European Heart Journal (2000) **21,** 296–305
Article No. euhj.1999.1744, available online at http://www.idealibrary.com on IDEAL®

Increased digitalis-like immunoreactive substances in patients with hypertrophic cardiomyopathy

T. Hayashi, Y. Ijiri[1], H. Toko, H. Shimomura, M. Okabe, F. Terasaki, Y. Kitaura and K. Kawamura

The Third Division, Department of Medicine, Osaka Medical College, and [1]Department of Pharmacy, Osaka Medical College Hospital, Takatsuki, Osaka, Japan

Aims Although increased digitalis-like immunoreactive substances have been found in cases of hypertension and heart failure, no information is available about digitalis-like immunoreactive substances in patients with hypertrophic cardiomyopathy. We investigated digitalis-like immunoreactive substances in the plasma and biopsied specimens of patients with hypertrophic cardiomyopathy.

Methods and Results In 40 patients with hypertrophic cardiomyopathy (27 with the non-obstructive type and 13 with the obstructive type), the plasma concentration of digitalis-like immunoreactive substances was studied by fluorescence polarization immunoassay. Right ventricular endomyocardial biopsy specimens were analysed immunohistochemically, using a monoclonal antibody against digoxin. An increase in digitalis-like immunoreactive substances of more than 0·2 ng . ml^{-1} in plasma was found in six of 27 patients with non-obstructive hypertrophic cardiomyopathy (22·2%) and five of 13 with obstructive hypertrophic cardiomyopathy (38·4%). Under light microscopy, positive staining against the antibody was observed heterogeneously on some cardiocytes. In non-obstructive hypertrophic cardiomyopathy, digitalis-like immunoreactive substances in the plasma correlated with the left atrial dimension and inversely with the cardiac index. In obstructive hypertrophic cardiomyopathy, plasma and myocardial digitalis-like immunoreactive substances were positively correlated; they also correlated with left ventricular end-diastolic pressures. Under electron microscopy, digitalis-like immunoreactive substances were detected at the sarcolemma in the free wall, T-tubules, intercalated discs and Z-bands of cardiocytes.

Conclusions Increased digitalis-like immunoreactive substances in plasma and cardiocytes, which may have been caused by pressure and/or volume overload, were found in patients with hypertrophic cardiomyopathy. Digitalis-like immunoreactive substances may act on the sarcolemma of cardiocytes and be transported into the cytoplasm.
(Eur Heart J 2000; 21: 296–305)

Key Words: Digitalis-like immunoreactive substances, hypertrophic cardiomyopathy, immunohistochemistry, ultrastructure.

Introduction

In 1994, Hamlyn reported the existence of endogeneous ouabain, corresponding to digitalis-like factors, which are Na^+-K^+-ATPase inhibitors and may participate in the volume regulation of cells and tissues of mammals[1]. However, the digitalis-like immunoreactive substances were found to be heterogeneous and may be involved in food ingestion. To date, increased concentrations of digitalis-like immunoreactive substances have been found under conditions of hypertension, cardiac failure, renal insufficiency and hepatic disorder, and also in the blood of pregnant women, fetuses and newborns[2–7].

Hypertrophic cardiomyopathy is characterized by abnormal stiffness of the left ventricle with resultant impaired ventricular filling. The most characteristic anatomical finding is inappropriate myocardial hypertrophy that occurs without clear cause, such as aortic stenosis or hypertension. In addition, progression of hypertrophic cardiomyopathy to left ventricular dilatation and dysfunction without a gradient may occur in 10 to 15% of patients[8,9].

No information is currently available regarding the existence or the role of digitalis-like immunoreactive substances in patients with hypertrophic cardiomyopathy. We hypothesized that digitalis-like immunoreactive substances increase in hypertrophic cardiomyopathy,

Revision submitted 6 April 1999, and accepted 1 June 1999.

Correspondence: Tetsuya Hayashi, MD, PhD, The Third Division, Department of Medicine, Osaka Medical College, 2–7 Daigakumachi, Takatsuki City, Osaka 569-8686, Japan.

0195-668X/00/040296+10 $35.00/0

Table 1 Summary of data from patients with hypertrophic cardiomyopathy

	HNCM			HOCM			z	*P*
	n	mean	SD	n	mean	SD		
Age	27	54·70	8·85	13	56·76	9·91	− 0·792	0·428
DLIS (ng . ml^{-1})	27	0·14	0·17	13	0·33	0·43	− 1·146	0·252
LAD (cm)	27	4·15	0·59	13	4·60	0·82	− 1·679	0·093
IVSd (cm)	27	1·35	0·49	13	1·74	0·68	− 1·927	0·054
PWd (cm)	27	1·07	0·19	13	1·08	0·17	0·148	0·882
Dd (cm)	27	4·73	0·54	13	4·32	0·46	2·389	0·017
Ds (cm)	27	2·87	0·51	13	2·47	0·33	2·619	0·009
FS (%)	27	40·15	7·47	13	41·62	7·49	− 0·680	0·496
Ao (mmHg)	26	124·96	18·86	13	130·54	29·96	− 0·612	0·541
LVsys (mmHg)	26	124·85	18·34	13	156·85	47·07	− 1·909	0·056
LVedp (mmHg)	27	12·26	6·91	13	16·00	6·79	− 1·825	0·068
C.I. (l . min^{-1} . mm^{-2})	25	2·87	0·69	13	2·82	0·57	0·046	0·963
Diameter (μm)	27	15·08	2·41	13	15·60	1·72	− 0·823	0·410
Disarray	26	1·77	0·76	13	2·00	0·91	− 0·980	0·327
Fibrosis	27	1·59	0·75	13	2·08	0·64	− 1·908	0·056
Score	20	1·89	0·92	11	2·21	0·93	− 0·668	0·504

