aus der Klinik für Anästhesiologie und Intensivmedizin
Direktor: Univ.-Prof. Dr. med. Michael Wendt
der Medizinischen Fakultät
der Ernst-Moritz-Arndt-Universität Greifswald

# Expression und Regulation aktiver Arzneimitteltransportproteine im Herzen

## Habilitationsschrift

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| | |
|---|---|
| Dekan: | Prof. Dr. rer. nat. Heyo K. Kroemer |
| 1. Gutachter: | Prof. Dr. med. Michael Wendt, Greifswald |
| 2. Gutachter: | Prof. Dr. med. Thea Koch, Dresden |
| 3. Gutachter: | Prof. Dr. med Dr. h.c. Michael Georgieff, Ulm |
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**INHALT**

# 1. EINLEITUNG

Aktiver Transport stellt einen physiologischen Mechanismus dar, Stoffe energieabhängig über Membrangrenzen zu transportieren und damit deren Konzentration beiderseits der Membran zu verändern. Neben passiver bzw. unterstützter Diffusion, sowie carriervermitteltem Transport verkörpert aktiver Transport ein seit vergleichsweise kürzerer Zeit bekanntes Phänomen, das nicht nur auf die Zellmembran (und damit die zelluläre Homöostase von Substraten) beschränkt ist, sondern auch intrazellulär an einer ganzen Reihe von physiologischen Prozessen beteiligt ist. Systematisch werden die heute bekannten Transportproteine entweder der solute carrier Familie (SLC, http://www.bioparadigms.org/slc/intro.asp) oder den ABC-Transportern (ATP-binding cassette, http://nutrigene.4t.com/humanabc.htm) zugeordnet.

Das Herz- Kreislaufsystem ist Ziel vielfältiger medikamentöser Interventionen, wobei abhängig von therapeutischer Breite und gewünschtem Effekt des jeweiligen Arzneimittels eine für den einzelnen Patienten ideale Dosierung oft schwer zu finden bzw. vorherzusagen ist. Direkt kardial angreifende Substanzen, die oral eingenommen werden, unterliegen der Aufnahme im Darm und dem first-pass-effect in der Leber. Bei kritischen bzw. potenziell kardiotoxischen Substanzen werden zur Therapieoptimierung routinemäßig Serumspiegelbestimmungen durchgeführt. Eine intravenöse Gabe, etwa im Op. oder auf der Intensivstation macht die Wirkung vorhersagbarer, eliminiert jedoch trotzdem bei weitem nicht alle Variabilität, vor allem nicht bei schwer kranken Patienten.

Primäres Ziel der vorliegenden Arbeit war die Untersuchung des Vorkommens von Arzneimitteltransport im menschlichen Herzen als einer vorstellbaren Ursache einer variablen Wirkung von Arzneistoffen bei konstantem Serumspiegel. Einhergehend mit der Identifikation einzelner Transporter sollten daraufhin die Variabilität sowie regulatorische Einflussgrößen identifiziert werden, was sowohl krankheitsbedingte als auch genetische Ursachen einschließt. Da ABC-Transporter auch physiologische Substrate und somit Einfluss auf deren Wirkung haben sollte darüber hinaus geklärt werden, inwieweit eine kardiale Expression einzelner dieser Membranproteine nachgewiesen werden kann, um im Anschluss daran wiederum mit einem geeigneten Modell die Variabilität der Expression untersuchen zu können.

## 2. STAND DER FORSCHUNG

### 2.1. P-Glykoprotein

Auslöser für die Entdeckung des aktiven Transports war die Suche nach der Ursache erworbener Arzneimittelresistenz von Tumoren, die 1976 zur Erstbeschreibung von P-Glykoprotein in Colchizin-resistenten Ovarialkarzinomzellen des chinesischen Hamsters führte (Juliano 1976). Ausschlaggebend für die Entdeckung war die Eigenschaft dieser Zellen, auch für eine Vielzahl anderer Substrate weniger empfindlich zu sein als Zellen, die keine Colchizin-Resistenz besaßen. P-gp wurde in der Folge als 170 kDa großes Transmembranprotein identifiziert, das zur Gruppe der ABC-Transportproteine gehört (Abb.1).

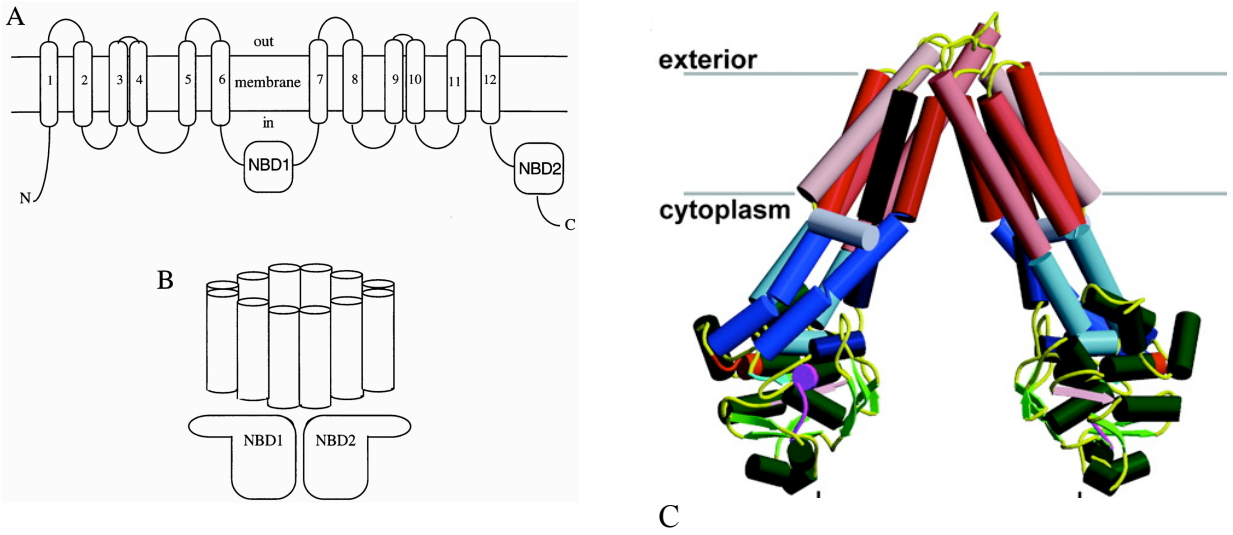


**Abb. 1: P-Glykoprotein-Modelle.** A: Struktur von P-Glykoprotein, 12 Transmembrandomänen (1-12) und 2 ATP-Bindungsstellen (NMB: nucleotide binding domain), B: vereinfachte Darstellung der räumlichen Anordnung (nach (Jones 2000)), C: Kristallstruktur von P-gp, Transmembrandomänen: rot, ATP-bindende Domänen: grün. (nach (Seigneuret 2003))

Gemeinsames Merkmal von ABC-Transportern ist der aktive ATP-abhängige Transport zum Teil sehr unterschiedlicher Substrate über eine Membrangrenze, hier die Zellmembran. 12 bzw. 6 (half-transporter) Transmembrandomänen bilden einen zentralen Porus und sind auf

der zytosolischen Seite mit 2 bzw. einer (half-transporter) ATP-Bindungsstelle verbunden. 1986 wurde P-gp in humanen Tumorzellen nachgewiesen (Ueda 1986). Der Transport von Chemotherapeutika gegen einen Konzentrationsgradienten aus Tumorzellen heraus konnte durch Vorbehandlung induziert werden (erworbene Resistenz), was auf regulatorische Aktivität als wichtigem Charakteristikum vieler ABC-Transporter hinweist (Signoretti 1997). Parallel zur Expression an der Grenze zwischen Blutstrom und Tumorgewebe wurden ABC-Transporter sehr früh auch in Leber und Niere als wichtigen Exkretionsorganen sowie im Darm gefunden (Arias 1990; Hori 1993; Saitoh 1995), wo sie durch Auswärtstransport von Umwelttoxinen und Xenobiotika offensichtlich einen maßgeblichen Anteil zur Aufrechterhaltung der Integrität des Körpers leisten. In besonders sensiblen Organen sind ABC-Transporter als Bestandteil der bekannten Barrieren des Körpers gefunden worden, zu deren bekannter Schrankenfunktion sie durch den Transport einmal in den Organismus gelangter Substanzen offenbar maßgeblich beitragen (Darm, Plazenta, Gehirn) (MacFarland 1994; Kusuhara 1997; Greiner 1999). Dies impliziert einen Einfluss der Expression von Arzneimitteltransportern auf die tatsächlich wirksame Konzentration eines Arzneistoffs innerhalb des Zielorgans unabhängig von einem gemessenen oder tatsächlichen Plasmaspiegel.

