Expression and Localization of the Multidrug Resistance Protein 5 (MRP5/ABCC5), a Cellular Export Pump for Cyclic Nucleotides, in Human Heart

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The multidrug resistance protein 5 (MRP5/ABCC5) has been recently identified as cellular export pump for cyclic nucleotides with 3',5'-cyclic GMP (cGMP) as a high-affinity substrate. In view of the important role of cGMP for cardiovascular function, expression of this transport protein in human heart is of relevance. We analyzed the expression and localization of MRP5 in human heart [21 auricular (AS) and 15 left ventricular samples (LV) including 5 samples of dilated and ischemic cardiomyopathy]. Quantitative real-time polymerase chain reaction normalized to β -actin revealed expression of the *MRP5* gene in all samples (LV, 38.5 ± 12.9 ; AS, 12.7 ± 5.6 ; $P < 0.001$). An MRP5-specific polyclonal antibody detected a glycoprotein of ~190 kd in crude cell membrane fractions from these samples. Immunohistochemistry with the affinity-purified antibody revealed localization of MRP5 in cardiomyocytes as well as in cardiovascular endothelial and smooth muscle cells. Furthermore, we could detect MRP5 and ATP-dependent transport of [³H]cGMP in sarcolemma vesicles of human heart. Quantitative analysis of the immunoblots indicated an interindividual variability with a higher expression of MRP5 in the ischemic ($104 \pm 38\%$ of recombinant MRP5 standard) compared to normal ventricular samples ($53 \pm 36\%$, $P < 0.05$). In addition, we screened genomic DNA from our samples for 20 single-nucleotide polymorphisms in the *MRP5* gene. These results

indicate that MRP5 is localized in cardiac and cardiovascular myocytes as well as endothelial cells with increased expression in ischemic cardiomyopathy. Therefore, MRP5-mediated cellular export may represent a novel, disease-dependent pathway for cGMP removal from cardiac cells. (Am J Pathol 2003; 163:1567–1577)

The intracellular levels of the second messenger 3',5'-cyclic GMP (cGMP) are controlled by the rate of cGMP synthesis by guanylyl cyclases and by the rate of cGMP elimination. In addition to metabolic degradation by phosphodiesterases, active cGMP export as an elimination route has been observed in many cell types.^{1–4} The multidrug resistance protein 5 (MRP5/ABCC5) represents the first molecularly identified ATP-dependent export pump for cyclic nucleotides with cGMP as a high-affinity substrate (K_m value of 2 μ mol/L) and cAMP as a low-affinity substrate.⁵ Other members of the MRP/ABCC family, which represents a subgroup of the ATP-binding cassette (ABC) transporter superfamily, are export pumps for amphiphilic anions, especially for conjugates of lipophilic compounds with glutathione, glucuronate, or sulfate, as shown for MRP1/ABCC1⁶ and for the apically localized MRP2.^{7–9} MRP4/ABCC4, which is closely related to MRP5, has been recently shown to mediate like MRP5 the efflux of cyclic nucleotides, however, with a lower affinity for cGMP compared to MRP5.^{10–12}

MRP5 expression in human heart is important, because several features of cGMP as a second messenger of nitric oxide (NO) have emerged in heart, not only in the regulation of the vascular smooth muscle tone^{13–15} but also in the regulation of cardiac contractility.^{16,17} A NO/cGMP-mediated negative inotropic effect seems to play a role in inflammatory myocardial dysfunctions¹⁷ as well as in protection of the cardiomyocytes in ischemic preconditioning. For the latter function it was suggested that among other factors endothelial cells release NO, which

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diffuses to the cardiomyocytes to increase cGMP-mediated resistance to a subsequent ischemic stress.^{18,19} Furthermore, cGMP has been discussed to play a role as a negative regulator of cardiomyocyte hypertrophy.^{20,21} Data from single heart mRNA samples on commercial multiple tissue Northern blots or in RNase protection assays used in screening studies indicated the presence of MRP5 mRNA in heart.^{22–24} MRP5 mRNA expression has also been recently shown in porcine coronary artery.²⁵

In the present study we show the expression, localization, and function of MRP5 in human heart by means of real-time polymerase chain reaction (PCR), immunoblotting, immunohistochemistry, and transport assays in isolated sarcolemma vesicles. In addition, we investigated the interindividual variation of the MRP5 expression levels in auricular and ventricular samples, including ventricular samples from patients suffering from ischemic or from dilated nonischemic cardiomyopathy (DCM). To assess possible genetic factors in the variability of the expression we screened genomic DNA from our samples for single-nucleotide polymorphisms (SNPs) in the *MRP5* gene.

