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Evidence of aldosterone synthesis in human myocardium in acute myocarditis☆

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ABSTRACT

Background: Myocarditis may be self-limited but has been identified as an important contributor to downstream cardiomyopathy. Aldosterone mediates myocardial damage in various conditions, but has not been considered specifically as a therapeutic target for inflammatory damage in acute myocarditis. We sought to demonstrate local aldosterone synthesis in human myocardium affected by acute myocarditis.
Methods: We evaluated myocardial samples obtained via endomyocardial biopsy (EMB) for expression of CYP11B2, the final and key enzyme for aldosterone synthesis, from patients with acute myocarditis and from stable heart transplant recipients with no evidence of rejection as negative controls. Excised adrenal glands from patients with aldosterone-secreting adenomas were used as positive controls. An experienced cardiovascular pathologist blinded to clinical information rated CYP11B2 stains as negative, positive, or borderline, also recording location of any CYP11B2-positivity.
Results: Sixteen patients' EMB samples showing definite acute myocarditis were identified (50% female). CYP11B2 was positive in 13/16 cases (81%), typically showing diffuse intracardiomyocyte cytoplasmic staining, vs. 2/16 borderline stains in transplant controls ($p < 0.001$ myocarditis vs. negative controls). All 3 adrenalectomy samples stained positive for CYP11B2 (diffuse intracellular staining). Importantly, no myocarditis or transplant patients were on aldosterone antagonist therapy at the time of biopsy.
Conclusions: In this proof-of-concept study, myocardium from patients with acute myocarditis demonstrates evidence and high prevalence of local aldosterone synthesis by immunohistochemistry that showed high accuracy, specificity, and sensitivity. Aldosterone warrants consideration as a specific target for therapy in patients with myocardial damage due to inflammation towards strategies that reduce downstream complications.

1. Introduction

Myocarditis is a disease defined by inflammation of the myocardium; while reported prevalence varies [1–3], it affects individuals worldwide of both sexes and across all age groups [2–5]. There are no specific treatments recommended for patients with mild symptoms without ventricular dysfunction, since the acute episode often resolves spontaneously [6]. However, up to 30% of patients with biopsy-proven myocarditis will go on to develop dilated cardiomyopathy (DCM) with heightened risk of heart failure, arrhythmias and death [5–8].

While many pathways are known to contribute to the progression from myocarditis to DCM [2,6,9], the role of aldosterone is less explored. Aldosterone, typically produced in the adrenal cortex, promotes inflammation and fibrosis [10–12]. Aldosterone synthesis requires the enzyme aldosterone synthase (CYP11B2) whose messenger ribonucleic acid (mRNA) is absent in normal human myocardium [13] but present (though not localized) in end-stage heart failure myocardium obtained at time of cardiac transplantation [14].

CYP11B2 mRNA has been identified in hypertensive and infarcted rat myocardium [15,16], though intramyocardial aldosterone synthesis has never been evaluated in acute human myocarditis. In this work, we tested the hypothesis that local aldosterone synthesis occurs within the cardiomyocytes of patients with acute myocarditis, seeking a novel

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therapeutic target to ideally reduce post-myocarditis DCM and heart failure.

2. Methods

2.1. Study population

Patients admitted to The Ohio State University Wexner Medical Center with acute myocarditis confirmed by endomyocardial biopsy (EMB) and immunohistochemistry (IHC) were retrospectively identified with approval of the Institutional Review Board for human subjects research. Review of clinical slides by an experienced cardiovascular pathologist (P.B.) confirmed that cases met established histopathological criteria of: "inflammatory infiltrate of the myocardium with necrosis and/or degeneration of adjacent myocytes not typical of the ischemic damage associated with coronary artery disease" [3,4]. Diagnosis was supported by IHC stain for CD3, CD4, CD20, and CD68 to further characterize inflammatory and cellular infiltrate. Acute myocarditis was defined clinically based on cardiovascular symptoms without coronary artery disease by angiography and with at least one of the following: serum troponin-I elevation, ECG findings suggestive of cardiac injury, or abnormal left ventricular function by echocardiogram or cardiac magnetic resonance [17].

