Prevention of experimental autoimmune myocarditis by hydrodynamics-based naked plasmid DNA encoding CTLA4-Ig gene delivery

Short title

Prevention of EAM by plasmid DNA encoding CTLA4-Ig

Satoru Abe[1] MD, Haruo Hanawa[1] MD, Manabu Hayashi[1] MD, Tsuyoshi Yoshida[1] MD, Satoru Komura[1] MD, Ritsuo Watanabe[1] MD, Hui Lie[1] MD, He Chang[1] MD, Kiminori Kato[1] MD, Makoto Kodama[1] MD, Hiroki Maruyama[2] MD, Mikio Nakazawa[3] PhD, Junichi Miyazaki[4] MD, Yoshifusa Aizawa[1] MD

Division of Cardiology[1], Division of Clinical Nephrology and Rheumatology[2], Niigata University Graduate School of Medical and Dental Science, Niigata, Japan, Department of Medical Technology[3], School of Health Science, Niigata University School of Medicine, Niigata, Japan, Division of Stem Cell Regulation[4], Osaka University Graduate School of Medical Science, Osaka, Japan

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Address correspondence to:

Satoru Abe, MD, First Department of Internal Medicine, Niigata University School of Medicine, Asahi-machi 1-754, Niigata, 951-8510, Japan.

E-mail: maro747@yo.rim.or.jp

ABSTRACT

Background: Rat experimental autoimmune myocarditis (EAM) was a T cell-mediated disease, which resembled the giant cell myocarditis seen in humans. Soluble CTLA4 improves some autoimmune diseases by blocking costimulatory signals on T cell. We investigated the effect of hydrodynamics-based naked plasmid DNA encoding CTLA4-immunoglobulin (Ig) gene delivery.

Methods and Results: Lewis rats were immunized with cardiac myosin and treated with hydrodynamic-based transfection, namely a rapid tail vein injection of a large volume of pCAGGS encoding CTLA4-Ig chimera solution on Day 0. The vector-derived CTLA4-Ig mRNA expressions were mainly detected in the liver and plasma CTLA4-Ig protein levels were maintained at about 2 μg/ml during the experiment period. On Day 17, the ratio of heart to body weight, the amount of mRNA of atrial natriuretic peptide and the inflammatory areas in CTLA4 group were significantly lower than in the Control group treated with empty plasmid. Maximum rate of intraventricular pressure rise and decline (dp/dt), minimum dp/dt, LVEDP and central venous pressure improved significantly after treatment with CTLA4-Ig. On day 14, expressions of IL-2 in popliteal lymphnodes in the CTLA4-Ig group were significantly lower than in the Control group.

Conclusions: Hydrodynamics-based transfection of plasmid encoding CTLA4-Ig chimera dramatically prevented EAM.

1 Key Words

2 gene therapy, dilated cardiomyopathy, cytokine, pCAGGS

3

Introduction

We have shown previously that experimental autoimmune myocarditis (EAM), induced in the rat, was a T cell-mediated disease, which resembled the giant cell myocarditis seen in humans, and that long-term administration of anti-$\alpha\ \beta$ T cell receptor (TCR) antibody prevented the progression of EAM.[1-3] T cells recognise peptide-major histocompatibility complex (MHC) complexes on antigen-presenting cell (APC) by TCR.[4] Then, costimulatory molecules, for example CD28, CD4 or CD8 on T cells and CD80 (B7-1), CD86 (B7-2) or ICAM-1 on APC, play important roles to enhance adhesion of T cells to APC and transduce a costimulatory signal.[5] In particular, engagement of the CD28 molecule with its ligand B7 provides an essential costimulatory signal without which full activation of T cells cannot occur.[6] Exposure of T cell clones to antigen complexes with MHC through TCR in the absence of the costimulatory signal induces a state of clonal anergy.[7] CTLA-4 is 44kD protein which is similar to CD28 and CTLA-4 exhibits about twenty times greater binding affinity for B7 than CD28.[8] Therefore, CTLA4-Immunoglobulin (Ig) chimera protein can strongly inhibit the engagement of the CD28 with B7.[9] Matsui Y et al, have reported that adenovirus vectors containing CTLA4-Immunoglobulin (Ig) improved the pathological findings in the hearts of EAM subjects, through their ability to block T cell costimulatory signals.[10] However, adenoviruses are thought to cause viral myocarditis and adenovirus vectors themselves may influence the immunological mechanism and cardiac function on EAM.[11,12] Morerover,

tragic death by devastating inflammatory reaction to the adenoviral vector in a clinical trial for ornithine transcarbamylase deficiency was reported.[13] Therefore, adenoviruses are difficult to use for myocarditis treatment clinically. On the other hand, gene transfer by a naked plasmid vector is easier and safer than a virus vector and does not generally cause infection.[14,15] Nevertheless, a shortcoming of gene transfer by plasmid was the inability to express transgene adequately. However, it has recently been reported that hydrodynamics-based gene delivery by naked plasmid vector, namely a rapid tail vein injection of a large volume of plasmid DNA solution, could induce a sustained high-level of the encoded protein.[16-18] The serum levels of the relevant proteins acquired by this novel transfer method were high enough to suggest that it had potential as a strategy for gene therapy.[17,18] The vector-derived mRNA expression was mainly detected in the hepatocytes[17,18] and only the encoded protein was secreted. This, together with the fact that no immunological reaction occur, is thought to allow the role of the protein to be evaluated alone. Furthermore, modified hydrodynamics-based gene delivery by naked plasmid vector, for example retrograde hepatic or coronary venous delivery, may be clinical gene therapy method in the future.[19] In the study discussed here, we report the effects of hydrodynamics-based naked plasmid DNA encoding CTLA4-Ig gene delivery on preventing the development of EAM in rats. CTLA4-Ig protects rats from development of EAM as evidenced by a significant reduction of the histological cardiac cellular infiltrate and improvement in the hemodynamic status.