Materials and Methods

Human Tissue Samples

Auricular samples were taken from 21 patients (Caucasians, 19 males and 2 females, 47 to 79 years old) undergoing open heart surgery for aorto-coronary bypass with the approval from the local ethics committee. The 15 ventricular samples were taken from excised heart left ventricle during orthotopic heart transplantation as described before.²⁶ These included samples from nonfailing hearts (NFs). These hearts were obtained from potential donors, without evidence of heart disease on medical history. Echocardiography showed normal fractional shortening and no evidence of regional wall motion abnormalities or valve disease. Valves were taken and used for human homografts. Myocardium was used for experimental purposes. Patients died from intracerebral hemorrhage or head injury. Hearts from patients suffering from ICM and from patients with DCM ($n = 5$, each) were obtained from heart transplantations because of heart failure. In patients with dilated cardiomyopathy coronary arteries were found without significant atherosclerotic lesions on cardiac catheterization. Patients with ICM had a history of one or more myocardial infarctions and three vessel diseases in all cases. Coronary artery disease was confirmed by cardiac catheterization before heart transplantation. In all cases, previous coronary bypass operations were performed. Medical therapy of patients suffering from ICM and DCM consisted of digitalis, diuretics, nitrates, and angiotensin-converting enzyme inhibitors. Tissue samples were immediately snap-frozen in liquid nitrogen or fixed in 4% paraformaldehyde.

RNA Isolation and Analysis

Total RNA was isolated from 50 mg of frozen tissue homogenate using a RNeasy Mini extraction kit (Qiagen,

Hilden, Germany). For real-time PCR, 200 ng of total RNA was reverse-transcribed using random hexamers and the TaqMan reverse transcription reagents (Applied Biosystems, Weiterstadt, Germany). Real-time PCR were set up with 8 ng of reverse-transcribed RNA for MRP5 and β -actin assay and 82.5 ng for SMRP, which was described as a splicing variant of MRP5.²⁷ Intron-spanning primers for MRP5, which detected both MRP5 and SMRP, and primers specific for SMRP were as follows: MRP5F 5'-CACCATC-CACGCCTACAATAAA-3', MRP5R 5'-CACCGCATCGCA-CACGTA-3', and the probe MRP5-TM, 5'-6FAM-GCTTG-GTTGTCATCCAGCAGCTCCTG XTP (GenBank accession number: NM.005688); SMRP-F, 5'-AGGGCGTACACT-CACGTAGCA-3', SMRP-R 5'-ATGACCCTGGGCTTC-GATCT-3' and the probe SMRP-TM 5'-6FAM-CAGCCACT-GAGGCTTCTGAGAGGGACTTTA-XTP (GenBank accession number: AB005659). Amplification reactions of β -actin were performed using the predeveloped TaqMan assay reagents endogenous control kit. PCR products were amplified with the TaqMan universal PCR mastermix (50°C, 2 minutes; 95°C, 10 minutes; followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute) and analyzed on a real-time PCR cycler (SDS 7700, Applied Biosystems). For quantification, fluorescence intensities were plotted against PCR cycle numbers. The amplification cycle displaying the first significant increase of the fluorescence signal was defined as threshold cycle (C_T). The C_T value of each sample was compared to the C_T values of the standardization series, which consisted of the cloned *MRP5* PCR-fragment in pGem-Teasy (Promega, Mannheim, Germany) resulting in a quantification of copy numbers mRNA. MRP5 and SMRP expression levels were normalized with respect to the stable expressed housekeeping gene β -actin. The average C_T for β -actin in samples from DCM was 26.56 (SD, 0.87), for ICM 26.50 (SD, 0.73), and for NF samples 25.79 (SD, 0.83), indicating no significant difference between healthy and diseased human heart. Data are expressed as ratio of MRP mRNA/ β -actin mRNA $\times 10^3$.

MRP5 Genotype

Genomic DNA from all samples were screened for SNPs by direct sequencing. PCR primers were designed based on the sequence of the *MRP5* gene from the GenBank (AC068644) to yield fragments covering the SNPs at the positions listed in Table 1. The amplification products were directly sequenced according to the manufacturer's instructions using the BigDye Terminator ready reaction mix (Applied Biosystems), purified with the QIAquick kit system on a Qiagen BioRobot 9600, and loaded onto an ABI3700 capillary sequencer (Applied Biosystems). PolyPhred (version 2.1), a software package that utilizes the output from Phred, Phrap, and Consed was used to identify single nucleotide substitutions.^{28,29}

Antibodies

The AMF antibody was generated against the deduced C-terminal sequence AMFAAAENKVAVKG specific for

Table 1. Genotype and Allele Frequencies of MRP5 Single Nucleotide Polymorphism and Genotype-Related mRNA Expression Levels

