complex (MHC), and T cells recognize it through T cell epitope and activate (differentiate to effector T cells) (step 1). B cells, which are specific to AngII, phagocyte HBc-AngII and present T cell epitope of HBc-AngII to T cells through MHC. Then, B cells differentiate to plasmacytes and produce antibodies with help of activated T cells (effector T cells) (step 2). These steps are required to produce antibodies efficiently.
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