Methods

Preparation of EAM

Male Lewis rats were purchased from Charles River Japan Inc. (Atsugi, Japan). They were maintained in our animal facilities until 8 weeks of age. Whole cardiac myosin as antigen was prepared from the ventricular muscle of porcine hearts as previously described.[1] It was dissolved in a solution of 0.3 mol/l KCl at a concentration of 10 mg/ml and emulsified with an equal volume of complete Freund's adjuvant supplemented with 10 mg/ml of Mycobacterium tuberculosis H37RA (Difco, Detroit, Michigan, USA). On Day 0, each rat received a single immunization at two subcutaneous sites on the foot, with a total of 0.2 ml of the emulsified preparation (Fig. 1). Throughout the study, all animals were treated in accordance with our institute's guidelines for animal experiments.

Plasmid DNA for gene transfer

To create plasmids for plasma concentration analysis, we first constructed a plasmid pCAGGS-Ig-glucagon Tag (Glu-tag) with SwaI and NotI restriction sites using polymerase chain reaction (PCR).[20] The first PCR products were amplified from rat spleen cDNA using KOD Plus DNA polymerase (TOYOBO, Osaka, Japan) and the primers (5'-gaGAATTCATTTAAATgagaGCGGCCGCcgtgcccagaaactgtg-3' with SwaI

and NotI restriction sites and 5'-tcaaccactgcacaaaatcttgggctttacccggagagtgggagagact-3') (Uppercase letters indicate restriction enzyme sites). The final PCR product inserts were then amplified from the diluted products of the first PCR reaction with the primers (5'-gaGAATTCATTTAAATgagaGCGGCCGCcgtgcccagaaactgtg-3' with SwaI and NotI restriction sites and 5'-gagagagaGAATTCtcaggtattcatcaaccactgcacaaaatcttgggc-3'). These products were inserted into the pCAGGS vector using EcoRI sites. Escherichia coli JM109 competent cells were then transformed and recombinant plasmids were isolated using a Quantum Prep Plasmid Maxiprep kit (Bio-Rad Laboratories, Hercules, California, USA). Next, in order to construct the pCAGGS-rat CTLA4-Ig-Glu-tag, rat CTLA4 cDNAs were amplified from phytohemagglutinin-stimulated splenocyte cDNA using the primers (5'-gaGAATTCATTTAAATggcttgtcttggactccagagg-3' and 5'- gcagcatcGCGGCCGCgtctgaatctgggcatggttctgg-3') and then inserted into the pCAGGS-Ig-Glu-tag using SwaI and NotI sites. The recombinant plasmids were isolated as described above.

In the next stage we constructed a plasmid pCAGGS-CTLA4-Ig for EAM treatment. The PCR products amplified from rat spleen cDNA using the primers (5'-gaGAATTCATTTAAATgagaGCGGCCGCcgtgcccagaaactgtg-3' with SwaI and NotI restriction sites and 5'-gagagagaGAATTCactctggggtcatttacccggagagtgggag-3' as described above were inserted into pCAGGS vector using EcoRI sites (pCAGGS-rat Ig). E. coli JM109 competent cells were then transformed and recombinant

plasmids were isolated as above. To construct the pCAGGS-CTLA4-Ig, rat CTLA4 cDNAs were amplified as described above and then inserted into pCAGGS-rat Ig using SwaI and NotI sites.

Gene transfer of plasmid DNA

The effect of CTLA4-Ig on EAM was evaluated using previously published methods on Day 17, as follows. EAM rats were rapidly injected into tail vein on Day 0 either plasmid DNA pCAGGS-CTLA4-Ig (n=9), or empty plasmid as a control (n=9) at a dose of 800 μ g per rat, in a volume of 20 ml (approximately 80 ml/kg body weight) within 10 seconds (Fig. 1). In order to evaluate the mechanism of CTLA4-Ig effect, EAM rats prepared by the above-mentioned method, pCAGGS-CTLA4-Ig (n=6), or empty plasmid as a control (n=6). EAM rats were sacrificed on Day 14 and popliteal lymphnodes were taken. In addition, in order to measure the serum concentration of the protein, normal rats were injected with pCAGGS-rat CTLA4-Ig-Glu-tag as described above (n=3) (Fig. 1). We compared with a rapid tail vein injection of a large volume and a injection into the tibialis anterior muscles by electroporation.[21] Rats (n=4) were anesthetized with diethyl ether and injected as follows. Aliquots of 100 μ l of plasmid DNA (pCAGGS-rat CTLA4-Ig-Glu-tag) at 2 μ g/ μ l in phosphate-buffered saline were injected 4 times (total amount of DNA was 800 μ g per rat) into the bilateral tibialis anterior muscles using a disposable insulin syringe with a 27-gauge needle. A pair of electrode needles with a gap of 5 mm was inserted into the muscle to a depth of 5

mm to encompass the DNA injection sites, and electrical pulses were delivered 4 times at 100 V using an electrical pulse generator (Electro Porator CUY21; TR Tech). In order to evaluate the organs expressing vector-derived CTLA4-Ig mRNA, normal rats (n=3) were rapidly injected into tail vein with plasmid DNA pCAGGS-CTLA4-Ig-Glu-tag.

Plasma chimeric protein measurement

Blood samples were taken Days 1, 4, 10, 13 and 16 after hydrodynamics-based gene transfection or a injection into the tibialis anterior muscles by electroporation. Glucagon concentrations were measured using a glucagon radioimmunoassay (RIA) Kit (DAIICHI RADIOISOTOPE LABS, Tokyo, Japan).[22] Chimeric protein concentrations were calculated using the following formula: (chimeric protein concentration) = (actually measured glucagon concentration) x (chimeric protein molecular weight) / (whole glucagon molecular weight).[20]

Hemodynamic study

On Day 17, for the surgical procedure to measure the hemodynamic parameters, rats were anesthetized initially with 2% isoflurane in oxygen and then the concentration was reduced to 0.5% to minimize the hemodynamic effect. Mean arterial pressure (mean AP) was recorded through a catheter introduced into the right femoral artery. Central venous pressure (CVP) was recorded through a catheter introduced into the

confluence of the vena cava with the right jugular vein. A catheter-tip transducer was inserted into the left ventricle from the right carotid artery to measure the peak left ventricular pressure (LVP) and left ventricular end-diastolic pressure (LVEDP). The rate of intraventricular pressure rise and decline (±dP/dT) was measured with a differential amplifier. Heart rate (HR) was calculated from electrocardiograms. All hemodynamic parameters were recorded on a thermostylus recorder after a stabilizing period of 10 min.