For negative controls we identified stable, post heart transplant (HTx) patients who had undergone routine follow-up EMB that showed no signs of rejection (score = 0) per International Society of Heart and Lung Transplantation guidelines [18]. For positive controls, we used adrenal gland tissue obtained at adrenalectomy from patients with primary aldosteronism.

Clinical and demographic data including symptoms at presentation, medications, electrocardiographic (ECG) findings, laboratory values, and imaging findings were recorded for patients in both the myocarditis and negative control groups.

2.2. CYP11B2 immunohistochemistry

Paraffin-embedded tissue was sectioned at 4 μm and placed on positively-charged slides. Slides were then placed in a 60 °C oven for 1 h, cooled, deparaffinized and rehydrated through xylene and graded ethanol solutions to water. All slides were quenched for 7 min in a 3% hydrogen peroxide aqueous solution to block for endogenous peroxidase. Slides were stained with the Dako (Agilent Technologies) Link 48 Autostainer Immunostaining System with the following antibody: CYP11B2 Polyclonal Antibody from Thermo Fisher Scientific. All incubations on the Autostainer were performed at room temperature. Slides were counterstained in hematoxylin and eosin (H&E) (Dako/Agilent #SK203), dehydrated through graded ethanol solutions, cleared with xylene, and coverslipped. Review of each patient's slides was performed by an experienced cardiovascular pathologist (P.B.) blinded to clinical information. Stains were scored based on percent positivity as: 0 (no evidence of staining or borderline i.e. non-convincing staining), 1+ (<25% myocytes staining positive), 2+ (26–50% staining positive), 3+ (51–75% staining positive) and 4+ (76–100% staining positive). Location of any CYP11B2 positivity was also recorded. Number of leukocytes per square millimeter was determined from review of H&E slides. Grade of any myocyte hypertrophy was scored as: 0 = none, 1 = mild, 2 = moderate, 3 = severe.

2.3. Statistical methods

Data are presented as mean ± standard deviation (SD) or as median and interquartile range (IQR) for continuous variables and as proportions for categorical variables. Comparison of continuous variables and proportions was performed with two-tailed *t*-test and Fisher exact test, respectively. The sample size required to detect a possible significant difference in CYP11B2 immunostaining between cases and controls was calculated based on power analysis assuming a theoretic prevalence of positive straining of 80% and 20%, in cases and controls, respectively, with pre-specified values for $1-\beta = 0.8$ and type I error rate, $\alpha = 0.05$. A prevalence of 20% for positive staining in the control group was adopted as a conservative value based upon the fact that previous studies show that aldosterone synthesis in the healthy myocardium is virtually absent [13,14]. Twelve subjects for each group were required to satisfy the above power analysis parameters. With 16 subjects enrolled for each group, the actual power and type I error rate of the study were $1-\beta = 0.91$ and $\alpha = 0.018$, respectively. Statistical significance was set at two tailed $p < 0.05$. IBM SPSS Statistic 21.0 (Chicago, IL, USA) was used for all statistical analyses.

3. Results

Thirty-eight patients who had undergone EMB with suspicion for acute myocarditis were identified over the prior 8 years. We excluded 19 cases where EMB yielded no findings of myocarditis or non-specific findings. Another 3 cases were excluded because of borderline findings such as mild inflammatory infiltrates without evidence of myocyte necrosis. Of the 16 cases of definite biopsy-proven acute myocarditis, 15 were lymphocytic dominant and one showed giant cells (5 days was the median time from symptoms to EMB).

Demographic and clinical information of the study population are shown in Table 1. As expected subjects with acute myocarditis were younger than post-HTx patients, mean age 37 ± 16 and 52 ± 7 years, respectively; and the prevalence of CV risk factors was lower in acute myocarditis subjects (Table 1). The age of 3 subjects with primary aldosteronism whose excised adrenal tissue was used as positive controls was 45 ± 14.1 years.