Der Einfluss von ABC-Transportern auf die Wirkung von Arzneimitteln kann auf vielerlei Weise zustande kommen. Als Beispiel für eine Induktion gilt etwa die verstärkte Expression von P-gp nach der Gabe antiretroviraler Substanzen (Huang 2001), ein bekannter Inhibitor ist Chinidin (Fromm 1999). Neben derartiger Regulation eines Transporters durch zeitgleich verabreichte Substanzen im Sinne einer Arzneimittelinteraktion (Kang 2001) ist bekannt, dass verschiedene ABC-Transporter einer genetischen Variabilität unterliegen sowie ihre Aktivität von Krankheitsprozessen beeinflusst werden könnte (Kerb 2001). Darüber hinaus ist ein Einfluss auf physiologische Exkretionsaufgaben denkbar (Mazzanti 1994; Gerloff 1998).

Neben externen Substaten wie Medikamenten und Xenobiotika konnten inzwischen auch eine Reihe endogener Substrate von ABC-Transportern identifiziert werden, deren Transport über Membrangrenzen Einfluss auf intra- bzw. extrazelluläre Konzentrationen haben kann. Beispiele hierfür sind zyklischen Nukleotide, die mit unterschiedlicher Affinität von MRP4 und MRP5 transportiert werden (Jedlitschky 2000), Steroidhormone (Kim 2004), sowie

Opioide (Oude Elferink 2001).

Die Beschreibung des Herzglykosids Digoxin als P-gp-Substrat 1992 ermöglichte neue Überlegungen zur Ursache unterschiedlicher therapeutischer Effizienz gleicher Dosen bei unterschiedlichen Patienten, wobei zuerst das Augenmerk ausschließlich auf der Aufnahme im Darm stand (de Lannoy 1992). Hinweise für eine therapeutisch relevante kardiale Expression von P-gp ergaben sich durch Untersuchungen an MDR1(-/-) Mäusen, deren kardiale Aufnahme von intravenös appliziertem Doxorubicin gegenüber Kontrolltieren deutlich erhöht war (van Asperen 1999). Am isolierten Rattenherzen konnte darüber hinaus gezeigt werden, dass die Aufnahme von Idarubicin, einem P-gp-Substrat, durch die gleichzeitige Gabe von P-gp-Inhibitoren gesteigert werden konnte (Weiss 2002). Diese Befunde bilden den Ausgangspunkt der vorliegenden Arbeit.

## 2.2. Regulation von P-Glykoprotein

Zu einer möglichen Regulation von P-gp im Herzen ist gibt es keine publizierten Arbeiten. Die verfügbaren Daten beziehen sich ausschließlich auf Tierexperimente und beleuchten nicht spezifisch das Herz. Die einzige inzwischen publizierte Studie zu einer möglichen krankheitsabhängigen Regulation von P-gp kommt am Rattenmodell für die tachykardiebedingte Herzinsuffizienz zu einem negativen Ergebnis (Sims 2004). Studien zur krankheitsabhängigen Transporterregulation an anderen Organen adressieren etwa Entzündungsvorgänge. Eine Induktion von P-gp durch pro-inflamatorische Zytokine konnte an Rattenhepatozyten sowie Endothelzellen der Blut-Hirn-Schranke nachgewiesen werden (Hirsch-Ernst 1998; Bauer 2006).

## 2.3. Breast Cancer Resistance Protein (BCRP ABCG2)

ABCG2, das mit nur 6 Transmembrandomänen und einer ATP-Bindungsstelle zur Gruppe der half-transporter gehört und funktionelle Einheiten als Homotetramer bildet (Xu 2004) wurde in einer Reihe von Krebszelllinien (zuerst in Mammakarzinomzellen) sowie als Vermittler der charakteristischen Transporteigenschaften einer Reihe von Stammzelllinien identifiziert (Bunting 2002). Sein bisher bekanntes physiologisches Vorkommen in menschlichem Gewebe

beschränkt sich auf Kolon, Dünndarm, Gallengangsepithel, Brustdrüse, Endothelzellen von Herzgewebe und Gehirn (Maliepaard 2001; Zhang 2003). Interessanterweise wurde ABCG2 kürzlich zudem in kardialen Progentorzellen der Maus gefunden, die im adulten Herzen Stammzellcharakteristiken aufweisen (Martin 2004). Eine Identifizierung dieses Transporters in unseren Vorhof- und Ventrikelproben würde also sowohl für die Klärung der physiologischen Bedeutung von ABCG2 im Herzen als auch potenziell für die Verlaufsbeobachtung nach Stammzelltherapie Auswirkungen haben können.

### 2.4. Multidrug resistance Associated Protein 5 (MRP5, ABCC5)

Neben externen Substraten spielen körpereigene Substanzen für verschiedene ABC-Transportproteine eine große Rolle. MRP5, das ebenfalls zu den an der Zellmembran exprimierten ABC-Transportproteinen gehört, war der erste identifizierte Vertreter dieser Gruppe, der zyklische Nukleotide transportiert (Jedlitschky 2000). Die höchste Affinität besitzt MRP5 dabei für zyklisches Guanosinmonophosphat (cGMP), einen wichtigen second messenger des NO-cGMP pathways zur Kontraktilität von Muskelzellen, der als molekulares Target der Arzneimittelindustrie zunehmende Bedeutung erlangt (Abb. 2).

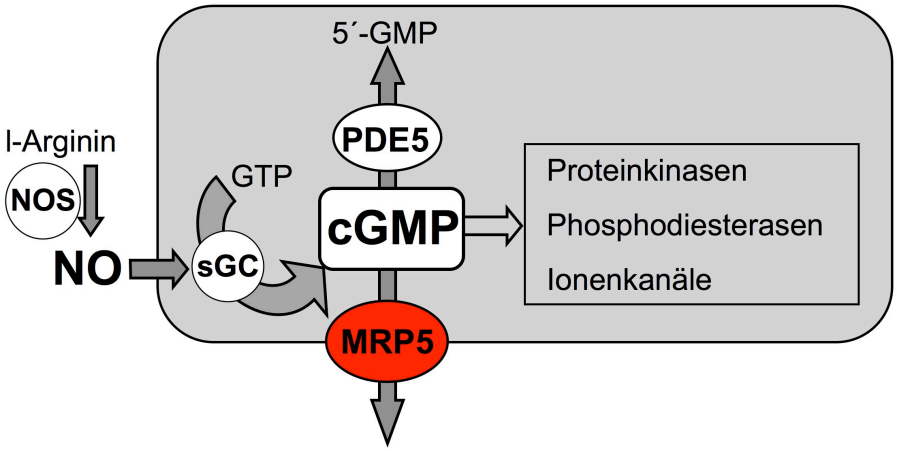


**Abb. 2: vereinfachtes Schema der intrazellulären cGMP-Homöostase.** Stickstoffmonoxid (NO), das durch NO-Synthase (NOS) synthetisiert wird, löst in der Zelle die Produktion von zyklischem Guanosinmonophosphat (cGMP) aus Guanosintriphosphat (GTP) durch lösliche Guanylatzyklase (sGC) aus. cGMP, das unter anderem die Kontraktilität von Muskelzellen beeinflusst, wird vor allem durch Phosphodiesterase 5 (PDE5) abgebaut, jedoch auch durch MRP5 nach extrazellulär transportiert.