Histopathology

After the hemodynamic study, a blood sample was obtained from the inferior vena cava. The heart on Day 17 was removed and cleaned of the surrounding tissues and atrium. The heart weight (HW) was measured and the ratio of HW to body weight (HW/BW) was calculated. For the histological evaluation, the hearts were sectioned at three levels and the middle one of the ventricles was fixed in 10% formalin. Paraffin-embedded tissues were cut and stained with hematoxylin-eosin and Azan-Mallory. In the biventricular cardiac cross section, the ratio of inflammatory area was computed by digital image processor software, Lumina Vision and Mac SCOPE (Mitani Co. Maruoka, Fukui, Japan), by means of the differences in color. The results were presented as the ratio of the inflammatory area to the whole biventricular section.

Quantitative RT-PCR

Total RNA was isolated from one third (apex side) of the rat's ventricle on Day 17 using Trizol (Life Technologies, Tokyo, Japan). cDNA was synthesized from 5 μ g of total RNA with random primers. Construction of the plasmid with rat atrial natriuretic peptide (ANP) cDNA as a standard sample for quantitative RT-PCR for ANP was as previously described.[23] Briefly, to construct the standard plasmid, cDNAs of ANP were amplified using the primers (5'-atggatttcaagaacctgctagac-3', 5'-gctccaatcctgtcaatcctac-3') from rat heart cDNA. The amplified cDNAs were directly inserted into the pGEM-T vector and the recombinant plasmids were purified. cDNA and diluted plasmid were amplified with the same primer used for making the plasmid and LightCycler-FastStart DNA Master SYBR Green I (Roche, Indianapolis, Indiana, USA) by LightCycler. The absolute copy numbers (molecules mRNA/ μ g of total RNA) of all the samples were calculated by the LightCycler software using this plasmid standard curve. In order to evaluate the mechanism of CTLA4-Ig, total RNA was isolated from popliteal lymphnodes of EAM on Day 14 and expressions of IL-2 and γ actin mRNA were similarly examined using the primers (IL-2, 5'-ctgagagggatcgataattacaaga-3', 5'-attggcactcaaatttgttttcag-3' ; γ actin, 5'-agccttccttcctgggcatggagt-3', 5'-tggaggggcctgactcgtcatact-3').

Vector-derived CTLA4-Ig mRNA expression

Total RNA was isolated from livers, kidneys, hearts, lungs and

spleens twenty four hours after rapid injection with pCAGGS-rat CTLA4-Ig-Glu-tag into tail vein. Expressions of vector-derived CTLA4-Ig mRNA were similarly examined using the primers (5'-tctgactgaccgcgttactccca-3' in seaquences of pCAGGS, 5'-gtgaggttcactctgctttcatta-3' in sequences of rat CTLA4) by LightCycler.

Statistical analysis

Data obtained from quantitative RT-PCR, HW, BW, myocarditis area and hemodynamic parameters were presented as the mean ± SD. Date of concentration of IL-1RA-Ig-Glu-tag were expressed as mean ± SEM. Statistical comparisons were performed by the Student non-paired t tests or one-way ANOVA and Bonferroni's multiple comparison test. The differences were considered significant at $p<0.05$.

Results

Plasma rat CTLA4-Ig-Glu-tag protein levels

Plasma CTLA4-Ig-Glu-tag protein levels, calculated by using Glu-tag, increased, peaking at 3550 ± 336 ng/ml (mean ± SEM) on Day 1 after hydrodynamics-based gene delivery by naked plasmid vector, namely a rapid tail vein injection of a large volume of plasmid DNA solution. Levels were maintained until Day 16 (1810 ± 400 ng/ml on Day 4, 3090 ± 629 ng/ml on Day 7, 2590 ± 270 ng/ml on Day 10, 2980 ± 321 ng/ml on Day 13

and 2300 ± 397 ng/ml on Day 16). However, plasma CTLA4-Ig-Glu-tag protein levels after a injection into the tibialis anterior muscles by electroporation were under 3.5 ng/ml, which was the sensitivity threshold, at all times (Fig. 1).

Vector-derived CTLA4-Ig mRNA expression in various organs

Because plasma CTLA4-Ig-Glu-tag protein levels were highest on Day 1 after hydrodynamics-based gene delivery, various organs were examined at the time. We detected the transgene-derived CTLA4-Ig mRNA by quantitative real-time PCR in the liver, heart, lungs, and kidney of rats that had been injected with a 20-ml volume of 800 μg of pCAGGS-rat CTLA4-Ig-Glu-tag (Fig 2). Among the organs examined, the level of CTLA4-Ig gene expression in the liver was the highest.

CTLA4-Ig treatment for EAM

EAM rats were treated with pCAGGS (Control group, n=9) or pCAGGS CTLA4-Ig (CTLA4 group, n=9). The parameters measured on Day 17 were compared between the two groups and the results of the statistical analysis are summarized in Table 1 and displayed in Fig.s 3 to 6. The results for HW, BW and HW/BW are shown in Fig. 3. BW was larger, while HW and HW/BW were smaller, in the CTLA4 group compared with the Control group; these differences were all statistically significant ($p<0.01$). The ratio of inflammatory area was significantly smaller

($p<0.05$) in the CTLA4 group compared with the Control group (Fig. 4A, 4B). There was no significant difference in HR between the two groups. However, the mean AP, LVP and the absolute value of +dP/dT or –dP/dT were significantly larger in the CTLA4 group compared with the Control group ($p<0.01$ in each case). LVEDP and CVP were significantly smaller in the CTLA4 group compared with Control group ($p<0.05$ for both) (Fig. 5).

Expression of ANP mRNA in EAM hearts

The expression of ANP mRNA in the heart is an important response in the modulation of cardiac function.[23] The level of ANP mRNA in the CTLA4 group ($0.80x10^6 \pm 0.96x10^6$ molecules/μg total mRNA) was significantly lower ($p<0.01$) compared with levels in the Control group ($7.70x10^6 \pm 4.41x10^6$ molecules/μg total mRNA) (Fig. 6).