Detailed characteristics of subjects presenting with acute myocarditis are shown in Table 2. The most common presenting symptoms were chest pain and dyspnea. Troponin-I was elevated in all subjects, ranging from 0.12 to 70.1 ng/ml. Most relevant electrocardiographic changes at presentation were ST-T segment abnormalities (ST depression, T-wave inversion), ventricular tachycardia or bradyarrhythmia. Importantly, none of the patients with myocarditis and in the negative control group were on aldosterone antagonist therapy at admission. Left ventricular ejection fraction tended to be reduced in most of the subjects with acute myocarditis (median 32%, IQR 28%). Cardiac magnetic resonance was performed in 12 subjects; and in 10 there were findings consistent with acute myocarditis including elevated signal on T2-weighted images or T2 mapping plus presence of mid to epicardial damage on late gadolinium enhancement images [19].

H&E staining showed evidence of myocyte damage and inflammatory infiltrate consistent with diagnosis of acute myocarditis in all 16 cases according to Dallas criteria. IHC was positive in all nine cases where it was performed (Table 2). Biopsies from transplanted hearts were negative for evidence of any rejection in all 16 subjects of the negative control group (score 0) [18].

All three adrenal tissue samples from patients with primary aldosteronism (positive controls) stained positive for CYP11B2. In the acute myocarditis group, 13 cases (81%) stained positive for CYP11B2, evident as diffuse intracytoplasmic staining in the cardiomyocytes: 4 cases were given a score of 4+ (76–100% of myocytes staining positive), 3 cases had a score of 3+ (51–75% positive), 3 cases had a score of 2+ (26–50% positive), and 3 cases had a score of 1+ (<25% positive). Two cases were labeled as borderline and one case did not stain positive for this enzyme. On the other hand in the control group, only two cases showed borderline staining, whereas the remaining 14 cases were completely negative. The difference between the two groups was highly significant, even if considering the two borderline post-HTx cases as staining positive ($p < 0.001$). Fig. 1 shows side-by-side

Table 1
Population characteristics.

	Myocarditis group (16)	Negative controls (16)	p value
Age, years	37 ± 16	52 ± 7	0.003
Female, N (%)	8 (50)	3 (19)	0.135
RACE (AA), N (%)	7 (44)	2 (12)	0.113
HTN, N (%)	8 (50)	10 (62)	0.722
DM, N (%)	2 (12)	9 (56)	0.023
Smoking, N (%)	2 (12)	0 (0)	0.484
HLP, N (%)	4 (25)	14 (87)	0.001
CKD, N (%)	0 (0)	7 (44)	0.007
BB, N (%)	8 (50)	1 (6)	0.015
ACE-i, N (%)	4 (25)	1 (6)	0.333
ARB, N (%)	0 (0)	0 (0)	1.000
MCRA, N (%)	0 (0)	0 (0)	1.000
Statin, N (%)	3 (19)	13 (81)	0.001
ASA, N (%)	5 (31)	14 (87)	0.003
SBP, mm Hg	113 ± 18	132 ± 21	0.007
DBP, mm Hg	75 ± 11	73 ± 11	0.590
HR, bpm	75 ± 16	91 ± 12	0.003
BMI, kg/m^2	29.8 ± 5.5	28.0 ± 6.4	0.394

Notes: Data are presented as mean ± SD or N (%). $p < 0.05$ considered significant. AA: Afro-American; HTN: hypertension; DM: diabetes; HLP: hyperlipidemia; CKD: chronic kidney disease; BB: beta blockers; ACE-i: angiotensin converting enzyme inhibitors; ARB: angiotensin II receptor blockers; MCRA: mineralocorticoid receptor antagonists; ASA: acetylsalicylic acid; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate.

Table 2
Characteristics of subjects with acute myocarditis.