Position from ATG	Location	Genotype	Frequency (%)	Allele	Frequency (%)	mRNA $\pm$ SEM
-1995	Prom5	GG	44.4	G	69.4	3.1 $\pm$ 0.5
		GA	50.0	A	30.6	3.4 $\pm$ 0.5
		AA	5.6			5.4
-1826	Prom5	TT	45.0	T	70.0	3.0 $\pm$ 0.4
		TC	50.0	C	30.0	3.4 $\pm$ 0.5
		CC	5.0			5.4
-1684	Prom4	TT	80.0	T	90.0	3.5 $\pm$ 0.4
		TA	20.0	A	10.0	2.9 $\pm$ 0.6
		AA	0.0			—
-1210	Prom3	CC	23.8	C	54.8	3.9 $\pm$ 0.5
		CT	61.9	T	45.2	2.9 $\pm$ 0.4
		TT	14.3			4.3 $\pm$ 0.7
-798	Prom3	CC	81.0	C	90.5	3.4 $\pm$ 0.4
		CA	19.0	A	9.5	2.9 $\pm$ 0.6
		AA	0.0			—
36156	Exon8	AA	33.3	A	64.3	3.1 $\pm$ 0.5
		AG	61.9	G	35.7	3.2 $\pm$ 0.4
		GG	4.8			5.4
36251	Intron8	GG	85.7	G	92.9	3.4 $\pm$ 0.3
		GA	14.3	A	7.1	3.0 $\pm$ 0.8
		AA	0.0			—
39270	Exon9	TT	21.1	T	55.3	3.7 $\pm$ 0.7
		TC	68.4	C	44.8	3.0 $\pm$ 0.4
		CC	10.5			4.2 $\pm$ 1.3
39557	Intron9	AA	84.2	A	92.1	3.4 $\pm$ 0.4
		AG	15.8	G	7.9	2.4 $\pm$ 0.4
		GG	0.0			—
39563	Intron9	AA	85.0	A	92.5	3.3 $\pm$ 0.3
		AC	15.0	C	7.5	3.2 $\pm$ 1.1
		CC	0.0			—
39569	Intron9	wt/wt	30.0	wt	60.0	3.2 $\pm$ 0.5
		wt/del	60.0	del	40.0	3.1 $\pm$ 0.4
		del/del	10.0			4.2 $\pm$ 1.3
39595	Intron9	GG	85.0	G	92.5	3.4 $\pm$ 0.4
		GT	15.0	T	7.5	2.4 $\pm$ 0.4
		TT	0.0			—
50138	Exon12	TT	23.5	T	55.9	3.8 $\pm$ 0.6
		TC	64.7	C	44.1	3.1 $\pm$ 0.5
		CC	11.8			4.9 $\pm$ 0.5
50423	Intron12	AA	20.0	A	53.3	4.1 $\pm$ 0.7
		AG	66.7	G	46.7	3.2 $\pm$ 0.5
		GG	13.3			4.9 $\pm$ 0.5
66276	Intron19	GG	38.1	G	66.7	3.0 $\pm$ 0.4
		GA	57.1	A	33.3	3.4 $\pm$ 0.4
		AA	4.8			5.4
74866	Intron24	GG	38.1	G	66.7	3.0 $\pm$ 0.4
		GC	57.1	C	33.3	3.4 $\pm$ 0.4
		CC	4.8			5.4
75087	Exon25	CC	85.7	C	92.9	3.3 $\pm$ 0.4
		CT	14.3	T	7.1	3.3 $\pm$ 0.6
		TT	0.0			—
97688	3'UTR	CC	95.2	C	97.6	3.4 $\pm$ 0.3
		CG	4.8	G	2.4	1.6
		GG	0.0			—
97827	3'UTR	AA	33.3	A	59.5	3.1 $\pm$ 0.5
		AG	52.4	G	40.5	3.2 $\pm$ 0.5
		GG	14.3			4.3 $\pm$ 0.7
97950	3'flanking	AA	85.7	A	92.9	3.5 $\pm$ 0.3
		AC	14.3	C	7.1	2.1 $\pm$ 0.3
		CC	0.0			—

The positions of SNPs given correspond to positions in the genomic sequence of *MRP5* (AC068644) with the first base of the ATG first codon set to 1 (NM_005688). mRNA is given as ratio of MRP5 mRNA/ β -actin relative to an internal standard. Expression levels were statistically not different (Kruskal-Wallis test, Mann-Whitney *U*-test).

human MRP5 at the Deutsches Krebsforschungszentrum, Heidelberg, as described.^{5,30} Affinity purification of the AMF antibody and the preimmune serum was performed as described previously using MRP5-overexpressing V79 cells.^{30,31} Additionally, the following monoclonal antibodies were used: smooth muscle actin (clone 1A4, DAKO, Hamburg, Germany), anti-CD34 (Novocast, Loxo GmbH, Dossenheim, Germany), and anti-desmin (clone D33, DAKO).

Preparation of Crude Membrane Fractions

Heart samples (0.1 to 0.3 g) were homogenized during thawing in homogenization buffer (5 mmol/L Tris/HCl, 250 mmol/L sucrose, and 0.1 mmol/L ethylenediaminetetraacetic acid) supplemented with protease inhibitors (0.1 mmol/L phenylmethyl sulfonyl fluoride, 0.3 μ mol/L aprotinin, and 1 μ mol/L pepstatin) using a Potter-Elvehjem homogenizer. The suspension was then centrifuged at $9000 \times g$ for 20 minutes at 4°C. The resulting postnuclear supernatant was withdrawn and centrifuged at $100,000 \times g$ for 30 minutes at 4°C. The pellets containing the crude membrane fractions (microsomes) were resuspended in Tris buffer (50 mmol/L, pH 7.4).