Expression of IL-2 mRNA in popliteal lymphnode

EAM on Day 14 is thought to be in progress of myocarditis and it was demonstrated that concanavalin A activated T cells in lymphnode on Day 14 elicited severe myocarditis.[3] mRNA of IL-2 known as T cell growth factor was significantly suppressed by hydrodynamics-based gene delivery of pCAGGS rat CTLA4-Ig (Fig. 7).

Discussion

We examined the effect of CTLA4-Ig on EAM using gene therapy with naked plasmid vector. We found that CTLA4-Ig improved dramatically not only the histopathological finding but also cardiac function of EAM. In previous studies, we have investigated the protective effect of antibody for TCR and cytokines in EAM.[2,25] This therapy has the advantage that specific immunologic tolerance can be achieved by blocking costimulatory signals in spite of lack of exact information of the autoantigen. Blockade of the CD28-B7 pathway with CTLA4-Ig has been demonstrated previously as a useful strategy in autoimmune disease.[26-29] In this study, it was demonstrated that gene expression of IL-2 in EAM popliteal lymphnode was suppressed by CTLA4-Ig. IL-2 known as a Th1 cytokine is thought to play an important role in progression of EAM.[30] This suppression of IL-2 mRNA may be one of the mechanism of CTLA4-Ig effect.

The CTLA4-Ig chimeric protein consists of rat CTLA4 and rat IgG1 Fc portions and Shioji reported that a large amount of the immunoglobulin Fc portions were effective for the prevention of EAM.[31] However, in this study, the serum level of CTLA4-Ig achieved by hydrodynamics-based transfection was only 1/1000 that of the native serum immunoglobulin level. We have used the hydrodynamics-based method to treat plasmids inserted into various kinds of Ig chimeric protein, but some therapies were ineffective in the prevention of EAM (data not shown). In the study reported here, the fc portion of immunoglobulin was considered to be ineffective.

Previous studies demonstrated that the intravenous administration of adenoviral vectors encoding a CTLA4-Ig chimeric protein could successfully ameliorate experimental allergic encephalomyelitis,[32] murine systemic lupus erythematosus,[33] murine collagen-induced arthritis,[34] nephritis in a murine lupus model,[35] and EAM.[10] Gene therapy by adenoviral vector is a powerful technology. However, compared with virus-mediated gene transfer systems, the introduction of an exogenous gene into cells in the form of naked DNA has many obvious advantages. Preparation of DNA has become routine in many laboratories and since it is chemically and biologically stable, no sophisticated storage conditions are required. Large quantities of highly purified plasmid DNA can be obtained easily and inexpensively. Wolff JA, et al reported the successful expression of a reporter gene in muscle[15] and Aihara H and Miyazaki J reported that gene transfer into muscle by electroporation in vivo was one hundred times more efficient than simple intramuscular DNA injection.[36] We first examined the effect on EAM of CTLA4-Ig gene transfer into muscle by electroporation. However, its effect was weak and hemodynamic differences between the Control group and CTLA4 group were not significant (data not shown). Therefore, in the current study we tried hydrodynamics-based transfection and have demonstrated that it clearly prevented the development of EAM. Plasma CTLA4-Ig-Glu-tag protein levels by hydrodynamics-based transfection were about one thousand times higher than by intramuscular transfection with electroporation. We demonstrated that the transgene-derived CTLA4-Ig mRNA was detected by quantitative real-time PCR in the liver, heart, lungs, spleen and kidney of

rats that had been treated with hydrodynamic-based transfection. Among the organs examined, the level of CTLA4-Ig gene expression in the liver was the highest. Hydrodynamics-based transfection is an easy and powerful technology. There was no significant difference in the hemodynamics on Day 17 after immunization between the Control group and non-treated EAM group (data not shown), which indicates that the initial temporal volume overload did not affect the hemodynamic status of EAM. This technology remedies the shortcoming of gene transfection using adenovirus, as outlined above. Matsui et al, demonstrated therapy with adenovirus vectors containing CTLA4-Ig to EAM.[10] In their study, blockade of T cell costimulation by CTLA4-Ig prevented the induction and progression of EAM, as shown in their histological findings, HW/BW ratio and cellular and hormonal immune response. Our data also indicated that a therapeutic administration of pCAGGS-CTLA4Ig prevented the induction of EAM, and improved not only the histological findings but also the hemodynamic status. Five out of the nine rats in the CTLA4-Ig group had few or no inflammatory findings. These results together suggest that pCAGGS-CTLA4-Ig is as effective as adenovirus vector in preventing the induction of EAM.

We demonstrated that intravenous injections of plasmids with cDNA encoding CTLA4-Ig protected Lewis rats from developing EAM as evidenced by a significant reduction to the histological cardiac cellular infiltrate and an improvement in the hemodynamic status.

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Figure Legends

Fig. 1.

Plasma rat CTLA4-Ig-Glu-tag protein levels after hydrodynamics-based gene delivery (open circle), namely a rapid tail vein injection of a large volume of pCAGGS-rat CTLA4-Ig-Glu-tag or intramuscular gene delivery (closed circle) into the tibialis anterior muscles by electroporation. Each bar represents mean ± SEM. Open arrow, immunization; solid arrow, gene transfer; open arrow head, evaluation of mRNA in popliteal lymphnode; solid arrow head, physiological and histological evaluation.

Fig. 2.

Absolute vector-derived CTLA4-Ig mRNA per 1 μ g total RNA in liver, heart, kidney, lung and spleen of normal rat twenty four hours after hydrodynamics-based gene delivery of pCAGGS-rat CTLA4-Ig-Glu-tag (n=3). Each bar represents mean ± SD. + $p<0.01$ differences between the two groups. Statistical comparisons were performed by one-way ANOVA and Bonferroni's multiple comparison test.

Fig. 3.

Effects of pCAGGS-CTLA4-Ig on body weight (BW), heart weight (HW), and HW/BW ratio on Day 17 after immunization. Each bar represents mean ± SD. + $p<0.01$ differences between the two groups. Statistical comparisons were performed by the Student non-paired t tests.

Fig. 4.