Ein weiteres Substrat von MRP5 mit niedrigerer Affinität ist cAMP. MRP4, das kürzlich ebenfalls als Transporter zyklischer Nukleotide indentifiziert wurde, besitzt eine weitaus geringere Affinität für cGMP (Chen 2001), so dass in einem vereinfachte Schema des NO-cGMP pathways MRP5 einen bisher unbekannten Weg darstellen könnte, den intrazellulären cGMP-Spiegel zu erniedrigen. Bisherige Vorstellung war, dass der intrazelluläre cGMP-Spiegel einzig von der Synthese durch Guanylatzyklasen (GC) und dem Abbau durch Phosphodiesterase (vor allem PDE5) abhinge. Der Nachweis einer Aktivierung von MRP5 in einem Modell mit aktiviertem NO-cGMP-Pathway fehlte. Eingeschränkte kardiale Kontraktilität ist ein Charakteristikum der Sepsis (Court 2002), wobei der intrazelluläre cGMP-Spiegel offenbar eine maßgebliche Rolle spielt (Kumar 1999). Darüber hinaus ist ein aktivierter NO-cGMP pathway in der Sepsis als ein Mechanismus der kardialen Depression identifiziert worden (Senzaki 2001). Auf der Suche nach einem geeigneten Modell fiel unsere Wahl auf zwei Schockmodelle, die beide gut charakterisiert waren (Maier 2004). Hinsichtlich der Regulation von MRP5 durch Sepsis gab es für andere Organe sowohl Studien, die eine induzierende als auch solche die eine inhibierende Wirkung beschreiben (Cherrington 2004; Donner 2004).

# 3. EIGENE BEFUNDE

## 3.1. Lokalisation der P-gp-Expression im menschlichen Herzen

Ausgangspunkt der Untersuchungen zur Expression und Regulation von ABC-Transportproteinen im menschlichen Herzen war die Vorstellung, dass eine Vielzahl unerklärter Wirkungsunterschiede bei Patienten nur mit Hilfe einer regionalen Veränderung der wirksamen Menge von Arzneimitteln zu erklären ist. Ein wichtiger Hinweis in dieser Richtung war die Identifikation lokal im Herzen aktiver Isoenzyme des Cytochrom-P450-Systems durch Thum und Mitarbeiter (Thum 2000). Mittels des JSB-1-Antikörpers konnte in einem Set menschlicher Herzen P-Glykoprotein identifiziert werden, wobei sich die Lokalisation hauptsächlich auf das Endothel von Arteriolen und Kapillaren beschränkte (Pfeil, Abb. 3). Eine Schrankenfuktion durch P-gp, das im Blut befindliche Substrate vom eigentlichen Myokard fernhält, erscheint somit in Analogie zu bekannten Schranken im menschlichen Körper denkbar (Meissner 2002).

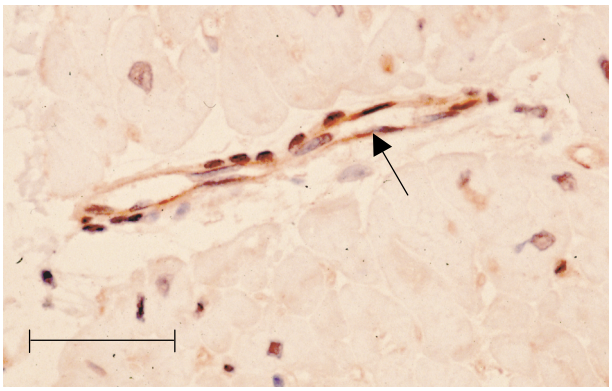

**Abb. 3: Lokalisation von P-gp- im menschlichen Ventrikelmyokard.**
Pfeil: Endothel, Größenmarkierung: 100 µm

Zusätzlich zum Protein konnte mittels in-situ-Hybridisierung ebenfalls die RNA für das MDR1-Gen in entsprechnder Lokalisation nachgewiesen werden, die für P-Glykoprotein kodiert (Abb. 4).

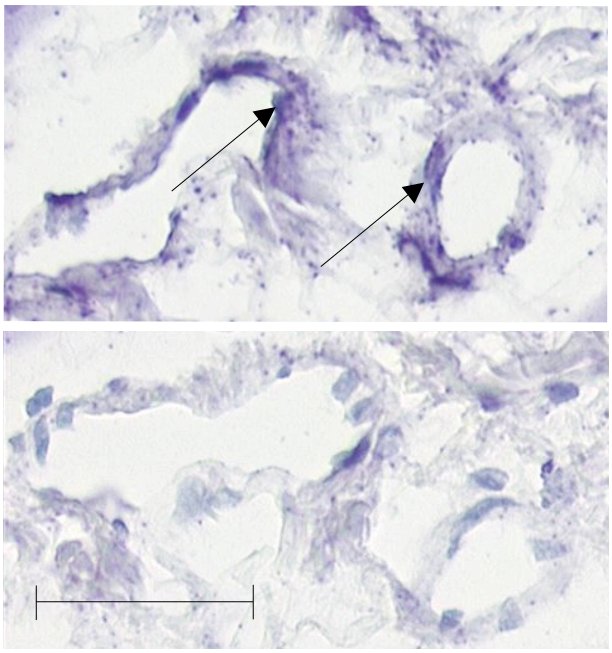

**Abb. 4: Lokalisation von MDR1 mRNA im menschlichen Ventrikelmyokard.**
Antisense cRNA-Proben (oben), Pfeile: Endothel, sense-Proben als Kontrolle (unten). Größenmarkierung: 100 µm

Im Rahmen einer Folgestudie an Gewebeproben aus dem Herzvorhof (s.u.) wurde gezeigt, dass P-Glykoprotein nicht nur im Ventrikel, sondern auch im Vorhof exprimiert wird (Abb. 5) (Meissner 2004).

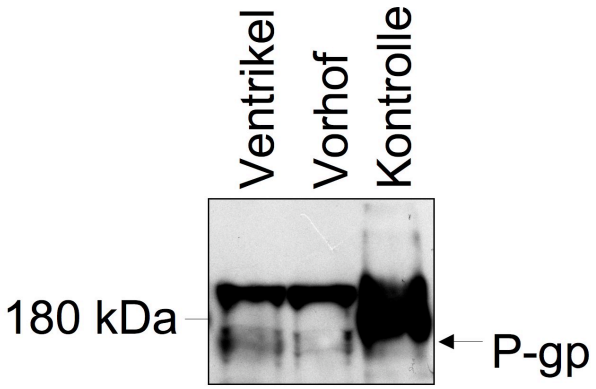


**Abb. 5: Western Blot von P-Glykoprotein in gereinigten Membranen.**
Anti-P-gp C219 detektiert P-gp als 170 kDa-Bande in Vorhof- und Ventrikelgewebe. Kontrolle: P-gp überexprimierende Leukämiezellen.

### 3.2. Einfluß von Kardiomyopathien auf die P-gp-Expression

Innerhalb untersuchten Proben fiel eine substanzielle Variabilität auf, die zur Ausangshypothese passte und mit anderen Ergebnissen zur Expression von P-Glykoprotein übereinstimmte. Da neben Herzgewebeproben ohne Herzinsuffizienz (non-failing) auch

Proben von Kardiomyopathie-Herzen zur Verfügung standen, konnten quantitative Unterschiede sowohl auf RNA- als auch Proteinebene untersucht werden. Bezogen auf 18S rRNA als Housekeeping-Gen ergab sich eine signifikant niedrigere MDR1-mRNA-Expression bei dilatativer Kardiomyopathie gegenüber sowohl gesunden als auch Herzen mit ischämischer Kardiomyopathie (Abb. 5, 6) (Meissner 2002).

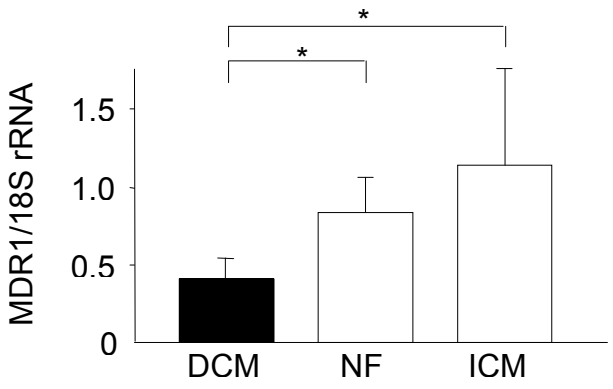


**Abb. 5: quantitative Bestimmung von MDR1 mRNA mittels real-Time-PCR.**
Herzgewebeproben mit dilatativer Kardiomyopathie (DCM) zeigen eine signifikant geringere MDR1 mRNA-Expression als gesunde (NF) oder Herzen mit ischämischer Kardiomyopathie (ICM).