HNCM=non-obstructive type; HOCM=obstructive type; DLIS=plasma concentration of digitalis-like substances; LAD=left atrial dimension; IVSd=diastolic ventricular septal thickness; PWd=diastolic posterior wall thickness; Dd=diastolic left ventricular dimension; Ds=systolic left ventricular dimension; FS=fractional shortening; Ao=aortic pressure; LVsys=left ventricular systolic pressure; LVedp=left ventricular end-diastolic pressure; C.I.=cardiac index. Note that Disarray, Fibrosis, and Score were evaluated semiquantitatively (see text).

when myocardial hypertrophy and cardiac failure (diastolic failure with/without systolic failure) are observed. We investigated digitalis-like immunoreactive substances in the plasma and biopsied specimens of patients with hypertrophic cardiomyopathy, and compared them with the haemodynamic data.

Methods

Patients

The subjects were 40 patients, aged 55·3 ± 9·1 (range 35–75, 10 females and 30 males), who were diagnosed with hypertrophic cardiomyopathy by echocardiography combined with Doppler recordings[8,9] and underwent cardiac catheterization; 27 patients had the non-obstructive type of hypertrophic cardiomyopathy (age 54·7 ± 8·8) and 13 the obstructive type (age 56·7 ± 9·9) (Table 1). The diagnosis of obstructive hypertrophic cardiomyopathy was confirmed by the existence of abnormal systolic anterior motion of the anterior mitral leaflet and systolic pressure gradient within the body of the left ventricle at rest[9].

Ten patients with idiopathic arrhythmia, ranging in age from 44 to 72 (56·4 ± 6·33) years, who showed no signs of organic cardiac dysfunction at echocardiography and who underwent cardiac catheterization including right ventricular biopsy, constituted the control group. Five patients (age 55·2 ± 3·83) undergoing coronary bypass surgery, in whom needle biopsies were taken from the right ventricle, served as additional controls. Tissue was removed from areas of normal contractility and a normal blood supply, which is as close as possible to normal human myocardium[10,11].

None of the subjects enrolled in this study had hypertension or had been prescribed digitalis or spironolactone, which was confirmed by careful history taking and from clinical charts.

Plasma concentration of digitalis-like immunoreactive substances

Plasma from patients obtained on the day or a few days prior to cardiac catheterization was analysed by fluorescence polarization immunoassay (Abbot TDX™, Abbott Laboratories, Chicago, U.S.A.)[12,13], to determine the concentration of digitalis-like immunoreactive substances. If there was more than 0·2 ng . ml^{-1} of digitalis-like immunoreactive substance in the plasma this was considered to be positive since the detection limit by fluorescence polarization immunoassay was 0·2.

Histology

Specimens were obtained via endomyocardial biopsy from the right ventricle from the 40 patients with hypertrophic cardiomyopathy and from the 15 control patients.

Every specimen was separated into two. For light microscopic study, the specimens were fixed in 10% formaldehyde, embedded in paraffin, and cut in 4 μm thick sections. The tissue sections were stained with haematoxylin and eosin and Mallory-azan, and examined by light microscopy. Measurement of the transverse

diameter of cardiocytes and semiquantitative analyses of myofibre disarrangement and collagen proliferation were conducted following methods previously published[14]. Briefly, each histological change was graded 0 to 4+ under light microscopy, according to the severity and the extent of the findings: grade (0), no apparent significant change; (1+), minimal degree; (2+), moderate degree; (3+), marked degree; (4+), excessively marked degree.

For the immunohistochemical light microscopic study, additional sections were obtained from the paraffin block from 31 patients with hypertrophic cardiomyopathy (20 cases were of the non-obstructive type and 11 of the obstructive type), ranging in age from 40 to 73 (54·6 ± 12·2) years. In seven cases of non-obstructive hypertrophic cardiomyopathy and two cases of obstructive hypertrophic cardiomyopathy, the biopsied specimens were too small to obtain additional sections. The sections were placed on silanized glass slides (No. S3003, DAKO Japan, Kyoto, Japan), and the slides were dried, dewaxed in xylene, and rehydrated in graded concentrations of ethanol. For quenching endogenous peroxidase activity, the slides were incubated in 3% peroxide for 10 min. After incubation with normal blocking serum, the sections were incubated overnight at 4 °C with monoclonal anti-digoxin antibody (MAB515, Chemicon International, Temecula, CA and #5111, Transformation Research, Framingham, MA, U.S.A.). After serial washing with phosphate-buffer solution, the slides were incubated with biotinylated secondary antibody for 45 min at room temperature. The sections were then allowed to react with Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA, U.S.A.) for 30 min. After incubation in the peroxidase substrate solution (Vectastain 3′3′-diaminobenzidine substrate kit, Vector Laboratories, Burlingame, CA, U.S.A.), the slides were counterstained with Mayer-Hematoxylin, cleaned, and mounted for light microscopy[15].

For the electron microscopic study, the specimens were fixed in 4% paraformaldehyde containing 0·25% glutaraldehyde and 4·5% sucrose, and embedded in LR White (London Resin Company, Hampshire, U.K.). Ultrathin sections were mounted on formvar/carbon support film grids (200 Ni/50, Electron Microscopy Sciences, Washington, PA, U.S.A.), and incubated in normal blocking serum. The sections were incubated overnight at 4 °C with 10 nm gold conjugated monoclonal anti-digoxin antibody (GM-38-10, EY laboratories, San Meteo, CA, U.S.A.). After double staining with uranyl acetate and lead citrate, the sections were examined with a Hitachi H-7000 electron microscope. Close accumulation of five or more gold particles, based on a comparison with the control, was considered to be a positive reaction for digoxin. Positive reactions were also confirmed when a similar accumulation of particles was seen at the same site in serial sections [16].