Fig. 4A, Transverse section of hearts. No therapy group (left), pCAGGS-treated Control group (middle), pCAGGS-CTLA4 Ig-treated group (right). Azan-Mallory staining. Fig. 4B, The ratio of inflammatory area to whole section in the Control group and CTLA4 group. Each bar represents mean ± SD. + $p<0.01$ differences between the two groups. Statistical comparisons were performed by the Student non-paired t tests.

Fig. 5.

Hemodynamic parameters on Day 17 after immunization. Each bar represents mean ± SD. Statistical comparisons were performed by the Student non-paired t tests.

* $p<0.05$, + $p<0.01$ differences between the two groups.

HR (heart rate); CVP (central venous pressure); AP (arterial pressure); LVP (left ventricular pressure); LVEDP (left ventricular end-diastolic pressure); dP/dT (rate of intraventricular pressure rise and decline).

Fig. 6.

Absolute number of atrial natriuretic peptide (ANP) mRNA per 1 μ g of total RNA in the two groups. Each bar represents mean ± SD. + $p<0.01$ differences between the two groups. Statistical comparisons were performed by the Student non-paired t tests.

Fig. 7.

Copy numbers of IL-2 mRNA / copy numbers of γ actin in popliteal lymphnodes of the two groups. Each bar represents mean ± SD.

* $p<0.05$ differences between the two groups. Statistical comparisons were performed by the Student non-paired t tests.

Fig. 1.

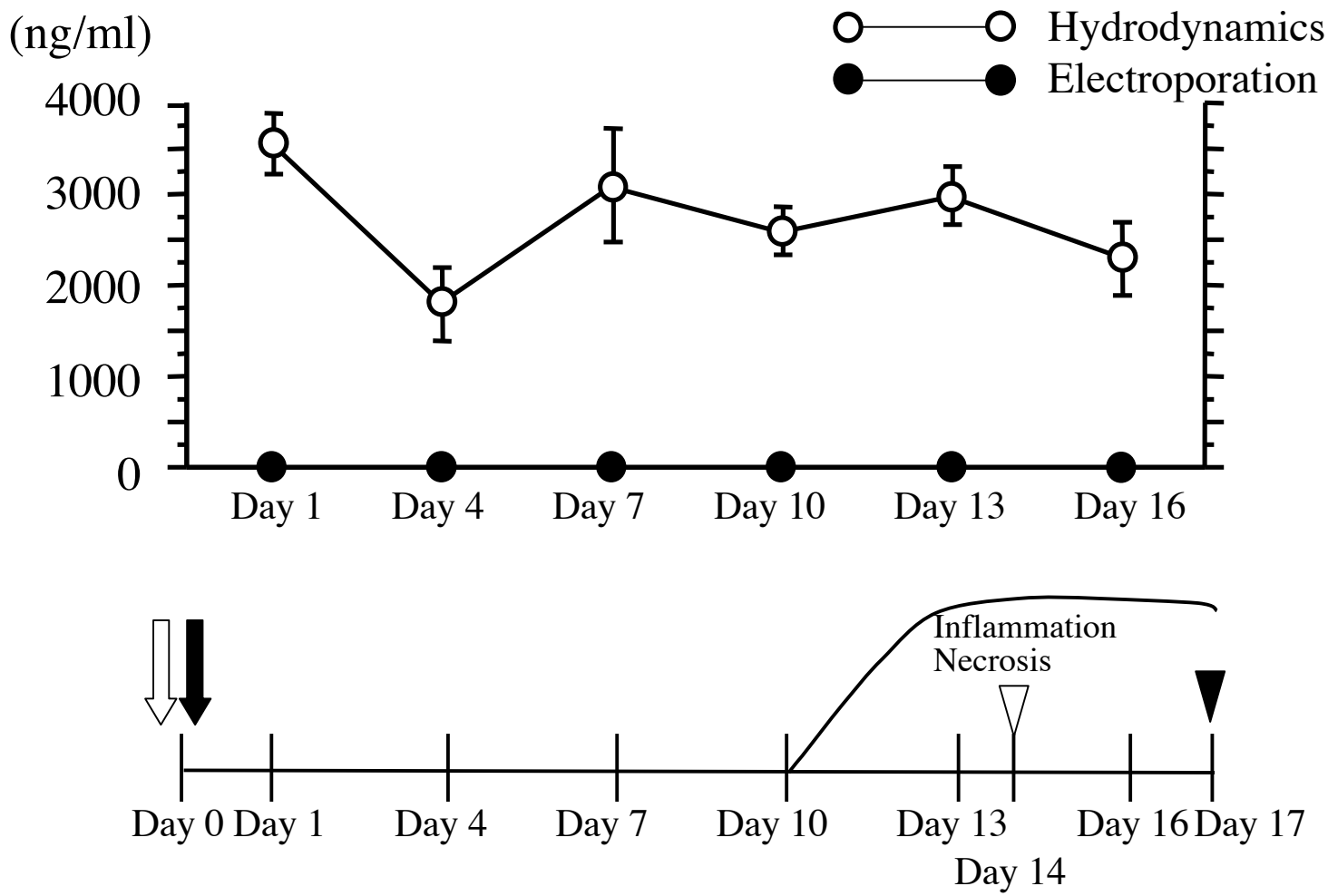

(ng/ml)
Hydrodynamics
Electroporation
4000
3000
2000
1000
0
Day 1
Day 4
Day 7
Day 10
Day 13
Day 16
Inflammation
Necrosis
Day 0
Day 1
Day 4
Day 7
Day 10
Day 13
Day 14
Day 16
Day 17

Fig. 2.