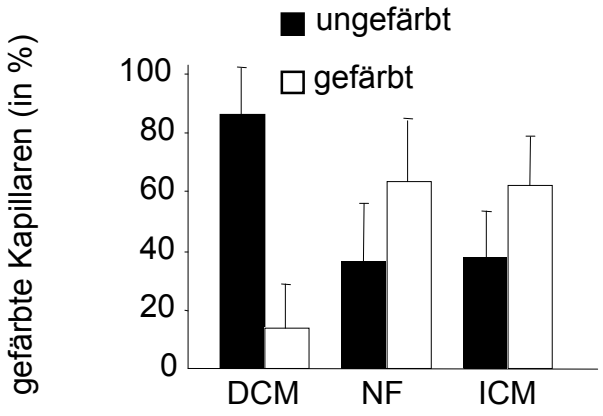


**Abb. 6: semiquantitative Auswertung des P-gp-Gehalts in immunhistochemischen Präparaten.**
Während Präparate mit dilatativer Kardiomyopathie (DCM) kaum gefärbte Gefäßanschnitte enthalten, liegt bei ischämischer Kardiomyopathie (ICM) und gesunden Herzen (NF) der gefärbte Anteil deutlich höher.

### 3.3. Regulation der P-gp-Expression durch genetische Einflüsse

Neben Medikamenten und Krankeitszuständen sind genetische Faktoren dafür bekannt, Einfluss auf die Expression von ABC-Transportproteinen zu haben. Das am besten untersuchte Beispiel dafür ist wiederum P-Glykoprotein, bei dem vor allem die beiden Polymorphismen in Exon 21 (2677 AT oder TT) und 26 (3435 CT) Anlass dafür gaben (Hoffmeyer 2000; Cascorbi 2001). Während das Ausmaß bzw. die Evidenz für den jeweiligen

Einfluss dieser beiden Loci umstritten ist wurden auch Studien publiziert, die eine Verbindung zwischen den beiden SNPs herstellen (Tanabe 2001; Johne 2002).

Die Ergebnisse in den 15 Ventrikelproben (s.o.) konnten zwar eine leicht reduzierte MDR1 mRNA-Expression in den Proben nachweisen, die den Exon 26 3435 TT Genotyp aufwiesen, dieser Befund war aber statistisch nicht signifikant. Zur eingehenden Untersuchung dieses Effekts wurde eine größere Studie initiiert, in deren Rahmen 51 Proben des rechten Vorhofs auf ihren MDR1 mRNA-Gehalt untersucht und mit ihrem jeweiligen Genotyp verglichen wurden (Abb. 7). Um eine eventuelle Kopplung der beiden Loci analysieren zu können wurde eine Haplotypanalyse vorgenommen, die jedoch ebenfalls keine Hinweise auf genetische Gründe für die beobachtete Variabilität der P-gp-Expression im menschlichen Herzen erbrachte (Abb. 8). Der in der Literatur kontrovers diskutierte Einfluss dieser beiden SNPs auf die P-Glykoprotein-Expression konnte somit für das menschliche Herz sowohl für jeden der beiden Loci einzeln als auch in Kombination verneint werden (Meissner 2004).

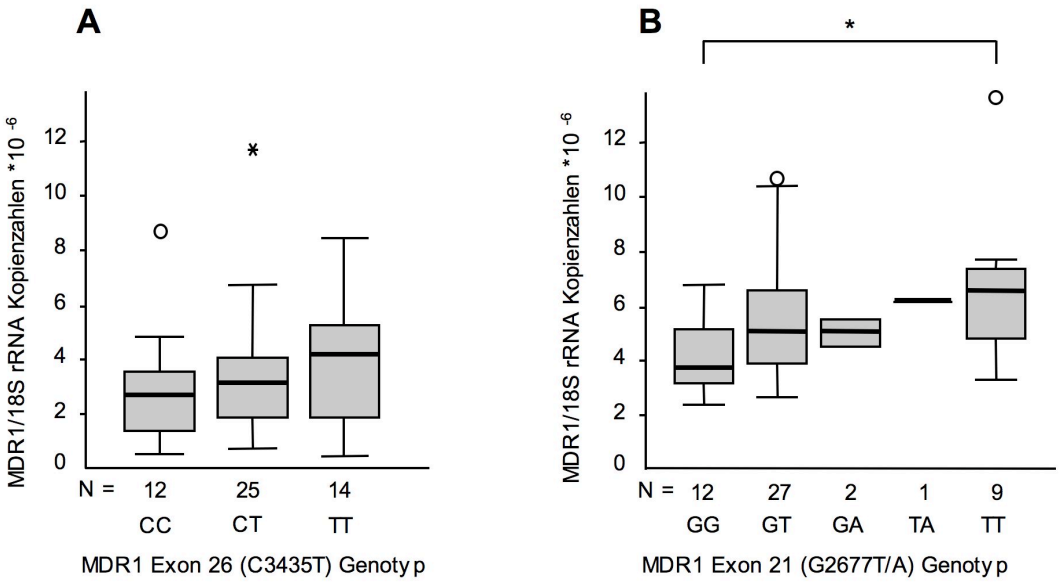


**Abb. 7: MDR1/18S rRNA Kopienzahlen in humanen Vorhofproben in Abhängigkeit vom Genotyp.** Während der Polymorphismus im Exon 26 keinen signifikanten Einfluss auf die Expressionsstärke hat (A), ist der Unterschied zwischen Wildtyp- und homozygot mutierten Proben für Exon 21 signifikant (B).

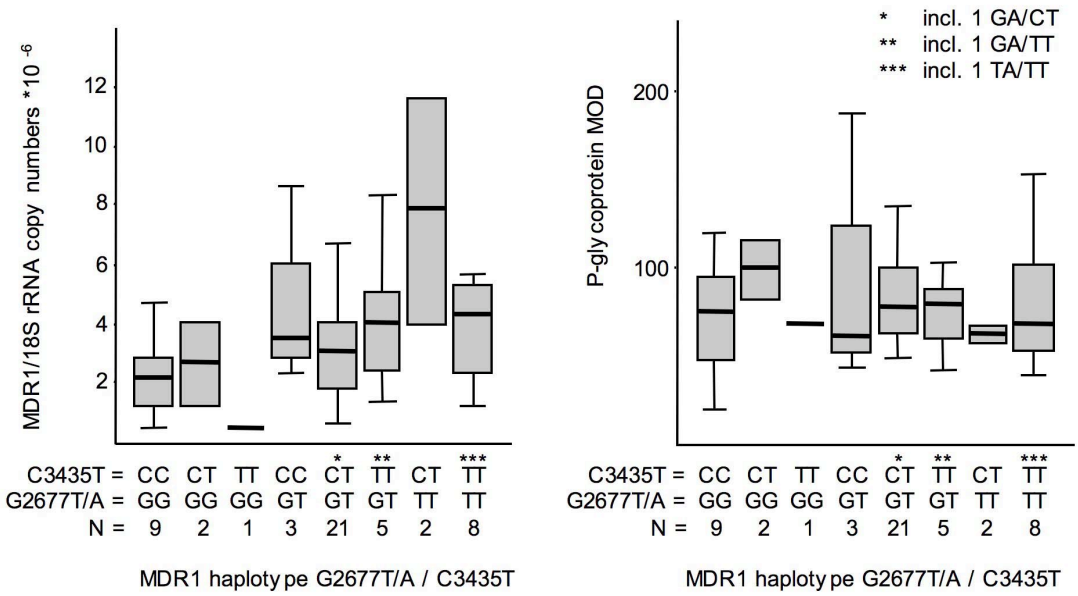


**Abb. 8: MDR1-Expression in Abhängigkeit vom Haplotyp (Exon 26 und 21).**

Weder die MDR1 mRNA-Expression (A) noch die semiquantitative Auswertung der P-gp Färbung in der Immunhistochemie (MOD: mean optical density, B) ergaben signifikante genetische Einflüsse auf die MDR1-Expression.