Histological specificity was checked by absorption tests using digoxin and bovine serum albumin. Specimens obtained from patients with dilated cardiomyopathy, who had been chronically treated with oral digoxin, were used as the positive control. Their data were reported elsewhere[17].

As negative controls for the immunohistochemical procedures, substitution of an identical concentration of non-immune IgG for the primary antibody and direct incubation in colloidal gold without primary antibody were adopted.

Semiquantitative analysis by immunohistochemistry

Following immunohistochemical analysis, the staining grade of digitalis-like immunoreactive substances was evaluated from 0 to 3+ under light microscopy in all specimens by three observers, who were blind towards the other available data. (Fig. 1). The average of the graded scores and the data of the plasma digitalis-like immunoreactive substance concentrations were compared with the haemodynamic, echocardiographic and histological parameters. All results were expressed as mean ± standard deviation (SD).

Statistical analysis

Statistical analysis was performed by the Fisher and the Wilcoxon methods, and the relationship between the scores of digitalis-like immunoreactive substances and each parameter was examined with linear regression analysis by the least squares method[17]. The results were considered statistically significant when $P<0·05$.

Results

Digitalis-like immunoreactive substances in plasma

None of the control patients was positive for digitalis-like immunoreactive substances in plasma. The plasma concentration of patients with hypertrophic cardiomyopathy ranged from 0 to 1·40 ng . ml^{-1}, with an average of 0·14 ± 0·17 in the non-obstructive types and 0·33–± 0·43 in the obstructive types (Table 1). An increase in digitalis-like immunoreactive substances of more than 0·2 ng . ml^{-1} was found in six of 27 patients with non-obstructive hypertrophic cardiomyopathy (22·2% and five of 13 with obstructive hypertrophic cardiomyopathy (38·4%). The incidence of positive digitalis-like immunoreactive substances in plasma was slightly higher in obstructive type patients than in non-obstructive type patients.

Although there was no significant difference in left ventricular systolic and end-diastolic pressures between those with and those without digitalis-like immunoreactive substances, the left ventricular pressures tended to be elevated in plasma-positive patients. The left atrial dimension in those with digitalis-like immunoreactive

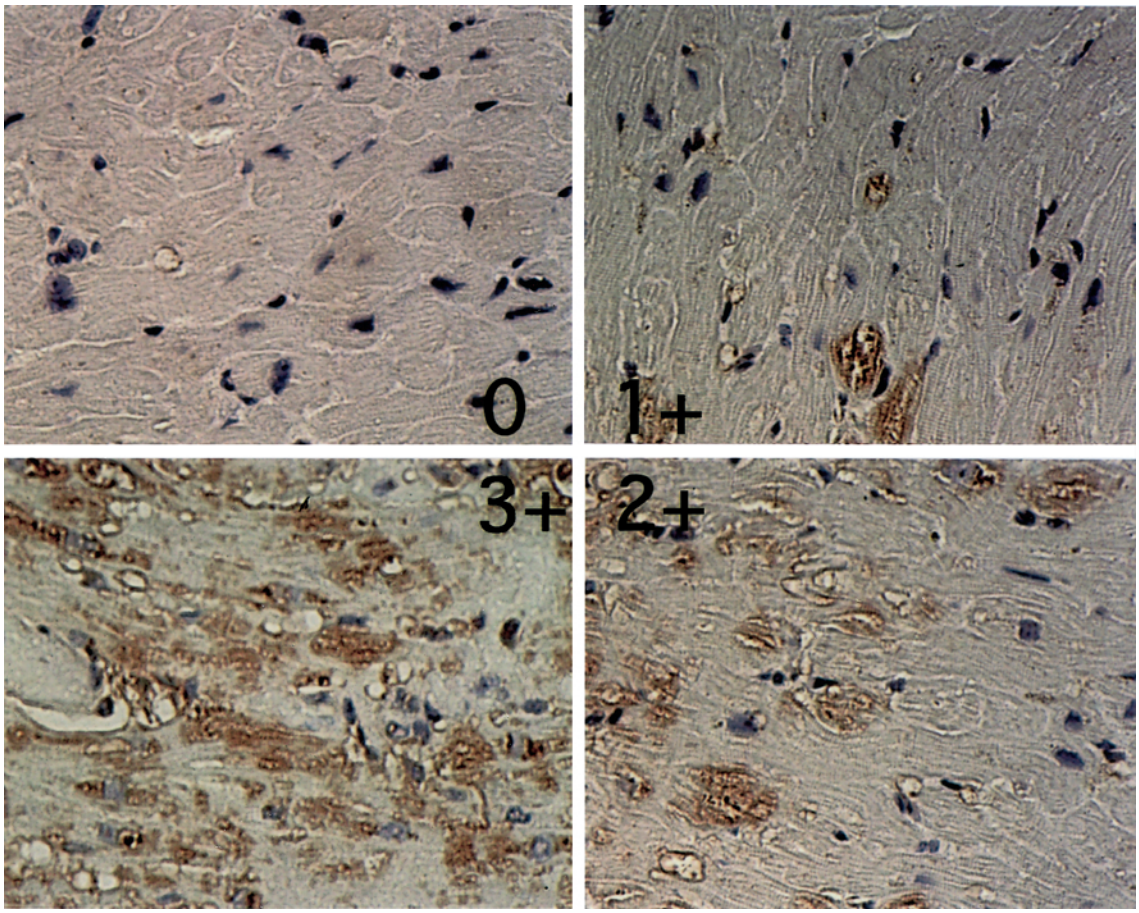

Figure 1 **Representative immunohistochemical staining grades of digitalis-like immunoreactive substances in hypertrophic cardiomyopathy patients. The immunoreactivity was evaluated semiquantitatively with staining grades from 0 to 3+ under light microscopy. Original magnification, × 100.**

substances (4·7 ± 0·6 cm) was significantly higher than in those without digitalis-like immunoreactive substances (4·1 ± 0·5 cm $P=0·01$).