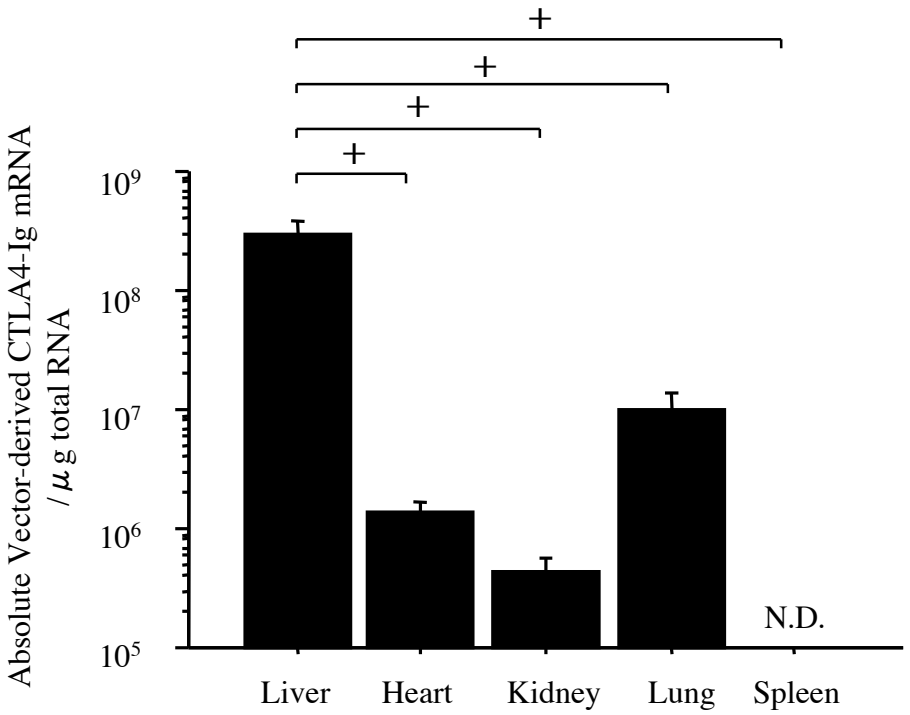

+
+
+
+
Absolute Vector-derived CTLA4-Ig mRNA / μg total RNA
10^9
10^8
10^7
10^6
10^5
N.D.
Liver
Heart
Kidney
Lung
Spleen

Fig. 3.

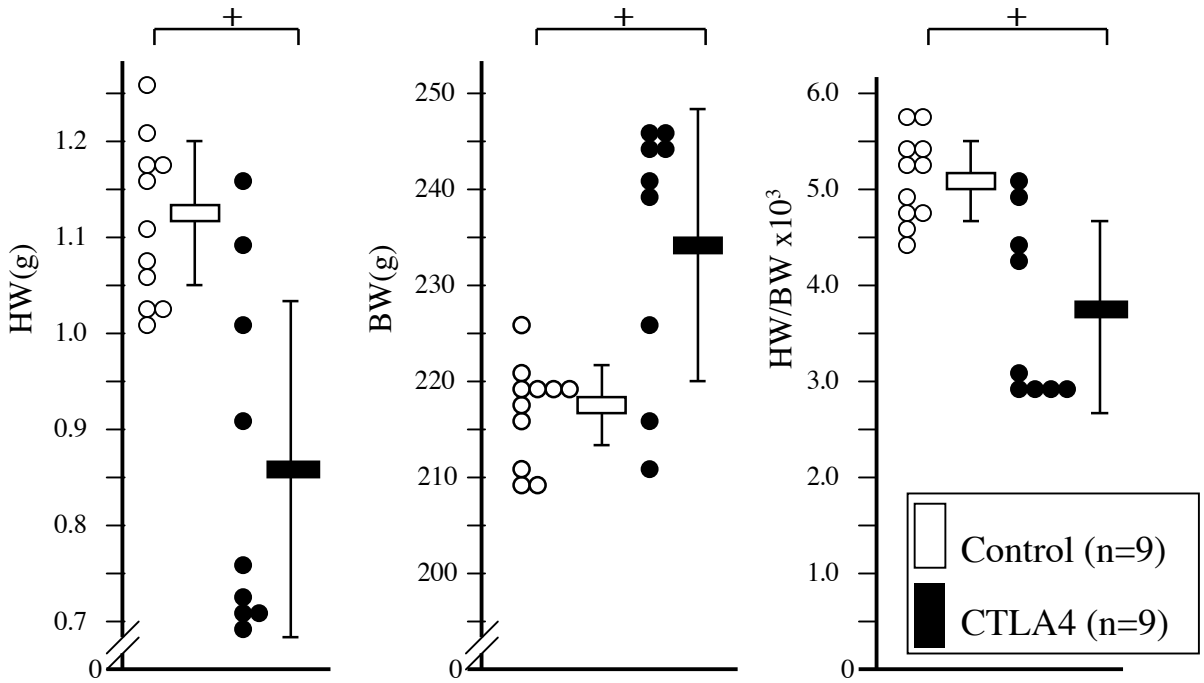
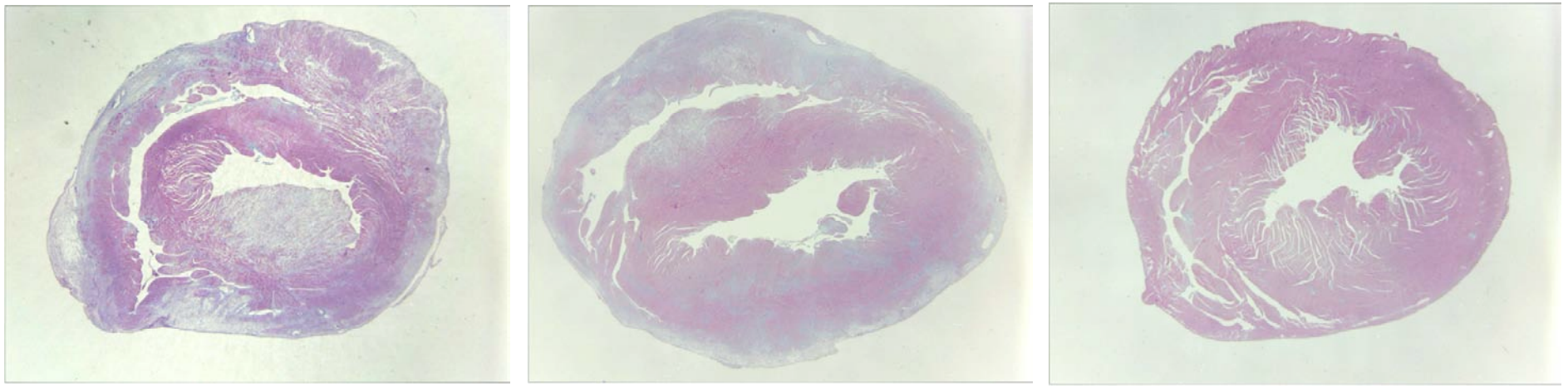

+
+
+
HW(g)
1.2
1.1
1.0
0.9
0.8
0.7
0
BW(g)
250
240
230
220
210
200
0
HW/BW x10³
6.0
5.0
4.0
3.0
2.0
1.0
0
Control (n=9)
CTLA4 (n=9)

Fig. 4.