### 3.4. Expression von ABCG2 im menschlichen Herzen

Die Expression von ABCG2 wurde in den vorhandenen Proben nachgewiesen, wobei parallel Vorhof- und Ventrikelgewebe untersucht werden konnte. In angereicherten Membranen beider Lokalisationen wurde eine ähnliche Lokalisation und Expressionsstärke gefunden (Abb. 9, 10). Die an sich für spezifische Stammzellen charakteristische Expression von ABCG2, die für deren Isolierung genutzt wird, ist somit – jedenfalls für das Herz – als Verlaufsparameter nach einer eventuellen Therapie mit diesen Zellen ungeeignet.

Die starke Variabilität wurde wiederum auf mögliche Ursachen hin untersucht, wobei es – in Analogie zur MDR1-Expression in den in der Population vorhandenen und untersuchten beiden single nucleotide polymorphisms keine Korrelation mit einer geänderten ABCG2-Expression gab, eine Aussage, die in Anbetracht der vorhanden Häufigkeiten eingeschränkt gültig ist. Um auszuschließen dass es sich hierbei um probenabhängige Veränderungen handelt wurde zudem untersucht, ob die ABCG2-Daten der Proben mit denen von MDR1

korrelieren, was nicht der Fall war. Insofern kann davon ausgegangen werden, dass die getroffenen quantitativen Aussagen zur ABCG2-Expression im menschlichen Vorhof gültig sind. Die krankheitsabhängige Expression in den zuvor beschriebenen Ventrikelproben ergab eine signifikant erhöhte Expression von ABCG2 bei beiden Kardiomyopathieformen im Vergleich zu gesundem Vergleichsgewebe, was mit den Aussagen für MDR1, jedoch nicht MRP5 übereinstimmt (Abb. 11). Zum Genotyp ergab sich wiederum kein Zusammenhang (Abb. 12)

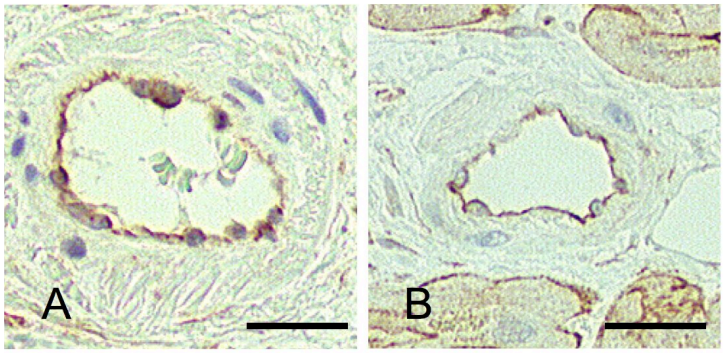


**Abb. 9: Immunhistochemie, Expression von ABCG2 im menschlichen Herzen.** Vorhof (A) und Ventrikel (B). Größenmarkierung: 25 µm

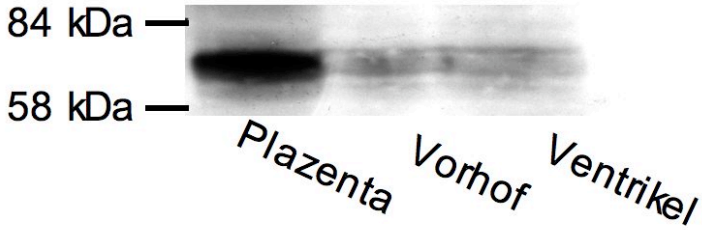


**Abb. 10: Western Blot, Expression von ABCG2 im menschlichen Herzen.** Nachweis als Bande bei 70 Kilodalton in isolierten Membranen.

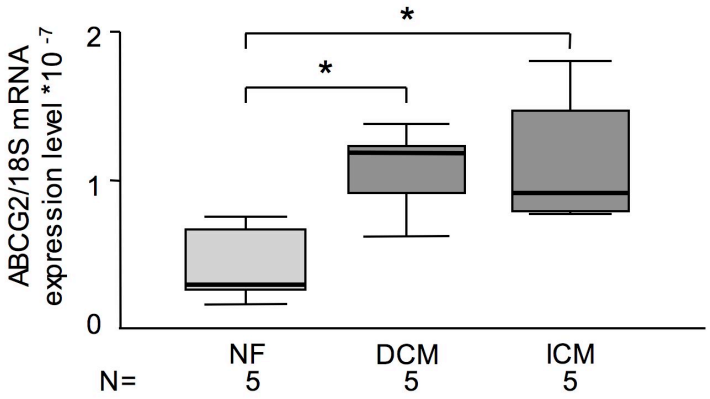


**Abb. 11: krankheitsabhängige ABCG2-Expression im menschlichen Herzen.** ABCG2 ist bei beiden Kardiomyopathieformen signifikant erhöht.

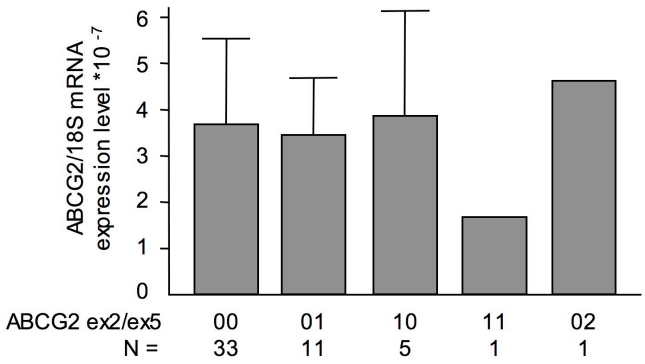


**Abb. 12: fehlende Abhängigkeit der kardialen ABCG2-Expression vom Genotyp.** Untersucht wurden Exon 2 (erste Zahl) und 5 (zweite Zahl); 0: homozygot Wildtyp, 1 heterozygot, 2 homozygot mutiert.

### 3.5. Expression und Lokalisation von MRP5 im menschlichen Herzen

Die kardiale Expression von MRP5 ist vor allem deshalb so interessant, weil cGMP als wichtiges Substrat für die kontraktile Funktion von Muskelzellen ausschlaggebend ist (s.o.). Ziel der Untersuchungen war deshalb der Nachweis von funktionellem MRP5 in entsprechender Lokalisation. In den oben bereits erwähnten Herzgewebeproben aus Vorhof und Ventrikel konnte MRP5 sicher nachgewiesen werden. Auffallend war seine Lokalisation nicht nur im Endothel, sondern auch in der glatten Gefäßmuskulatur sowie in Kardiomyozyten (Abb. 13). Zur Quantifizierung und Kontrolle wurden sowohl die Blots als auch die Gewebeschnitte mit Anti-Desmin gefärbt.

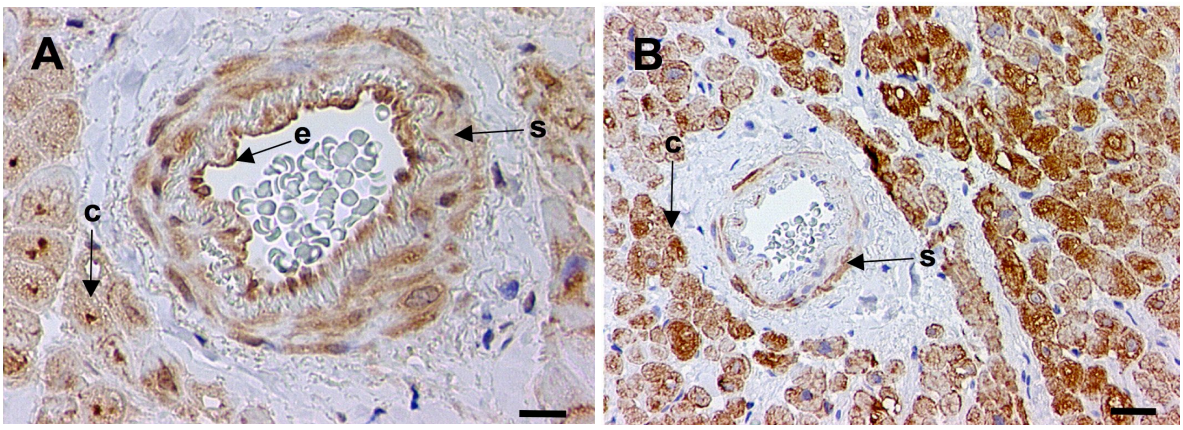


**Abb. 13: Lokalisation von MRP5 (A) und Desmin (B) im menschlichen Vorhofmyokard.**
Im Gegensatz zu Desmin findet sich MRP5 nicht nur in glatten Muskelzellen (s) und Kardiomyozyten (c) sondern auch im Gefäßendothel (e). Größenmarker: 5 µm (A) und 25 µm (B).