Histology

The diameter of cardiocytes and the semiquantitative data found by ordinary light microscopic study are shown in Table 1. There was no significant difference between non-obstructive and obstructive hypertrophic cardiomyopathy.

Immunohistochemistry

In controls, no 3′3′-diaminobenzidine products or gold accumulation was observed (Fig. 2). Under light microscopy, positive staining against the antibody was observed heterogeneously on some cardiocytes, both in non-obstructive and obstructive hypertrophic cardiomyopathy (Fig. 2). The number of cardiocytes with positive staining tended to be greater in patients with obstructive hypertrophic cardiomyopathy than in those with the non-obstructive type. Some patients who were negative for digitalis-like immunoreactive substances in the plasma showed positive staining immunohistochemically in the myocardium. Under electron microscopy, digitalis-like immunoreactive substances were detected at the sarcolemma in the free wall, T-tubules and intercalated discs. Digitalis-like immunoreactive substances were also observed in the cytoplasm adjacent to the intercalated discs, nuclei and Z-bands of cardiocytes (Fig. 3). The cell membranes of the endothelial cells of capillary vessels also presented positive digitalis-like immunoreactive substances.

Semiquantitative analysis by immunohistochemistry

Following immunohistochemistry, the staining grade of digitalis-like immunoreactive substances was evaluated (Fig. 1), and the average score is shown in Table 1. There was no significant difference in the score between non-obstructive and obstructive hypertrophic cardiomyopathy. Nine of 31 patients with hypertrophic cardiomyopathy, whose plasma and myocardium were studied

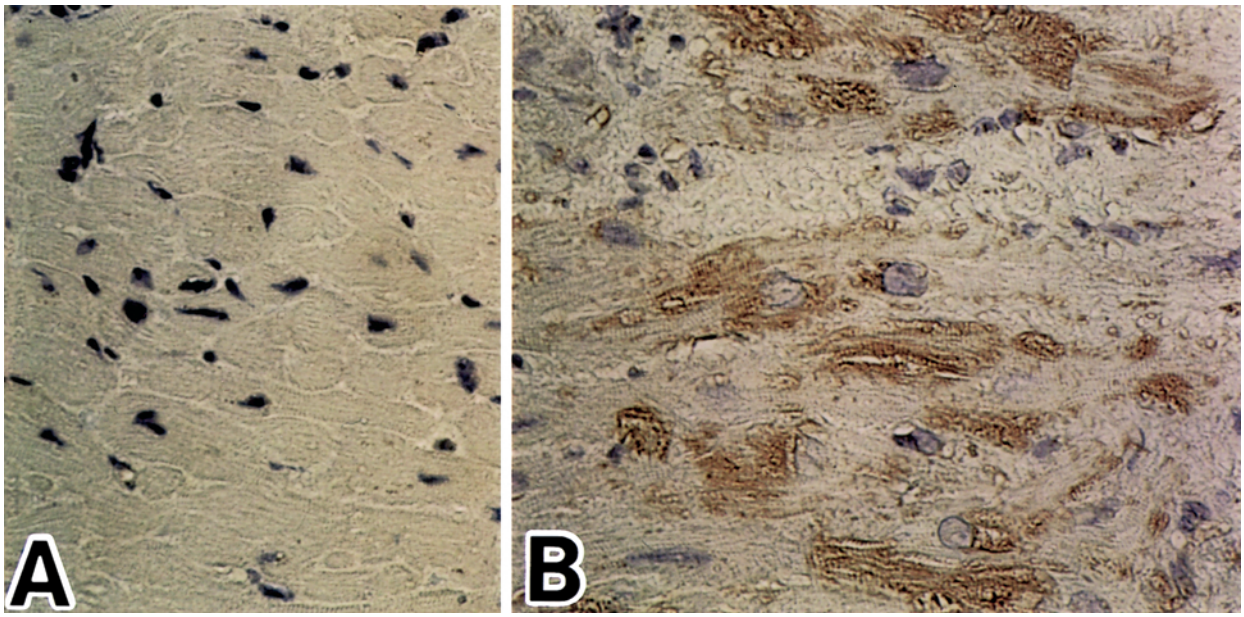

Figure 2 **Immunohistochemistry of digitalis-like immunoreactive substances. (A) No 3′3′-diaminobenzidine products are observed in the control group. (B) A representative positive case of non-obstructive hypertrophic cardiomyopathy. The positive reactivity is seen on the sarcolemma, cross-striae and intercalated discs of cardiocytes and capillary walls. Original magnification, × 100.**