#	Age (years)	Gender	Race	CV risks	PMH	Symptoms at presentation	NYHA class	Peak Tn-I (ng/ml)	CRP (mg/dl)	BNP (pg/ml)	ECG presentation	LVEF (%)	IHC	CYP11B2 staining	CYP11B2 stain score*	Myocyte hypertrophy score**	Time admission-biopsy (days)
1	33	M	AA	HTN, HLP	Negative	Dyspnea	2	70.1	74	300	VT	30	Positive	Positive	4	1	5
2	25	F	AA	None	Post-partum CMP, Von Willebrand disease	CP	1	4.81	NA	4	ST-T changes	26	NA	Negative	0	0	6
3	24	M	AA	None	Negative	CP, dyspnea	1	27.91	NA	NA	TWA	29	NA	Positive	3	1	5
4	30	F	AA	HTN	Negative	CP, cardiogenic shock	4	19.98	137	NA	Prolonged QT	15	Positive	Positive	3	1	3
5	22	M	C	None	Negative	CP	1	28.55	140	30	ST-T changes	45	Positive	Positive	3	0	1
6	21	F	C	None	Addison's disease, lupus anticoagulant	Cardiogenic shock	4	22.36	237	1201	ST-T changes	15	Positive	Borderline	0	0	15
7	56	M	C	None	Negative	Dyspnea	2	0.53	68	689	VT; junctional	29	NA	Positive	4	1	4
8	68	M	C	HTN, HLP	Negative	CP, dyspnea	1	0.15	NA	25	TWA	40		Positive	2	1	7
9	17	M	C	None	Negative	Syncope	1	0.27	0.8	42	RBBB	58		Positive	4	1	3
10	34	M	C	HTN	LVH	Dyspnea	2	0.32	NA	100	PAF	50	NA	Positive	4	2	8
11	63	F	AA	HTN, smoking, HLP	Negative	CP, dyspnea	3	0.12	24.2	413	ST-T changes	45		Positive	2	1	2
12	40	F	AA	None	Recurrent myocarditis, AV block s/p PPM-ICD	Dyspnea	2	3.23	NA	2106	ST-T changes	20	NA	Positive	1	0	12
13	21	F	AA	Smoking	Asthma	CP, dyspnea	2	11.02	NA	163	RBBB	50	Positive	Positive	2	1	3
14	55	F	C	HTN, DM	CHF; VA s/p ICD	Dyspnea	2	0.12	2.4	367	VT	35	Positive	Borderline	0	0	12
15	41	F	C	HTN	Negative	Cardiac arrest/VA	2	0.15	NA	144	VA	20	Positive	Positive	1	0	2
16	44	M	C	HTN, HLP	DVT	Dyspnea	2	0.33	NA	NA	TWA	46	NA	Positive	1	1	15

Notes: M: male: F: female; AA: Afro-American; C: Caucasian; HTN: hypertension; HLP: hyperlipidemia; DM: diabetes; LVH: left ventricular hypertrophy; PMH: relevant past medical history; IHC: immunohistochemistry; CMP: cardiomyopathy; PPM-ICD: pacemaker-defibrillator; DVT: deep vein thrombosis; CHF: chronic heart failure; NYHA: New York Heart Association; CP: chest pain; VT: ventricular tachycardia; VA: ventricular arrhythmia; PAF: paroxysmal atrial fibrillation; TWA: T-wave abnormality; RBBB: right bundle branch block; CRP: C-reactive protein; BNP: brain natriuretic peptide; LVEF: left ventricular ejection fraction. *CYP11B2 stains were scored from 0 to 4+ (see text), and **myocyte hypertrophy if present was deemed mild (1), moderate (2) or severe (3), otherwise absent (0).