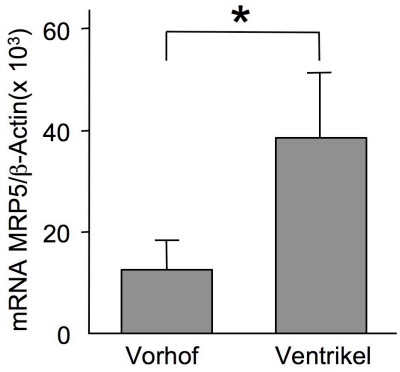


**Abb. 14: Expression von MRP5 mRNA in Vorhof- und Ventrikelgewebe im Vergleich.** Im Ventrikel findet sich eine signifikant stärkere MRP5 Expression.

Während sich im Ventrikel deutlich mehr MRP5 findet (Abb. 14), fällt doch auch hier eine große interindividuelle Variabilität auf. Auf der Suche nach Ursachen sind wiederum die Kardiomyopathieproben untersucht worden, bei denen es einen Trend zu verstärkter Expression bei ischämischer Kardiomyopathie gab (Abb. 15), der für die quantitative Auswertung des Western Blots (15B) und die semiquantitative Auswertung der Immunhistochemie für die Kardiomyozyten (15D) signifikant war.

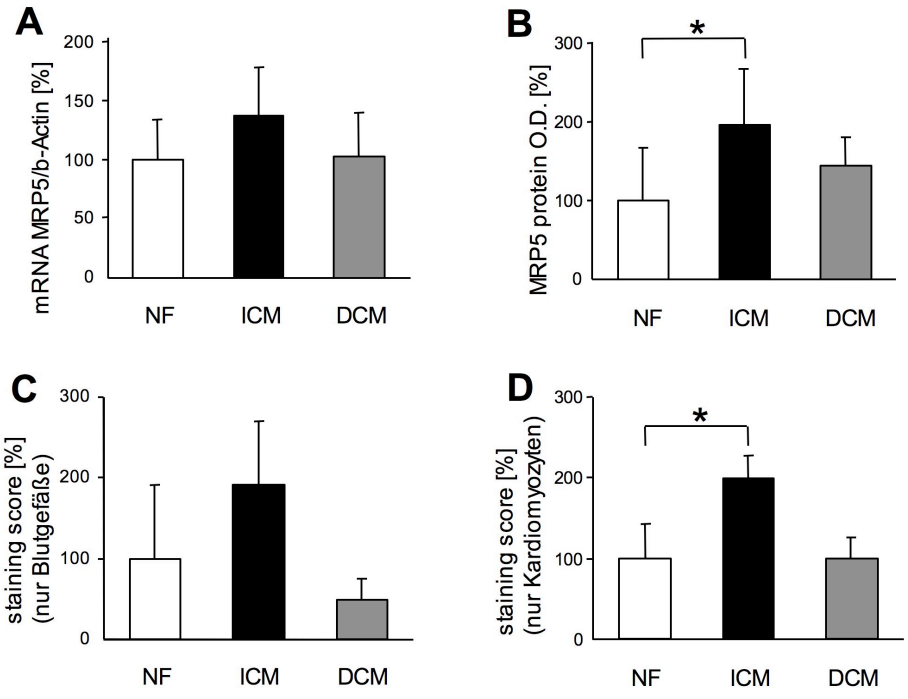


**Abb. 15: Expressionsstärke von MRP5 im menschlichen Ventrikelmyokard.** mRNA (A), Western Blot (B), semiquantitative Immunhistochemie für gefärbte Blutgefäße (C) und Kardiomyozyten (D).

### 3.6. Kardiale Regulation von MRP5 im Sepsismodell der Maus

In Analogie zu den oben beschriebenen Befunden am menschlichen Herzen konnte MRP5 bei der Maus morphologisch in den Endothelzellen, den glatten Gefäßmuskelzellen und in den Kardiomyozyten nachgewiesen werden (Abb. 16). Mittels Western Blot gelang ein qualitativer Proteinnachweis in allen Versuchsgruppen.

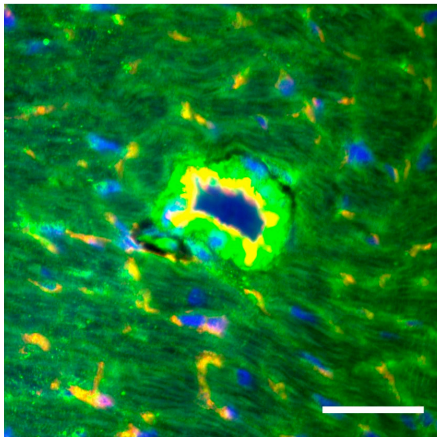

**Abb. 16: konfokalmikroskopische Aufnahme von MRP5 im Schweineherzen.** Anti-MRP5 AMF (grün) färbt Endothel-, Gefäß- und Herzmuskelzellen. Eine Endothelgegenfärbung (PECAM, rot) ist superimposed und resultiert in einer Gelbfärbung des Endothels. Blau: DNA-Färbung (TOTO). Größenmarkierung: 50 µm.

Die kardiale Expression von MRP5 mRNA war in beiden Sepsismodellen (LPS-Injektion und Colon-ascendens-stent Peritonits, CASP) nach 10 Stunden gegenüber den entsprechenden Kontrollen und nativen Proben signifikant reduziert (Abb. 17). Parallel dazu waren in beiden Modellen die Interleukin 6-Konzentrationen im Serum deutlich angestiegen. In Anbetracht der cGMP-Transportkapazität von MRP5 könnte ein reduzierter cGMP-Auswärtstransport somit einem erhöhten intrazellulären cGMP-Spiegel hervorrufen und potenziell zu einer verschlechterten Herzfunktion bei septischer Kardiomyopathie beitragen.

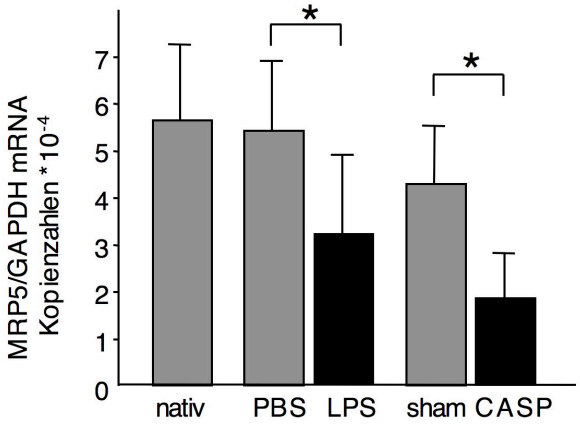


**Abb. 17: MRP5 mRNA-Veränderungen im Schweineherzen bei experimentellem Endotoxin- (LPS) bzw. multibakteriellem Schock (CASP).** Die MRP5 mRNA ist in beiden Modellen kardial signifikant reduziert.

## 4. DISKUSSION UND AUSBLICK

Das Herz wird zunehmend als Ort von Metabolismus und Transport wahrgenommen (Thum 2000; Chowbay 2003; Couture 2006; Solbach 2006). Dies ist aus mehreren Gründen sinnvoll. Zum einen wird deutlich, dass effektive Arzneimittelspiegel im Serum nicht unbedingt auf die wirksame Dosis an der Effektorzelle schließen lassen müssen, was sowohl Metabolismus als auch Transport als Mechanismus zur Ursache haben kann. In Anbetracht der Vielzahl der bekannten Zytochrome und ABC-Transporter ergeben sich entsprechende Mengen möglicher Angriffspunkte für eine individualisierte Arzneimitteltherapie, die eine Berücksichtigung von Genotyp und Krankheitszustand eines Patienten für eine möglichst ideale Dosierung zum Ziel hat. So praxisfern das im Moment noch klingen mag zeigen die Bemühungen der Industrie um schnellen Zugriff auf den individuellen Genotyp ausgewählter Zytochrome und ABC-Transporter doch in diese Richtung (Liljedahl 2003). Im Rahmen der Abschätzung kardiotoxischer Nebenwirkungen in der Chemotherapie maligner Tumoren und bei der Herzinsuffizienz zeichnen sich Lösungen für die Praxis ab, bei denen ABC-Transportproteine eine Rolle spielen werden (Cascorbi 2004). Die Untersuchung möglicher Drug-Drug Interaktionen am Herzen, die für verschiedene Medikamente im Tierversuch bereits erfolgreich demonstriert werden konnten (Kang 2001), wird in Zukunft an Bedeutung gewinnen, auch ohne auf spezielle individuelle Besonderheiten eingehen zu müssen.