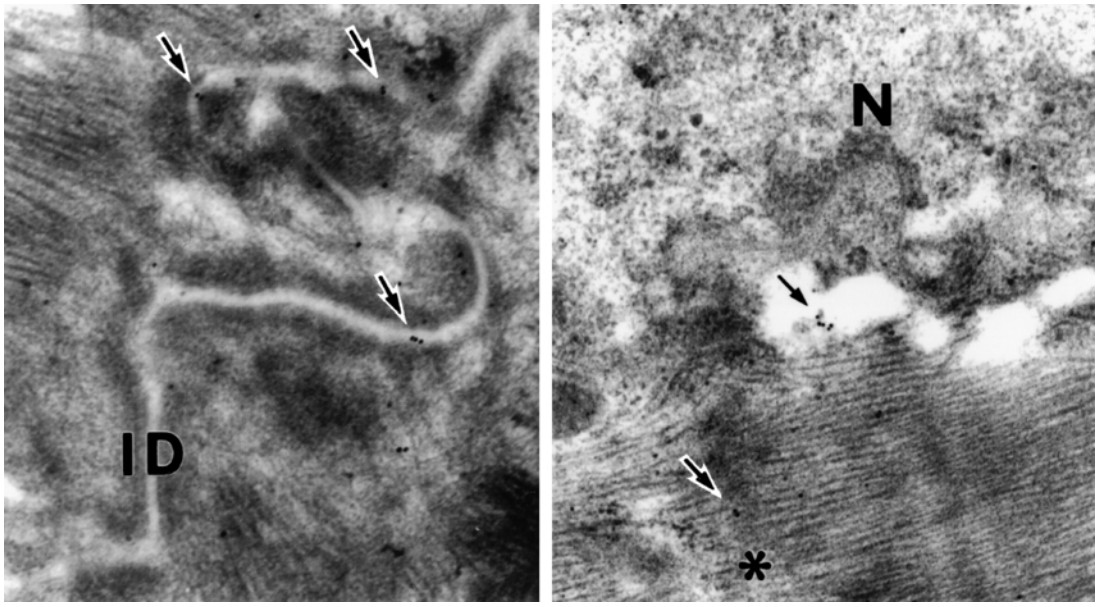

Figure 3 **Immunoelectron micrographs of digitalis-like immunoreactive substances. Gold particles (10 nm) (arrows) are seen on the intercalated disc (ID), and also in the cytoplasm adjacent to the nucleus (N) and Z band-like material (asterisk) (original magnification, × 30 000).**

simultaneously, had increased digitalis-like immunoreactive substances in the myocardium (average score more than 2·0); the number of patients with digitalis-like immunoreactive substances in plasma only was three (Table 2).

The correlation coefficients and *P* values of the plasma concentrations of digitalis-like immunoreactive substances, the graded scores, and all the other parameters for patients with non-obstructive and obstructive hypertrophic cardiomyopathy are shown in Tables 3 and 4, respectively. The coefficients are listed above the diagonal and the *P* values are below.

In non-obstructive hypertrophic cardiomyopathy, there was no correlation between the plasma concentration of digitalis-like immunoreactive substances and the graded score in biopsied specimens. However,

Table 2 Summary of the number of patients with hypertrophic cardiomyopathy whose plasma and myocardium were positive or negative for DLIS

	Plasma (−) Myocardium (−)	Plasma (−) Myocardium (+)	Plasma (+) Myocardium (−)	Plasma (+) Myocardium (+)
HNCM (n=20)	8	6	2	4
HOCM (n=11)	4	3	1	3
Total (n=31)	12	9	3	7

DLIS=digitalis-like immunoreactive substances; HNCM=non-obstructive type; HOCM=obstructive type; Plasma (+)=positive DLIS in plasma; Plasma (−)=negative DLIS in plasma; Myocardium (+)=positive DLIS in the myocardium; Myocardium (−)=negative DLIS in the myocardium.

plasma digitalis-like immunoreactive substances correlated with the left atrial dimension and were inversely correlated with the cardiac index (Table 3).

In obstructive hypertrophic cardiomyopathy, on the other hand, plasma digitalis-like immunoreactive substances correlated with the graded score, left atrial dimension and left ventricular systolic and end-diastolic pressures; the graded score also correlated with left ventricular end-diastolic pressures (Table 4).

A significant difference was also recognized as follows: the degree of myofibre disarrangement (Disarray) correlated with collagen proliferation (Fibrosis), Disarray was inversely correlated with fractional shortening in non-obstructive hypertrophic cardiomyopathy (Table 3) and correlated with left ventricular end-diastolic pressures. Fibrosis was inversely correlated with fractional shortening in obstructive hypertrophic cardiomyopathy (Table 4).

In the entire group of 40 patients with hypertrophic cardiomyopathy, the plasma concentration of digitalis-like immunoreactive substances correlated with Fibrosis and the left atrial dimension (P=0·023 and 0·001, respectively), and the graded score correlated with Fibrosis and left ventricular end-diastolic pressures (P=0·031 and 0·026, respectively).

Discussion

Identification of digitalis-like immunoreactive substances

A number of antibodies to digoxin or ouabain cross-react with steroid hormones. This is partly due to structural similarities in the epitope of the antigen. In several studies using the immunohistochemical technique, with an anti-digoxin monoclonal antibody, the digitalis-like immunoreactive substances were localized in the hypothalamus or the adrenal medulla[18–21]. We previously described a histochemical technique to localize the immunoreactive products of anti-digoxin monoclonal antibodies in patients who were chronically treated with digoxin[17].

Digitalis-like immunoreactive substances are known to inhibit the sodium pump of the mammalian cell membrane, and might be considered a new class of hormone participating in the volume regulation of cells and tissues of mammals[1]. In general, in situ fixation of the steroid hormone is difficult. Assuming that digitalis-like immunoreactive substances function like new steroid hormones, digitalis-like immunoreactive substances could be identified immunohistochemically, from a specific binding globulin responsible for transport[22,23]. Thus, immuno-positive findings against the anti-digoxin monoclonal antibody observed in this study are considered to be due to digitalis-like immunoreactive substances.

Digitalis-like immunoreactive substances in plasma

By fluorescence polarization immunoassay, 22·2% of non-obstructive and 38·4% of obstructive hypertrophic cardiomyopathy patients were positive for plasma digitalis-like immunoreactive substances. The difference in the incidence might be due to the greater elevation of left ventricular systolic and end-diastolic pressures in obstructive hypertrophic cardiomyopathy (Table 1). Statistical analysis revealed that plasma digitalis-like immunoreactive substances were correlated with the left atrial dimension both in non-obstructive and obstructive hypertrophic cardiomyopathy (Tables 3 and 4). Furthermore, in obstructive hypertrophic cardiomyopathy, plasma digitalis-like immunoreactive substances correlated with left ventricular systolic and end-diastolic pressures, suggesting that increased digitalis-like immunoreactive substances in plasma were a secondary phenomenon caused by pressure and/or volume overload.