Preparation of Sarcolemma Vesicles from Human Heart Tissue

Sarcolemma vesicles were prepared from auricular tissue samples according to the procedure described by Khananshvilii and colleagues³² with some modifications. In brief, the tissue was homogenized in incubation buffer (250 mmol/L sucrose and 10 mmol/L Tris/HCl, pH 7.4) supplemented with protease inhibitors (0.1 mmol/L phenylmethyl sulfonyl fluoride, 0.3 μ mol/L aprotinin, and 1 μ mol/L pepstatin) using a Potter-Elvehjem homogenizer (20 strokes, 1000 rpm). The homogenate was centrifuged at $9000 \times g$ for 15 minutes. The supernatant was saved, and pellets were homogenized once again. Combined supernatants were centrifuged at $100,000 \times g$ for 30 minutes, and the pellets were resuspended in incubation buffer and homogenized by 30 strokes with a tight-fitting Dounce B homogenizer. The membrane suspension was layered on 38% sucrose in 5 mmol/L of HEPES-KOH (pH 7.4) and centrifuged at $290,000 \times g$ for 90 minutes in a swing-out rotor. The turbid layer at the interface (sarcolemma fraction) was harvested, diluted with incubation buffer, homogenized again by 30 strokes with the tight-fitting Dounce B homogenizer, and washed by centrifugation at $100,000 \times g$. The resulting pellet was resuspended in incubation buffer and the membrane suspension was passed 20 times through a 27-gauge needle for vesicle formation. Membrane vesicles were frozen and stored in liquid nitrogen.

Deglycosylation by Peptide N-Glycosidase F

To prove glycosylation, membrane proteins (40 μ g) were denatured and treated with peptide N-glycosidase F (PN-

GaseF) using a N-Glycosidase F deglycosylation kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. In control samples the enzyme suspension was replaced by incubation buffer. Treated samples were lyophilized and subjected to immunoblot analysis.

Immunoblot Analysis

Crude membrane fractions or purified membrane vesicles were loaded onto a 7.5% sodium dodecyl sulfate-polyacrylamide gel after incubation in sample buffer at 37°C for 30 minutes. Purified membranes from MRP5-transfected V79 cells (V79/MRP5)⁵ were used as positive control for MRP5 expression. Immunoblotting was performed using a tank blotting system (Bio-Rad) and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Primary antibodies were diluted in Tris-buffered saline containing 0.05% Tween 20 and 1% bovine serum albumin to the following final concentrations: AMF serum, 1:1000; affinity-purified AMF, 1:250; and anti-desmin, 1:500. Secondary horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antibodies (Bio-Rad, München, Germany) were used at a 1:2000 dilution. The results of the immunoblotting were quantified by densitometric analysis using the Kodak ID scientific imaging systems software. The optical density (OD) of the 190-kD band in the samples was expressed as percentage of the OD of a defined amount of recombinant MRP5 prepared from the V79/MRP5 cells and related to the detection of desmin in the same blot to correct for possible variations in the proportions of connective tissue in the samples.

Vesicle Transport Studies

The ATP-dependent transport of [8-³H]cGMP (0.3 TBq/mmol; Hartmann Analytic, Braunschweig, Germany) into inside-out membrane vesicles was measured by rapid filtration through nitrocellulose filters essentially as described.⁵ Sarcolemma vesicles (140 μ g protein) were incubated in the presence of 4 mmol/L ATP, 10 mmol/L MgCl₂, 10 mmol/L creatine phosphate, 100 μ g/ml creatine kinase, and 4 μ mol/L [³H]cGMP, in incubation buffer containing 250 mmol/L sucrose and 10 mmol/L Tris/HCl, pH 7.4. The final incubation volume was 75 μ l. Aliquots (20 μ l) of the incubations were taken at the times indicated, diluted in 1 ml of ice-cold incubation buffer and filtered immediately through nitrocellulose filters (0.2- μ m pore size, presoaked in incubation buffer). Filters were rinsed with 5 ml of incubation buffer, dissolved in liquid scintillation fluid, and counted for radioactivity. In control experiments, ATP was replaced by an equal concentration of 5'-AMP. Rates of net ATP-dependent transport were calculated by subtracting values obtained in the presence of 5'-AMP as a blank from those in the presence of ATP and are given in pmol [³H]cGMP $\times$ mg protein⁻¹ (1 pmol $\times$ mg protein⁻¹ = 667 dpm). For studying the effect of an increased osmolarity of the extravesicular medium, the vesicles were preincubated

for 1 hour at 4°C in buffer containing 1 mol/L of sucrose or in standard incubation buffer containing 250 mmol/L of sucrose.

Immunohistochemistry

Paraffin-embedded tissue blocks were sectioned at a thickness of 2 μ m and mounted onto slides. The sections were deparaffinized and stained immunohistochemically with the AMF antiserum (dilution 1:100) or the affinity-purified AMF (dilution, 1:10). As a negative control for the specificity of the AMF antibody, preimmune serum affinity-purified in the same way was used at a dilution of 1:10. The monoclonal antibodies against smooth muscle actin, anti-CD34 and anti-desmin were used at dilutions of 1:25, 1:25, and 1:40, respectively. The staining was developed using the labeled streptavidin-biotin detection method (DAKO). For quantitative analysis of vascular MRP5 expression immunostained vessels were assessed in eight high-magnification fields (0.2 mm² per field). Positive vessels were counted and given a score of 1 to 3 for staining intensity. The number of immunopositive vessels was multiplied with the staining score reaching the final score for each case. Staining of the cardiomyocytes was evaluated accordingly.