Zum anderen stellen Befunde zur Variabilität von Transportern körpereigener Substrate und deren Regulation im Rahmen pathologischer Ereignisse unter Umständen völlig neue therapeutische Angriffspunkte zur Verfügung. Im Hinblick auf die kardiale MRP5-Expression bleiben dafür jedoch entscheidende Fragen offen. Zwar ist die Fähigkeit von MRP5 zum Transport von cGMP unstrittig (Jedlitschky 2000) und in unserer Studie an isolierten Membranen aus menschlichen Vorhöfen auch nachgewiesen worden (Dazert 2003). Falls man MRP5 jedoch einen relevanten Beitrag zur Regulation des intrazellulären MRP5-Spiegels zugestehen möchte, müsste zuerst geklärt werden, wie sich die transportierte Menge cGMP zur in der gleichen Zeit abgebauten und synthetisierten Menge cGMP verhält. Darüber hinaus ist immer noch unklar, wie sich die differenzierte Regulation aller an der cGMP-Homöostase beteiligten Faktoren verhält. Entsprechende Untersuchungen an den Herzgewebeproben der in dieser Arbeit beschriebenen Sepsisversuche laufen derzeit. Für eine Beteiligung NO-

unabhängiger Mechanismen an der septischen Kardiomyopathie, die cGMP gleichwohl einschließen könnten, sprechen kardiodepressive Eigenschaften von TNFα, die unabhängig von einer NO-Einfluss beschrieben wurden (Muller-Werdan 1997). In jedem Falle können solche Untersuchungen allenfalls einen umschriebenen Beitrag zur Aufklärung der komplexen Vorgänge des Krankheitsbildes der Sepsis liefern, dessen Bedeutung im klinischen Alltag eher zunimmt (Linde-Zwirble 2004). Schätzungen gehen von einem Anteil von 10% der akuten septischen Kardiomyopathie an allen Sepsistodesfällen aus.

Die derzeit bekannten kardial exprimierten Transportproteine schließen jedoch auch Vertreter ein, die in den hier vorgestellten Studien nicht berücksichtigt wurden. ABCA5 etwa wurde immunhistochemisch ebenfalls in Kardiomyozyten nachgewiesen, wo es im Gegensatz zu den hier beschriebenen sarkolemmal exprimierten Transportern in der Lysosomenmembran nachgewiesen wurde (Kubo 2005). Obwohl seine spezifische Funktion bisher unklar ist, konnte gezeigt werden, dass knockout-Mäuse eine Kardiomyopathie entwickeln, die morphologische Ähnlichkeiten mit den kardialen Befunden bei lysosomalen Defekten aufweist. Das ABC-Transportprotein ABCC9 (Sulfonylurea receptor 2, SUR2) wiederum wird zwar im Sarkolemm exprimiert, besitzt jedoch keine Transporteigenschaften im eigentlichen Sinn, sondern bildet einen Bestandteil des ATP-abhängigen Kaliumkanals, der an vielen Stellen des Körpers vorkommt. In Maus und Ratte ist SUR2 im Ventrikelmyokard sowie in Koronargefäßen beschrieben worden (Morrissey 2005).

Aufgrund der immer unübersichtlicher werdenden Menge neuer Arzneimittel und therapeutisch eingesetzter Substanzen erscheint die Untersuchung auf deren jeweiliges Potenzial als Substrat für kardiale ABC-Transportproteine zwingend. Die in der vorliegenden Arbeit zusammengefassten Studien sollten Anlass dazu geben, dabei nicht nur auf oral aufgenommene, sondern auch intravenös verabreichte kardial angreifende Substanzen zu achten, deren therapeutische Breite eng bzw. deren kardiale Toxizität hoch ist. Vieles spricht dafür, dass die besprochene Problematik auch aus anästhesiologischer Sicht nicht auf interindividuelle Dosierungsunterschiede von Betablockern und Herzglykosiden als P-gp-Substraten sowie die septische Kardiomyopathie beschränkt bleiben wird.

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## 6. EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder der Medizinischen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Habilitation eingereicht wurde.
Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel benutzt habe.

St. Louis, am 04.02.2007 Konrad Meissner

## 7. LEBENSLAUF

Konrad Meissner

geb. am 16.05.1972 in Naumburg/Saale
8555 Bryan Ave., St. Louis MO 63117, USA

Tel priv.: 001 314 601 2625
Tel dienstl.: 001 314 362 3661
Fax: 001 314 747 1070

meissnek@wustl.edu
www.anest.wustl.edu

Ausbildung/ berufliche Tätigkeit

| | |
|---|---|
| 07/1990 | Abitur in Meißen |
| 10/1991-10/1993 | Studium der Humanmedizin an der Universität Leipzig |
| 11/1993-05/1998 | Studium der Humanmedizin an der Ernst-Moritz-Arndt-Universität Greifswald |
| 10/1996-031997 | Forschungsaufenthalt im Dept. of Anesthesia, Research Laboratories, Brigham and Women´s Hospital, Harvard Medical School, Boston MA, USA (G.R. Strichartz) |
| 01/1998-02/1998 | PJ, Intensive Care Medicine, Beth Israel Deaconess Hospital; Emergency Medicine, Massachusetts General Hospital, Harvard Medical School, Boston MA, USA |
| 10/1998-03/2000 | AiP, Klinik für Anästhesiologie (M. Wendt) und Institut für Pharmakologie (H.K. Kroemer), EMAU Greifswald |
| 01/1999 | Dr. med. (magna cum laude) |
| 04/2000-04/2005 | Assistenzarzt, Klinik für Anästhesiologie (M. Wendt), und wiss. Arbeit im Institut für Pharmakologie (H. K. Kroemer), EMAU Greifswald |
| seit 04/2005 | Facharzt für Anästhesiologie |
| seit 08/2006 | Attending Anesthesiologist, Barnes-Jewish Hospital, Instructor of Anesthesiology, Dept. of Anesthesiology, Washington University, St. Louis MO, USA |

| | | |
|---|---|---|
| Stipendien/ Preise/ Drittmittel | 1992-1998 | Stipendium des Ev. Studienwerk Villigst e.V. Schwerte/Ruhr |
| | 1997 | 2nd prize, Free Poster Competition, XVI Annual ESRA Congress, London, UK |
| | 2000-2001 | Stipendium der Deutschen Gesellschaft für Kardiologie – Herz- und Kreislaufforschung, Düsseldorf, |
| | 2002, 2003 | Anschubfinanzierungen der Medizinischen Fakultät Greifswald (mit D. Pavlovic: „Entwicklung und Charakterisierung eines Notfallbeatmungsventils im Tiermodell“, „Wirkung steroidaler Muskelrelaxantien auf den Tonus glatter Bronchialmuskulatur“) |
| | 2002-2003 | Karl und Lore Klein-Stiftung, Oy-Mittelberg (mit H.K. Kroemer: „Molekularbiologische Untersuchungen zu Expression, Regulation und Funktion von Arzneimitteltransportern“) |
| | 2004 | Innovationspreis des Kultusministeriums Mecklenburg-Vorpommern (mit M. Wendt: “Serialisierte Infusion”) |
| | 2004-2007 | Förderung eines Gemeinschaftsantrags durch das BMBF (NBL3-Programm, mit Ch. Fusch und A. Popa-Wagner, Greifswald: „Transporter an Kompartimentgrenzen“) |
| | 2005 | Hans J. Dengler Preis für Klinische Pharmakologie der Paul-Martini-Stiftung, Berlin |
| | 2006-2007 | Förderung eines Projekts durch die Bayer AG (mit Th. Krieg, St. Felix und H.K. Kroemer: „Cardioprotective effects of PDE-5 inhibition with vardenafil through regulation of MRP membrane transporters“) |
| | 2006-2007 | Förderung eines Projekts zur Charakterisierung serialisiert zu verabreichender Pharmaka durch die Dräger AG, Lübeck (mit W. Weitschies, Greifswald), Diplomandenstelle |