The incidence of positive digitalis-like immunoreactive substances in plasma should be compared with other physiological and pathological conditions. Further investigation may be necessary.

The role of digitalis-like immunoreactive substances in hypertrophic cardiomyopathy

It is known that digitalis-like immunoreactive substances are Na^+-K^+-ATPase inhibitors and may

Eur Heart J, Vol. 21, issue 4, February 2000

Table 3 Statistical analysis in non-obstructive hypertrophic cardiomyopathy

	DLIS	LAD	IVSd	PWd	Dd	Ds	FS	Ao	LVsys	LVedp	C.I.	Diam	Disarray	Fib	Score
DLIS	#	0·456	−0·005	−0·299	0·157	0·285	−0·270	−0·233	−0·294	0·029	−0·543	−0·184	0·223	0·389	0·110
LAD	0·033	#	−0·087	−0·167	0·327	0·221	0·071	0·006	0·057	−0·025	−0·269	0·375	0·079	0·155	−0·004
IVSd	0·984	0·667	#	0·422	−0·339	−0·126	−0·280	−0·163	−0·138	−0·017	0·238	−0·027	0·250	0·189	−0·014
PWd	0·177	0·405	0·028	#	−0·371	−0·272	0·004	0·114	0·108	−0·127	0·198	0·082	−0·292	−0·169	0·056
Dd	0·486	0·096	0·084	0·057	#	0·673	0·011	−0·183	−0·237	−0·200	−0·001	0·310	0·278	0·158	−0·143
Ds	0·199	0·269	0·531	0·170	0·000	#	−0·653	−0·313	−0·327	−0·131	−0·333	0·000	0·405	0·067	0·002
FS	0·244	0·724	0·157	0·983	0·958	0·000	#	0·444	0·419	0·110	0·414	0·229	−0·412	0·043	0·093
Ao	0·310	0·975	0·428	0·580	0·372	0·119	0·023	#	0·875	0·145	0·325	0·202	−0·259	−0·106	0·305
LVsys	0·196	0·783	0·501	0·600	0·243	0·103	0·033	0·000	#	0·142	0·211	0·185	−0·292	−0·096	0·308
LVedp	0·899	0·902	0·934	0·528	0·317	0·516	0·585	0·481	0·489	#	0·055	−0·046	−0·194	−0·052	0·244
CI	0·013	0·193	0·251	0·343	0·997	0·103	0·039	0·113	0·311	0·792	#	0·306	0·011	0·199	−0·293
Diameter	0·412	0·054	0·894	0·684	0·116	0·998	0·251	0·323	0·366	0·822	0·137	#	0·123	0·168	−0·418
Disarray	0·330	0·700	0·219	0·148	0·169	0·040	0·036	0·201	0·147	0·343	0·957	0·550	#	0·461	−0·007
Fibrosis	0·074	0·440	0·344	0·398	0·431	0·742	0·831	0·605	0·642	0·799	0·571	0·403	0·018	#	0·294
Score	0·707	0·986	0·954	0·819	0·558	0·994	0·704	0·219	0·214	0·314	0·254	0·075	0·977	0·221	#

The correlation coefficients are listed above the diagonal and p values are below. DLIS=plasma concentration of digitalis-like substances; LAD=left atrial dimension; IVSd=diastolic ventricular septal thickness; PWd=diastolic posterior wall thickness; Dd=diastolic left ventricular dimension; Ds=systolic left ventricular dimension; FS=fractional shortening; Ao=aortic pressure; LVsys=left ventricular systolic pressure; LVedp=left ventricular end-diastolic pressure; CI=cardiac index; Diam=diameter; Fib=fibrosis. Note that Disarray, Fib, and Score were evaluated semiquantitatively (see text).

Table 4 Statistical analysis in obstructive hypertrophic cardiomyopathy

	DLIS	LAD	IVSd	PWd	Dd	Ds	FS	Ao	LVsys	LVedp	C.I.	Diam	Disarray	Fib	Score
DLIS	#	0·78	− 0·321	0·237	0·302	0·312	0·076	0·091	0·685	0·673	0·201	0·073	0·417	0·203	0·723
LAD	0·010	#	0·022	0·202	0·412	0·439	− 0·127	0·283	0·309	0·483	− 0·242	0·115	0·062	0·243	0·556
IVSd	0·366	0·943	#	− 0·029	− 0·129	0·137	− 0·357	− 0·122	− 0·022	0·169	− 0·661	0·261	0·529	0·537	0·174
PWd	0·510	0·509	0·926	#	− 0·004	0·285	− 0·195	0·414	0·367	0·057	0·318	0·176	0·091	0·385	0·016
Dd	0·397	0·162	0·675	0·989	#	0·366	0·032	− 0·177	0·125	− 0·386	− 0·064	0·213	− 0·509	− 0·314	0·146
Ds	0·380	0·133	0·656	0·345	0·219	#	− 0·765	0·415	0·486	0·000	− 0·180	0·243	0·161	0·456	0·410
FS	0·834	0·680	0·232	0·523	0·918	0·002	#	− 0·466	− 0·200	0·139	0·325	− 0·444	− 0·262	− 0·645	− 0·106
Ao	0·802	0·348	0·690	0·160	0·562	0·159	0·108	#	0·374	− 0·058	− 0·072	0·264	− 0·071	0·190	− 0·230
LVsys	0·029	0·305	0·942	0·217	0·685	0·096	0·512	0·208	#	0·220	0·336	0·433	0·392	0·152	0·235
LVedp	0·033	0·094	0·582	0·852	0·193	1·000	0·650	0·850	0·470	#	− 0·072	− 0·356	0·687	0·268	0·656
CI	0·578	0·426	0·014	0·290	0·836	0·557	0·278	0·816	0·262	0·815	#	− 0·005	− 0·150	− 0·367	− 0·175
Diameter	0·841	0·707	0·389	0·565	0·485	0·424	0·129	0·383	0·140	0·233	0·986	#	0·029	0·142	− 0·322
Disarray	0·230	0·841	0·063	0·767	0·076	0·599	0·387	0·819	0·185	0·010	0·625	0·924	#	0·485	0·570
Fibrosis	0·574	0·423	0·058	0·194	0·663	0·117	0·017	0·534	0·620	0·375	0·218	0·643	0·093	#	0·338
Score	0·043	0·075	0·608	0·962	0·669	0·211	0·756	0·496	0·487	0·028	0·607	0·334	0·067	0·310	#