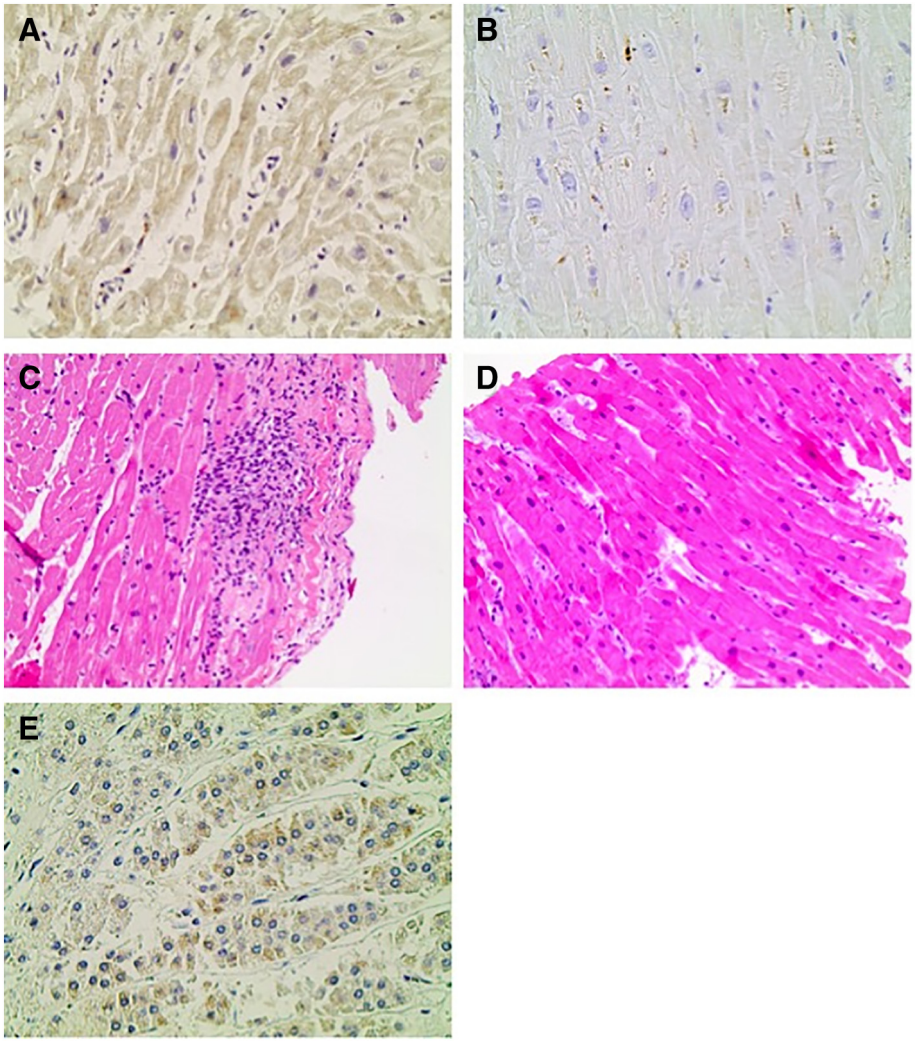

Fig. 1. Light microscopy of immunohistochemistry (CYP11B2) and Hematoxylin Eosin Staining. **Legend**: Panels A and B show side by side comparison of typical immunostaining targeted for CYP11B2 (aldosterone synthase). Panel A (acute lymphocytic myocarditis) demonstrates evidence of prominent positive staining localized within the cytoplasm of myocytes (diffuse brown coloration) as opposite to B (normal heart without evidence of rejection) where there is no staining uptake. Panels C and D are correspondent Hematoxylin Eosin Staining (H&E) of the same subjects. In C a patchy inflammatory infiltrate along with myocyte damage is present meeting Dallas criteria for acute lymphocytic myocarditis. Panel D demonstrates a normal myocardial tissue. Panel E is a slice of a cortical adrenal gland used as positive control which demonstrates diffusely positive staining targeted for CYP11B2. CYP11B2 and H&E slides are shown at original magnifications of 400× and 200×, respectively.

comparison of CYP11B2 immunostaining along with H&E slides of one case of acute lymphocytic myocarditis, one negative control and one positive control.