A



B

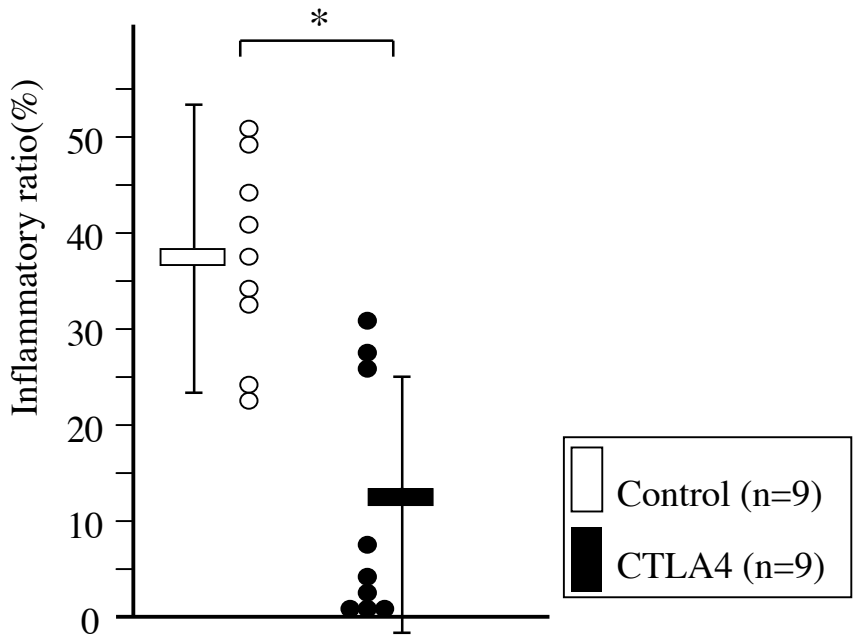


Fig. 5.

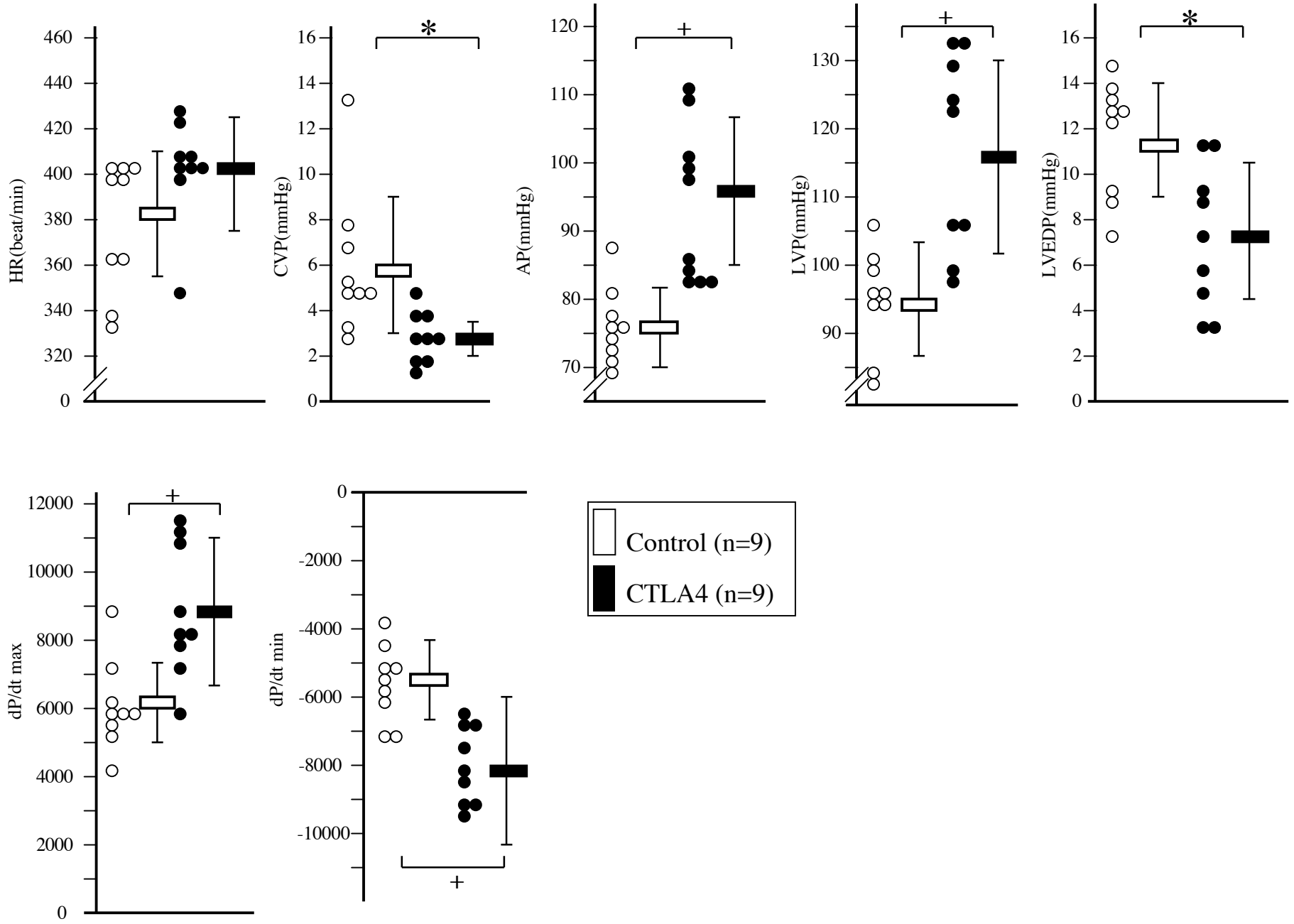

HR(beat/min)
460
440
420
400
380
360
340
320
0
CVP(mmHg)
16
14
12
10
8
6
4
2
0
*
AP(mmHg)
120
110
100
90
80
70
+
LVP(mmHg)
130
120
110
100
90
+
LVEDP(mmHg)
16
14
12
10
8
6
4
2
0
*
dP/dt max
12000
10000
8000
6000
4000
2000
0
+
dP/dt min
0
-2000
-4000
-6000
-8000
-10000
+
Control (n=9)
CTLA4 (n=9)

Fig. 6.