Statistical Analysis

The amounts of mRNA or protein were compared using the Mann-Whitney *U*-test; *P* < 0.05 was considered as significant. Expression in dependence of *MRP5* genotype was compared by Mann-Whitney *U*-test and Kruskal-Wallis test. Data are given as mean $\pm$ SD or SEM as indicated.

Results

MRP5 Expression in Auricular and Ventricular Tissue Samples from Human Heart

MRP5 mRNA levels were assessed by quantitative real-time PCR in 21 auricular and 15 left ventricular human heart samples. MRP5 mRNA was detectable in all samples with the auricular samples showing less MRP5 mRNA than the ventricular samples (Figure 1, inset). The interindividual variability of the mRNA contents of the 21 auricular samples is also shown in Figure 1. In addition, we analyzed whether an SMRP transcript, proposed to represent a short splicing variant of MRP5,^{27,33} was detectable in the human heart samples. SMRP mRNA could be detected by real-time PCR, however, only if the 10-fold amount of RNA was used in the assay (13.1 ± 15.1 in the auricular and 17.3 ± 21.4 in the ventricular samples).

Furthermore, immunoblots on crude membrane preparations from these samples were probed with the MRP5-specific antiserum AMF characterized before.^{5,30} The 190-kD MRP5 glycoprotein was detected in all samples, as shown in Figure 2 for ventricular crude membranes and in membranes from MRP5-transfected cells⁵ used as

Figure 1. Histogram (frequency distribution) of MRP5 mRNA levels in auricular samples of human hearts. MRP5 mRNA levels were determined by real-time PCR and calculated as relative amounts of ratio MRP5/ β -actin. **Inset:** Comparison of the MRP5 mRNA levels in the auricular (aur, *n* = 21) and ventricular (ventr, *n* = 5) heart samples. Mean values $\pm$ SD. *, Significant difference at *P* < 0.001.

a positive control and internal standard for protein quantification. The blots were also probed with a monoclonal antibody against desmin (Figure 2, bottom) as a marker protein for cardiomyocytes and vascular smooth muscle cells, which was used for normalization of the densitometrical quantification to correct for possible variations in the proportions of connective tissue in the samples. In accordance with the mRNA data, the auricular samples contained less MRP5 ($44 \pm 22\%$ recombinant MRP5 standard, *n* = 21) than the normal ventricular samples ($53 \pm 36\%$, *n* = 5). The observed protein levels in the auricular samples correlated to the mRNA levels determined by real-time PCR (*r* = 0.45, *P* < 0.04 for data normalized to β -actin). Because the AMF antiserum de-

Figure 2. Immunoblot analysis of MRP5 in crude membrane fractions prepared from human ventricular heart samples. MRP5 was detected with the anti-MRP5 antiserum AMF (**top left**) and the affinity-purified AMF antibody (AMF pur, **top right**) as a 190-kD protein in membrane fractions (25 μ g of total protein) from ventricular tissue from normal nonfailing hearts (NF) as well as from tissue from patients with dilated (DCM) and ischemic (ICM) cardiomyopathy. Membrane vesicles from MRP5-transfected cells (V79/MRP5, 10 μ g of protein) served as a positive control. **Bottom:** Detection of the muscle cell marker protein desmin on the same blot.

Figure 3. Immunohistochemical detection of MRP5 in human heart samples. **A–C:** Detection of MRP5 with the affinity-purified AMF antibody (**brown staining**). **Blue staining:** Counterstaining with hemalaun (nuclear staining). MRP5 staining was observed in endothelial and smooth muscle cells of arterioles (**A**), in auricular (**A, B**) as well as ventricular (**C**) cardiomyocytes, and in endocard (**B**). **D:** Negative control (purified preimmune serum). **E:** Smooth muscle cells and cardiomyocytes stained with anti-desmin antibody. **F:** Arterial endothelium stained with anti-CD34 antibody. e, endothelium; s, smooth muscle cells; c, cardiomyocytes; n, endocard. Scale bars: 25 μ m (**A, F**); 50 μ m (**B–E**).

tected additional bands in the cardiac samples, the polyclonal antibody was affinity purified as described before.³⁰ As shown in the upper right panel of Figure 2 the affinity-purified antibody showed a high specificity for the 190-kD MRP5 and thus provided a reliable tool for immunohistochemistry studies.