The correlation coefficients are listed above the diagonal and p values are below. DLIS=plasma concentration of digitalis-like substances; LAD=left atrial dimension; IVSd=diastolic ventricular septal thickness; PWd=diastolic posterior wall thickness; Dd=diastolic left ventricular dimension; Ds=systolic left ventricular dimension; FS=fractional shortening; Ao=aortic pressure; LVsys=left ventricular systolic pressure; LVedp=left ventricular end-diastolic pressure; CI=cardiac index; Diam=diameter; Fib=fibrosis. Note that Disarray, Fib, and Score were evaluated semiquantitatively (see text).

participate in the volume regulation of cells and tissues in mammals[1]. Delva *et al*. found a strong and significant correlation between plasma concentrations of digoxin-like immunoreactivity and haemodynamic variables which characterize the pulmonary circulation in patients with various cardiovascular diseases[24]. In the study by Bagrov *et al*., plasma Na^+-K^+-ATPase inhibitory activity was inversely correlated with the cardiac index and cardiac output[5]. Our study revealed that plasma digitalis-like immunoreactive substances and the cardiac index were inversely correlated in non-obstructive hypertrophic cardiomyopathy, whereas it did not exist in obstructive hypertrophic cardiomyopathy. This difference might be due to the small number of patients with obstructive hypertrophic cardiomyopathy.

Under immunoelectron microscopy, digitalis-like immunoreactive substances were detected at the sarcolemma in the free wall, T-tubules and intercalated discs, indicating the localized site of Na^+-K^+-ATPase in situ. According to a previous study, in the hypertrophied heart induced by pressure overload, the number of Na^+-K^+-ATPase units was reduced, although the affinity of the enzyme was unchanged[25]. The localization and expression of cardiac Na^+-K^+-ATPase in hypertrophic cardiomyopathy should be examined in detail.

Digitalis-like immunoreactive substances were also found in the cytoplasm of cardiocytes, such as the Z-bands near the site of intercalated discs. Cardiac glycosides may be sequestered or internalized, together with their binding sites, as a part of membrane turnover[26]. Thus, the immunoreactivity observed in cardiocytes indicates that digitalis-like immunoreactive substances were transported into the cytoplasm.

Some patients with hypertrophic cardiomyopathy, who were negative for digitalis-like immunoreactive substances in the plasma, showed positive staining immunohistochemically in the myocardium (Table 2). No characterization was detected in available clinical data of these patients. Further investigations should be conducted to explain this unusual finding.

Additional subjects

The statistical analysis of the histochemical results showed that Fibrosis correlated with Disarray ($P=0{\cdot}018$) and Disarray was inversely correlated with the fractional shortening ($P=0{\cdot}036$) in non-obstructive hypertrophic cardiomyopathy, and that Fibrosis was inversely correlated with fractional shortening ($P=0{\cdot}017$) in obstructive hypertrophic cardiomyopathy (Tables 3 and 4). The proliferation and subtypes of collagen may be important factors to understand the pathophysiology of hypertrophic cardiomyopathy[11].

Limitations of the study

In many cases, the biopsied specimens were too small to obtain additional sections for immunohistochemical analysis. We could not perform immunostaining in all cases of hypertrophic cardiomyopathy examined in this study.

We previously reported the incidence of plasma digitalis-like immunoreactive substances in patients with hypertensive heart disease, and the immunohistochemical study of the myocardium in hypertensive heart disease is also necessary[27]. Currently, endomyocardial biopsy specimens from patients with hypertensive heart disease are not available, and animal models should be studied in future examinations.

In this study, distantly related tissue, such as skeletal muscle, was not examined. It might be important to study other tissue in patients who showed positive digitalis-like immunoreactive substances in plasma and/or in the myocardium.

Conclusions

Increased digitalis-like immunoreactive substances in plasma and cardiocytes, which are correlated with pressure and/or volume overload, were found in patients with hypertrophic cardiomyopathy. Digitalis-like immunoreactive substances may act on the sarcolemma of the cardiocytes and be transported into the cytoplasm.

We thank Dr Hisao Yamada, Department of Anatomy, Shiga University of Medical Science, and Dr Hakuo Takahashi, Department of Clinico-Pathology, Kansai Medical University, for their thoughtful suggestions. This study was supported in part by a Grant from the Vehicle Racing Commemorative Foundation.