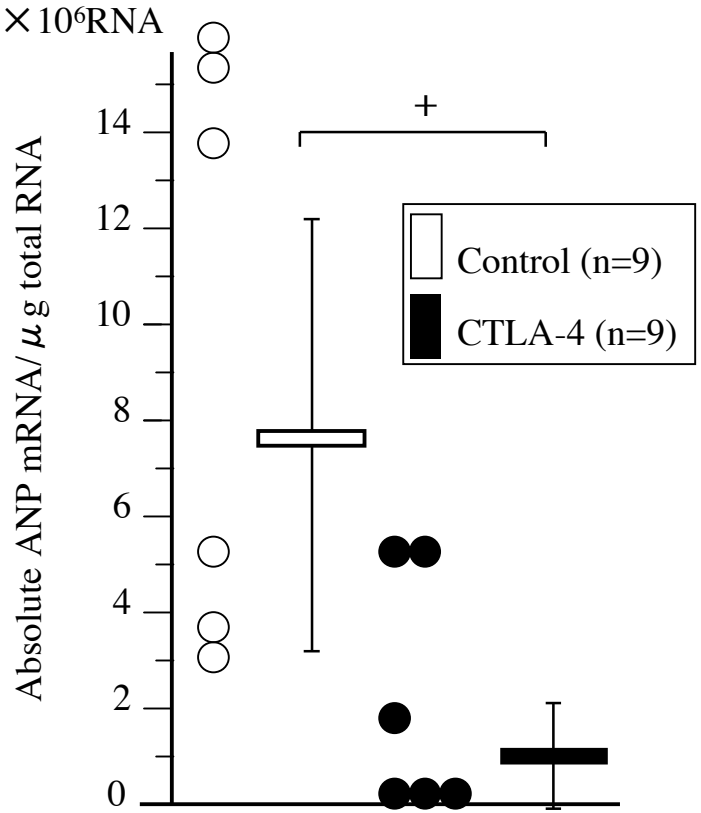

×10⁶RNA
Absolute ANP mRNA/μg total RNA
14
12
10
8
6
4
2
0
+
Control (n=9)
CTLA-4 (n=9)

Fig. 7.

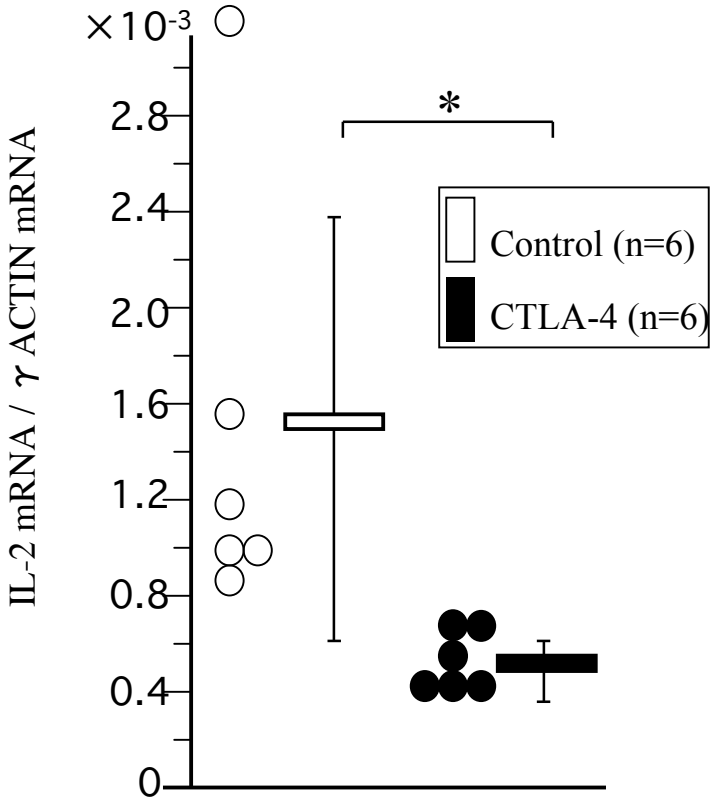

×10-3
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
IL-2 mRNA / γ ACTIN mRNA
*
Control (n=6)
CTLA-4 (n=6)

Table 1. Summary of results in the Control and CTLA4 groups.

	Control(n=9)	CTLA4(n=9)	
HW(g)	1.11 ±0.07	0.86 ±0.18	p<0.01
BW(g)	218±5.36	236±14.1	p<0.01
HW/BW_1000	5.08±0.42	3.73±0.95	p<0.01
Inflammatory Ratio(%)	38.6±12.3	12.1±13.91	p<0.05
Hemodynamic parameters			
HR (bpm)	377±27.8	402±25.3	
CVP(mmHg)	5.93±3.08	2.87±1.12	p<0.05
AP (mmHg)	76.8±5.74	94.6±11.6	p<0.01
LVP(mmHg)	95.1±7.73	117±14.5	p<0.01
LVEDP(mmHg)	11.7±2.62	7.38±3.28	p<0.05
dP/dT max	6034±1344	8833±1969	p<0.01
dP/dT min	-5654±1147	-8069±1266	p<0.01
mRNA of ANP (molecules / μg of total RNA)	$7.70x10^6$±$4.41x10^6$	$0.80x10^6$±$0.96x10^6$	p<0.01

Statistical significance between the Control group and CTLA4 group was determined by the Student's non-paired t test. HW (heart weight); BW (body weight); HR (heart rate); CVP (central venous pressure); AP (arterial pressure); LVP (left ventricular pressure); LVEDP (left ventricular end-diastolic pressure); dP/dT (rate of intraventricular pressure rise and decline); ANP (atrial natriuretic peptide).