Immunolocalization of MRP5

The localization of MRP5 in the heart was visualized by immunohistochemistry on deparaffinized sections of the auricular and ventricular tissue samples (Figure 3). Incubation with the affinity-purified AMF antibody resulted in

Figure 4. Relative MRP5 levels in ischemic (ICM) and dilated (DCM) cardiomyopathy compared to nonfailing hearts (NF). MRP5 mRNA levels (A) as well as protein levels detected by immunoblotting (B) or by immunohistochemistry (C, D) were quantified as described in Materials and Methods. In the immunohistochemistry MRP5 expression in the blood vessels (C) and in the cardiomyocytes (D) were evaluated separately. All data are given in percentage of mean NF $\pm$ SD ($n = 5$ for each group; *, significant difference at $P < 0.05$). OD, optical density.

staining of the cardiovascular endothelial and smooth muscle cells but not of the connective tissue as shown for an auricular arteriole in Figure 3A (brown staining). Staining was also observed in the auricular and ventricular cardiomyocytes (Figure 3, B and C) and in the endocard (Figure 3B) and capillary endothelial cells (Figure 3C). In control experiments with the purified preimmune serum no staining was observed (Figure 3D). For cell type identification, desmin was stained as cellular marker for the cardiomyocytes and smooth muscle cells and CD-34 as marker for the vascular endothelium (Figure 3, E and F).

Figure 5. Transport of cGMP into sarcolemma vesicles from human heart. Membrane vesicles (140 μ g of protein) were incubated with [3 H]cGMP (4 μ mol/L) in the presence of 4 mmol/L ATP (■) or 4 mmol/L 5'-AMP (◆) (left) and the vesicle-associated radioactivity was determined as described in Materials and Methods (mean values $\pm$ SD, $n = 3$). The rate of net ATP-dependent transport (right, ▲) was calculated by subtracting transport in the presence of 5'-AMP as a blank from transport in the presence of ATP. **Right inset:** Immunodetection of MRP5 in the sarcolemma vesicles (40 μ g of total protein) and V79/MRP5 membrane vesicles (20 μ g of protein) (left lanes) and in sarcolemma membranes after incubation with (+) or without (–) PNgaseF (right lanes). The AMF anti-MRP5 serum was used for staining.

Figure 6. Possible involvement of MRP5 in the regulation of the function of cardiovascular smooth muscle cells and cardiomyocytes via the NO/cGMP-signaling pathway. NO is formed by nitric oxide synthases in cardiovascular endothelial cells, eg, in response to an ischemic stimulus, and diffuses to cardiovascular smooth muscle cells as well as to the cardiac myocytes. In these cells it enhances the cellular levels of cGMP by stimulation of soluble guanylyl cyclases (sGC). Intracellular targets of the cGMP signal transduction pathway include cGMP-dependent protein kinases (PKGs), cGMP-gated ion channels, as well as cGMP-regulated cAMP phosphodiesterases (PDEs). The signal is terminated either by metabolic degradation of cGMP by PDEs or by ATP-dependent export of cGMP from the cell mediated by MRP5.

MRP5 Expression in ICM and DCM

The ventricular heart samples included samples from patients suffering from ICM or DCM ($n = 5$ each). As shown in Figure 4B, densitometrical quantification of the immunoblots indicated a significant higher MRP5 level in the ICM samples ($104 \pm 38\%$ of recombinant MRP5 standard) compared to the DCM ($76 \pm 20\%$ of recombinant standard) and normal (NF) samples ($53 \pm 36\%$ of recombinant standard, $P < 0.05$). In accordance, mRNA levels (Figure 4A) and scores in the quantitative immunohistochemistry evaluation (Figure 4, C and D) were highest in the ICM samples. Thereby, an increase in MRP5 staining was observed in the blood vessels (Figure 4C) as well as in the cardiomyocytes (Figure 4D). However, there was a considerable interindividual variability, indicated by the standard variations. In addition, a significant increase ($P < 0.025$) of the amount of SMRP transcript was observed in samples of patients with ICM (ICM, 42.3 ± 26.8 ; NF, 5.5 ± 4.8 ; DCM, 7.6 ± 7.9).

MRP5 Expression and cGMP Transport in Human Heart Sarcolemma Vesicles

To study MRP5 function in cardiomyocytes, isolated sarcolemma vesicles were prepared from the auricular tissue samples. ATP-dependent transport of [3 H]cGMP, which proceeded into the fraction of inside-out oriented vesicles, was studied during a 20-minute period (Figure 5). ATP-dependent transport (Figure 5, right) was calculated by subtracting the vesicle-associated radioactivity in the presence of 5'-AMP from the values obtained in the presence of ATP. ATP-dependent [3 H]cGMP accumulation at the cGMP concentration of 4 μ mol/L, was 9.1 ± 1.1 pmol $\times$ mg protein $^{-1}$ at 20 minutes (mean value $\pm$ SD, $n = 3$).

To determine whether the ATP-dependent uptake of [^3H]cGMP by the vesicles reflects transmembrane movement rather than binding to the membrane surface, the influence of the medium osmolarity was studied. An increase in osmolarity of the extravesicular medium would be expected to decrease the intravesicular volume without inhibiting nonspecific binding to the vesicle surface.³⁴ At a concentration of 1 mol/L of sucrose (outside the vesicles) the rate of ATP-dependent transport was only $13.4 \pm 1.7\%$ (mean $\pm$ SD, $n = 3$) of the value obtained under standard conditions with 250 mmol/L of sucrose. The vesicle-associated radioactivity in the presence of the control nucleotide 5'-AMP may consist of binding of [^3H]cGMP to the vesicle surface and filter membrane as well as of an ATP-independent transport into the inside-out or right-side-out vesicles. This radioactivity was only slightly affected by the shrinking of the vesicles ($66 \pm 16\%$ of the control values), indicating that it represents mainly binding.