References

[1] Hamlyn JM. Discovery of endogeneous ouabain; A new mammalian hormone. In: Bamberg E, Schoner W, eds. The Sodium Pump. New York: Springer, 1994: 722–31.
[2] Gruber KA, Whitaker JM, Buckalew VM Jr. Endogeneous digitalis-like substance in plasma of volume expanded dogs. Nature 1980; 287: 743–45.
[3] Schreiber V. Endogeneous digitalis-like immunoreactive factor(s) (endoxin, endocardin, edigen). Physiol Bohemoslov 1981; 30: 385–8.
[4] Graves SW, Brown B, Valdes R. An endogeneous digoxin-like substance in patients with renal impairment. Ann Intern Med 1983; 99: 604–8.
[5] Bagrov AY, Fedorova OV, Maslova MN, Roukoyatkina NI, Ukhanova MV, Zhabko EP. Endogeneous plasma Na, K-ATPase inhibitory activity and digoxin like immunoreactivity after acute myocardial infarction. Cardiovasc Res 1991; 25: 371–7.
[6] Pudek MR, Secombe DW, Whitefield MF, Ling E. Digoxin-like immunoreactivity in premature and full-term infants not receiving digoxin therapy. New Engl J Med 1983; 308: 904–5.
[7] Antolovic R, Sich B, Kirch U *et al*. Endogeneous 'Digitalis': proscillaridin A immunoreactivity, another compound of the group of endogeneous 'cardiac glycosides'? Therapeutic Research 1995; 17: 146–57.
[8] Maron BJ. Hypertrophic Cardiomyopathy. Curr Probl Cardiol 1993; 18: 639–704.
[9] Wynne J, Braunwald E. The cardiomyopathies and myocarditides. In: Braunwald E, eds. Heart Disease, 5th edn. Philadelphia: W.B. Saunders Co, 1997: 1404–63.

[10] Speiser B, Riess CF, Schaper J. The extracellular matrix in human myocardium: part I: Collagens I, III, IV, and VI. Cardioscience 1991; 2: 225–32.
[11] Hayashi T, Shimomura H, Terasaki F *et al*. Collagen subtypes and matrix metalloproteinase in idiopathic restrictive cardiomyopathy. Int J Cardiol 1998; 64: 109–16.
[12] Jolley ME. Fluorescence polarization immunoassay for the determination of therapeutic drug levels in human plasma. J Anal Toxicol 1981; 5: 236–40.
[13] Jolley ME, Stroupe SD, Schwenzer KS *et al*. Fluorescence polarization immunoassay. III. An automated system for therapeutic drug determination. Clin Chem 1981; 27: 1575–9.
[14] Noda S. Histopathology of endomyocardial biopsies from patients with idiopathic cardiomyopathy; quantitative evaluation based on multivariate statistical analysis. Jpn Circ J 1980; 44: 95–116.
[15] Hayashi T, James TN, Buckingham DC. Ultrastructure and immunohistochemistry of the coronary chemoreceptor in human and canine hearts. Am Heart J 1995; 129: 946–59.
[16] Hayashi T, Spry CJF, Kawamura K *et al*. Ultrastructural and immunohistochemical studies on myocardial biopsies from a patient with eosinophilic endomyocarditis. Cardiovasc Pathol 1996; 5: 105–12.
[17] Shimomura H, Hayashi T, Terasaki F, Hirota Y, Kitaura Y, Kawamura K. In situ localization of digoxin in hearts under digitalization: an ultrastructural and immunohistochemical study in humans and rats. Cell Vision 1996; 3: 364–9.
[18] Ihara N, Yuri K, Yamada H, Sano Y. Immunohistochemical studies on the distribution of endogeneous digitalis-like substance-containing neurons in the rat hypothalamus, with special consideration on the possibility of their coexistence with posterior lobe hormones. Arch Histol Cytol 1988; 51: 35–42.
[19] Yamada H, Ihara N, Takahashi H, Yoshimura M, Sano Y. Distribution of the endogeneous digitalis-like substance-containing neurons labelled by digoxin antibody in hypothalamus and three circumventricular organs of dog and macaque. Brain Res 1992; 584: 237–43.
[20] Takahashi H, Ihara N, Terano Y *et al*. Ouabain-like immunoreactive substances exist in the hypothalamus and the adrenal medulla in rats. Pathophysiology 1994; 1: 25–28.
[21] Yamada H, Naruse M, Naruse K *et al*. Histological study on ouabain immunoreactivities in the mammalian hypothalamus. Neurosci Lett 1992; 141: 143–6.
[22] Antolovic R, Linder D, Hahnen J, Schoner W. Affinity labelling of a sulfhydryl group in the cardiac glycoside receptor site of Na^+-K^+-ATPase by N-hydroxysuccinimidyl derivatives of digoxigenin. Eur J Biochem 1995; 227: 61–7.
[23] Antolovic R, Sich B, Kirch U *et al*. Endogeneous digitalis: proscillariden A immunoreactivity, another compound of the group of endogeneous cardiac glycosides? Therapeutic Research 1996; 17: 146–57.
[24] Delva P, Devynck M-A, Degan M *et al*. Correlation between plasma level of endogeneous digitalis-like substance and haemodynamic parameters measured during cardiac catheterisation. J Hypertens 1988; 6 (Suppl 4): S348–50.
[25] Nirasawa Y, Akera T. Pressure-induced cardiac hypertrophy: changes in Na^+, K^+-ATPase and glycoside actions in cats. Eur J Pharmocol 1987; 137: 77–83.
[26] Will PC, Longworth JW, Brake EM, Cook JS. Analysis of intracellular drug (ouabain) sequestration as a mechanism of detoxification. Mol Pharmocol 1977; 13: 161–71.
[27] Hayashi T, Shimomura H, Toko H, Okabe M, Terasaki F, Kawamura K. The role of digitalis-like factors in patients with hypertrophic cardiomyopathy (Abstr). Jpn Circ J 1997; 61: 592.