4. Discussion

In this study, we show for the first time evidence of intramyocardial aldosterone synthesis in the myocardium of patients with biopsy-proven acute myocarditis. Aldosterone synthase (CYP11B2), the key enzyme required to synthesize aldosterone, was present in cardiomyocytes in a very high proportion of EMB samples from patients with acute myocarditis as compared to myocardial tissue from stable post-HTx subjects with no evidence of rejection (negative controls). Importantly, none of the post-HTx patients were receiving mineralocorticoid antagonist drug therapy arguing against a potential source of false-negative result of immunostaining. Finally, positive staining in all adrenal gland tissue examined from patients with primary aldosteronism (positive controls) supports the validity of this technique. This in vivo demonstration of aldosterone synthesis in humans with myocarditis offers critical new evidence to support investigations of aldosterone-targeted treatment for the acute phase of a disease that contributes significantly to downstream cardiomyopathy and heart failure.

It is increasingly appreciated that aldosterone, beyond its traditional role in electrolyte and blood pressure regulation, directly influences the myocardium and vasculature. Aldosterone and mineralocorticoid receptor (MR) activation initiate an inflammatory response by increasing the generation of reactive oxygen species (ROS) [11]. MRs are expressed in cardiomyocytes, endothelial cells, vascular smooth muscle cells, adipocytes, and monocytes [11,20]. Administration of aldosterone promotes monocyte and macrophage infiltration and increased expression of inflammatory cytokines in the myocardium, vasculature, and kidney [11]. In the heart, perivascular inflammation is followed by the proliferation of fibroblasts and myofibroblasts, collagen production, perivascular fibrosis, and lastly, interstitial fibrosis [21,22].

Reverse transcription polymerase chain reaction (RT-PCR) studies of myocardium from normal and failing human hearts indicate aldosterone synthesis only in the latter [13,14], with some evidence obscured by concomitant malignancies [23]. The present study shows for the first time that cardiomyocytes in humans with acute, biopsy-proven myocarditis show evidence of aldosterone synthesis in inflamed myocardium. This represents a novel pathophysiological insight into the acute phase of this condition with potentially significant

therapeutic implications. Acute myocarditis, particularly when 'self-limited', does not mandate specific therapies other than supportive measures. However, in the subacute phase, adverse remodeling can progress to DCM that carries significant morbidity and mortality. Waiting to institute therapies once HF ensues, recognizing that at least 20% of DCM/heart failure may have resulted from antecedent myocarditis, means heightened risk of death and disability despite advances in medications, device therapies, circulatory support and transplantation; hence, new therapeutic approaches for the acute phase that ideally *prevent* downstream DCM are warranted [5–8,24].

Aldosterone antagonists are established therapy in patients with HF to reduce morbidity and mortality for systolic HF in patients with persistent NYHA functional class II to IV symptoms [24,25]. However, current guidelines do not consider aldosterone antagonist therapy for acute myocarditis without heart failure. A single preclinical study of encephalomyocarditis virus-inoculated mice showed that eplerenone treatment inhibited mast cell-derived proteinases, attenuated myocardial remodeling and fibrosis, and improved survival [26]. In humans with dystrophin-associated cardiomyopathy, a condition where the epicardial pattern of injury by late gadolinium enhancement cardiac magnetic resonance mirrors that seen in acute myocarditis [27], clinical trial data support efficacy of eplerenone added to background ACEI or ARB therapy while systolic function is still preserved [28,29]. Whether or not this translates to human myocarditis is unknown, though the target validation of this work would support clinical trials in this regard.

4.1. Limitations

The declining use and low sensitivity of EMB in acute myocarditis limited sample size despite screening over an 8-year period at a large referral center with advanced heart failure services. However, the study was carefully powered to detect significant differences in immunostaining between cases and controls. While the patients in this cohort were admitted primarily for acute myocarditis, there was significant concomitant LV systolic dysfunction and a few cases with heart failure by BNP elevation. Further studies are needed to know if aldosterone synthesis warrants therapeutic targeting in myocarditis regardless of LVEF and functional status. Finally, viral assays were not performed; further investigations exploring aldosterone synthase differences in viral from non-viral myocarditis are warranted.

5. Conclusions

Aldosterone synthase expression can be detected in high proportion in human myocardium during acute myocarditis. Clinical trials of aldosterone antagonist therapy for acute myocarditis warrant consideration towards reduced downstream HF/DCM.

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Conflicts of interests

Authors have no conflict of interests to disclose.

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