As shown in the immunoblot (Figure 5, right inset), MRP5 was enriched in these vesicles. The broad diffuse band, characteristic for glycosylated proteins, was shifted to a lower apparent molecular mass (~ 160 kD) after treatment with PNGaseF indicating the existence of asparagine-linked glycan chains (there are eight potential glycosylation sites in MRP5).

Polymorphisms in the MRP5 Gene

To assess if the variability in the MRP5 expression is because of genetic variations we screened the genomic DNA from our samples for SNPs. We identified 20 SNPs in these samples, 4 in exons, 8 in introns, 2 in the 3'-untranslated region (3'UTR), 1 in the 3'-flanking region, and 5 in the promoter region. Detailed information for nucleotide positions and substitutions as well as allele frequencies are given in Table 1. Recently, 76 SNPs in the *MRP5* gene and 8 in its 3'-flanking region have been identified in a Japanese population.³⁵ Most of the SNPs in introns, exons, and the 3'-flanking regions found in our samples were also identified in this study. The SNPs in exon 12 (position 50128), in the 3'-UTR (position 97827), and promoter region (position -1210) are identical with SNPs in the NCBI SNP database with accession numbers rs939336, rs562, and rs1520195, respectively. The three SNPs in the promoter region position -1995, position -1826, and position -1684 have not been published so far. The polymorphisms found in the coding regions are all silent mutations, ie, they would not cause substitution of an amino acid. Correlating the genotype with the mRNA expression data (Table 1, last column), a statistically significant effect of the genotype on the MRP5 expression could not be detected for either of these SNPs.

Discussion

In this study we describe the expression and localization of the ATP-dependent organic anion export pump MRP5 (ABCC5) in human heart on the mRNA as well as on the protein level. Immunohistochemistry revealed localization

of MRP5 in three different cell types in the heart: in vascular smooth muscle cells, in cardiomyocytes, and in vascular endothelial cells (Figure 3). Furthermore, we could detect MRP5 and ATP-dependent transport of cGMP, a high-affinity substrate of MRP5, in human sarcolemma vesicles (Figure 5). The physiological and pathophysiological functions of MRP5 remain still to be defined, however, the finding that it transports cyclic nucleotides, especially cGMP,⁵ suggests that it can affect the signal transduction role of cGMP by reducing its intracellular content in addition to the degradation by phosphodiesterases (Figure 6). In a recent study by Wielinga and colleagues³⁶ the influence of MRP5 on intracellular cGMP levels has been investigated in MRP5-transfected HEK293 cells. The authors detected enhanced cGMP efflux from the MRP5-transfected cells, but only an approximately twofold decrease in the cellular cGMP levels what may be because of the experimental setting. In addition to a role in the regulation of the intracellular cGMP levels, the MRP5-mediated export may have a paracrine-signaling function, since biological effects of extracellular cAMP and cGMP have been reported.^{37,38}

Assuming a role in cGMP elimination, there is a clear rationale for MRP5 expression in the vascular smooth muscle cells, where cGMP plays a key role in muscle relaxation.¹³⁻¹⁵ The detection of MRP5 in cardiovascular smooth muscle cells is in line with the localization of MRP5 in the genitourinary tract where MRP5 was detected in smooth muscle cells of corpus cavernosum, ureter, urethra, and bladder, as well as of blood vessels,³⁰ and in line with a recent study by Mitani and colleagues²⁵ showing MRP4 and MRP5 mRNA in porcine coronary and pulmonary artery. Interestingly, MRP5 staining was also observed in the cardiomyocytes (Figure 3). The specificity of the affinity-purified antibody used for this detection was confirmed by several control experiments before³⁰ and in this study by use of the purified preimmune serum (Figure 3). MRP5 protein and function was also detected in isolated human sarcolemma vesicles (Figure 5). ATP-dependent transport of cGMP in cardiomyocyte sarcolemma vesicles is a novel finding that further supports the concept of MRP5 as relevant component in the regulation of cardiac cGMP levels. A critical role for cGMP in cardiomyocytes is currently discussed for the phenomenon of preconditioning to ischemic tolerance in which brief episodes of a subtoxic insult induce a robust protection against the deleterious effects of subsequent prolonged ischemia.^{39,40} Among other factors NO, formed by endothelial cells, seems to play a prominent role in initiating and mediating the protective response of the cardiomyocytes (eg, reducing contractility and oxygen consumption).^{18,19,41} Thereby, cGMP as second messenger of NO may act in the cardiomyocytes by reducing the influx of cellular Ca^{2+} through L-type Ca^{2+} channels⁴² and by stimulating a cGMP-sensitive phosphodiesterase, such as PDE2, resulting in reduction of cAMP levels.^{18,41,43} cGMP might also have a particular role in dilated cardiomyopathy since it has been shown that NO attenuates hypertrophy in cardiomyocytes through cGMP formation.^{20,21} In view of cGMP formation

in cardiac muscle cells, it is interesting that the cGMP-specific phosphodiesterase PDE5 is expressed unequivocally in cardiovascular smooth muscle cells but expression in the cardiomyocytes is controversial. The major PDE activity in the human cardiac ventricle was shown to be calcium/calmodulin-dependent PDE1- and cGMP-regulated PDE2, both hydrolyzing cGMP as well as cAMP.⁴⁴ PDE5 was long assumed to be absent in the myocardium.⁴⁴ More recent studies have provided evidence for *PDE5* gene expression in the human heart,^{45,46} however, the PDE5 protein could not be detected in cardiomyocytes in an immunohistochemistry study with a PDE5-selective antibody.⁴⁷ In the absence of PDE5, the cGMP elimination route via export may have even more impact in these cells for signal termination under conditions of enhanced cGMP formation. Another interesting aspect, in which the MRP5/cGMP elimination pathway may be involved, is the control of cardiac glucose utilization. The intracellular glucose concentration in cardiomyocytes was recently shown to be regulated by cGMP.⁴⁸ The inhibitory effects of some cGMP analogs on glucose transport in myocytes could be because of the inhibition of the redistribution of glucose transporters from intracellular stores to the cell surface.⁴⁸

The expression of MRP5 in heart is considerably high, judged from the mRNA and immunoblot signals (Figures 1 and 2). Our quantitative analysis of MRP5 in the ventricular heart samples from ICM patients suggested an up-regulation of MRP5 under ischemic conditions (Figure 4). Despite the considerable interindividual variation and the relative low number of individuals the higher MRP5 expression was consistent in immunoblot, immunohistochemistry, and mRNA analysis and cannot be attributed to drug therapy, because there were no differences in drug therapy between DCM and ICM patients. This up-regulation could be related to the ischemic preconditioning with enhanced tissue cGMP levels demanding enhanced cGMP elimination. A direct role of cyclic nucleotides in the regulation of MRP4 and MRP5 expression has been hypothesized by Sampath and colleagues.⁴⁹ NO and cGMP have been shown to regulate expression of several genes through cGMP-dependent protein kinase (PKG)-mediated activation of transcription factors as AFT-1 and nuclear factor- κ B.^{50,51} Alternatively, MRP5 could be up-regulated together with other membrane transporters through a more general protective response to ischemia. Aside from regulation, expression of ABC transporters can be influenced by genetic factors. In an ongoing study including patients with thiopurine (eg, azathioprine, 6-mercaptopurine)-related toxicity and thiopurine methyltransferase wild-type a screening for *MRP5* polymorphisms was performed. Preliminary results indicate a possible association with 20 of a total of 95 identified SNPs within the *MRP5* gene that were therefore screened in the present study. A recent study from Hoffmeyer and colleagues⁵² reports a polymorphism in exon 26 of the *MDR1* gene, which affects expression of P-glycoprotein thereby modifying absorption of digoxin. In contrast, none of the 20 SNPs found in the *MRP5* gene and promoter region in this study did alter the expression (Table 1). It is therefore reasonable to assume that the

pronounced interindividual variability observed in our study is attributable to regulation rather than genetic factors.

We detected also a significant increase of the amount of the SMRP (short type of multidrug resistance protein) transcript in the ICM samples. The SMRP cDNA was cloned from a human lung cancer cell line²⁷ and turned out to be identical with the 3'-half of the full-length MRP5 cDNA.²⁴ A truncated protein of only 946 amino acids (instead of 1437 of MRP5) would result from this cDNA. SMRP mRNA was reported to be detectable in several human tissues³³ leading to the proposal that it represents a splicing variant of the MRP5 gene and that the encoded short form of the MRP5 protein has a physiological role.³³ We could also detect minor amounts of SMRP transcript in all samples. However, so far a respective truncated protein could not be shown to be present in any tissue and was also not detected by the antibody used in this study, which was raised against the C-terminus of MRP5⁵ and therefore should detect also the SMRP protein. Thus, the physiological relevance of the SMRP transcript remains to be clarified.

Regulatory functions of cGMP have also been described in vascular endothelial cells, eg, in the regulation of endothelial permeability.⁵³ Besides the export of cyclic nucleotides, MRP5 as organic anion export pump²⁴ may have a protective function against potential toxic compounds that can be pumped from the endothelial cells back into blood. MRP5 was shown to confer resistance to nucleobase and nucleoside analogs used extensively in anti-cancer and anti-viral therapy, by cellular export of the intracellularly formed respective nucleoside monophosphate.^{54,55} Variations in the MRP5 expression in the cardiovascular endothelium as well as in the cardiomyocytes may therefore influence the concentration of these compounds in the heart tissue. Cardiac expression of drug-metabolizing enzymes as cytochrome P-450 monooxygenases⁵⁶ as well as of the ABC transporter MDR1/P-glycoprotein²⁶ has been demonstrated and may have substantial implications for drug therapy, especially for the interindividual variability in cardiac effects and toxicity.

In conclusion, the expression of MRP5 in cardiac and cardiovascular myocytes as well as endothelial cells indicates the presence of ATP-dependent cGMP export as potential novel component and pharmacological target in the regulation of cardiac tissue cGMP levels. In addition, our data point to an enhanced expression in patients with ICM.

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