## 8. ANHANG

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ARTICLE

# Expression and Localization of P-glycoprotein in Human Heart: Effects of Cardiomyopathy

Konrad Meissner, Bernhard Sperker, Christiane Karsten, Henriette Meyer zu Schwabedissen, Ute Seeland, Michael Böhm, Sandra Bien, Peter Dazert, Christiane Kunert–Keil, Silke Vogelgesang, Rolf Warzok, Werner Siegmund, Ingolf Cascorbi, Michael Wendt, and Heyo K. Kroemer

Institut für Pharmakologie and Peter Holtz Research Center of Pharmacology and Experimental Therapeutics (KM,BS,CK,HMzS,SB,PD, CK–K,WS,IC,HKK), Klinik für Anästhesiologie (KM,MW), Institut für Pathologie, Ernst-Moritz-Arndt-Universität Greifswald (SV,RW), Greifswald, Germany, and Medizinische Klinik III, Universität des Saarlandes (US,MB), Homburg/S., Germany

**SUMMARY** ABC-type transport proteins, such as P-glycoprotein (P-gp), modify intracellular concentrations of many substrate compounds. They serve as functional barriers against entry of xenobiotics (e.g., in the gut or the blood–brain barrier) or contribute to drug excretion. Expression of transport proteins in the heart could be an important factor modifying cardiac concentrations of drugs known to be transported by P-gp (e.g., β-blockers, cardiac glycosides, doxorubicin). We therefore investigated the expression and localization of P-gp in human heart. Samples from 15 human hearts (left ventricle; five non-failing, five dilated cardiomyopathy, and five ischemic cardiomyopathy) were analyzed for expression of P-gp using real-time RT-PCR, immunohistochemistry, and in situ hybridization. Immunohistochemistry revealed expression of P-gp in endothelium of both arterioles and capillaries of all heart samples. Although P-gp mRNA was detected in all samples, its expression level was significantly reduced in patients with dilated cardiomyopathy. We describe variable expression of P-gp in human heart and its localization in the endothelial wall. Thus, intracardiac concentrations of various compounds may be modified, depending on the individual P-gp level. **(J Histochem Cytochem 50:1351–1356, 2002)**

**KEY WORDS**
P-glycoprotein
heart
drug transport
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IT IS INCREASINGLY RECOGNIZED that drug transport across biomembranes is facilitated by membrane proteins. Such drug transporters belonging to the ABC (ATP-binding cassette) family can influence the intracellular concentration and hence the action of many compounds in a variety of cells and tissues (Tishler et al. 1995; Drach et al. 1996; Schinkel et al. 1997; Wijnholds et al. 1997). The initial observation of therapeutic implications resulted from chemotherapy-resistant tumor cells, which had a high expression of the ABC transporter P-glycoprotein (P-gp), leading to low intracellular concentrations of cytotoxic drugs. Subsequent investigations described expression of P-gp and other transport proteins under physiological conditions in various cells (e.g., enterocytes, hepatocytes, endothelial cells of the blood–brain barrier). P-gp may serve as a functional barrier against drug entry (expression in the gut wall results in low absorption) or contribute to drug excretion (expression at the canalicular site of hepatocytes or tubule cells). Moreover, expression in endothelial cells of the blood–brain barrier protects against drug penetration into the CNS (Jette et al. 1993). Consequently, knockout mice devoid of P-gp activity have major alterations in drug disposition (enhanced absorption and high CNS concentrations of P-gp substrates; Kawahara et al. 1999; Schinkel 1997).

Expression of P-gp in humans reveals a wide interindividual variability. Both genetic and environmental factors have been identified that contribute to this

Correspondence to: Heyo K. Kroemer, PhD, Institut für Pharmakologie, Ernst-Moritz-Arndt-Universität Greifswald, Friedrich-Loeffler-Str. 23d, D-17489 Greifswald, Germany. E-mail: kroemer@uni-greifswald.de

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variability. For example, Hoffmeyer and co-workers (2000) identified a mutation in the P-gp encoding gene MDR1, which affected the bioavailability of the P-gp substrate digoxin. The antibiotic rifampin is an effective inducer of P-gp and other ABC transporters (e.g., MRP2; Greiner et al. 1999; Fromm et al. 2000), and this effect is mediated by a PXR-binding DR4 motif in an upstream enhancer of MDR1 (Geick et al. 2001).

Substrates for P-gp exhibit a wide structural diversity and cover a wide range of therapeutic indications. Notably, many cardiovascular active compounds are subject to drug transport by P-gp. Digoxin has been unequivocally identified as a P-gp substrate by experiments in cell lines, in animals, and in humans (de Lannoy and Silverman 1992). The disposition of several β-blocking drugs (e.g., talinolol, pafenolol) is modulated by P-gp (Westphal et al. 2000). Moreover, cardiotoxic drugs such as anthracyclines (e.g., doxorubicin) are P-gp substrates (Fardel et al. 1997) and accumulate in cardiac tissue of mdr−/− mice (van Asperen et al. 1999). Therefore, expression of P-gp in human heart may alter the intracardiac concentrations and hence the effects of therapeutic agents and cardiotoxic drugs.

We therefore investigated the expression, localization, and genotype of P-gp in human heart using immunohistochemistry (IHC), in situ hybridization (ISH), real-time RT-PCR, and RFLP. Here we describe variable expression of P-gp in the endothelial wall of cardiac blood vessels. Our data point to reduced expression of P-gp in patients with dilated cardiomyopathy.

## Materials and Methods

### Tissue Samples

After approval from the local ethics committee, heart tissue samples were taken from excised heart left ventricle during orthotopic heart transplantation due to end-stage heart failure and were immediately frozen in liquid nitrogen or fixed in 4% paraformaldehyde. Of the 15 subjects, five suffered from ischemic cardiomyopathy (ICM) and five from dilated non-ischemic cardiomyopathy (DCM). Medical therapy of patients suffering from DCM and ICM consisted of digitalis, diuretics, nitrates, and angiotensin-converting enzyme inhibitors. Tissue samples from five non-failing hearts (NF), which were not transplanted for surgical reasons or blood group incompatibility, served as controls.

The frozen tissue was homogenized using a vibration grinding mill (Mikro-Dismembrator S; B. Braun Biotech, Melsungen, Germany) for standard RNA and DNA preparation protocols. Fixed tissue was embedded in paraffin for IHC or ISH.

### MDR1 Genotype

Genomic DNA was prepared from 30 mg tissue using standard phenol/chloroform extraction. The genotype of each individual at the MDR1 exon 26 C3435T locus was determined using a polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assay according to Cascorbi et al. (2001). Briefly, after amplification of a 197-bp DNA fragment, the variant in exon 26 C3435T was discriminated in a subsequent restriction enzyme digest using Sau3AI. The PCR reactions were carried out in a Perkin–Elmer 9700 thermocycler. DNA fragments generated after restriction enzyme digestion were separated on a 3.5% agarose gel (low-temperature melting agarose:standard agarose 3:1). Restriction fragments were visualized after ethidium bromide staining of the agarose gel using a UV transilluminator (Kodak Digital Science Image Station 440CF; Eastman Kodak, Rochester, NY).

### RT-PCR Analysis of MDR1

Total RNA was isolated from 50 mg frozen tissue homogenate using a guanidinium isothiocyanate extraction kit (PeqLab; Erlangen, Germany), and subsequent DNase treatment, followed by spin column purification (Strataprep Total RNA Miniprep Kit; Stratagene, Amsterdam, The Netherlands).

For real-time RT-PCR, 200 ng of total RNA was reverse-transcribed using random hexamers and the TaqMan Reverse Transcription Reagents (Applied Biosystems; Weiterstadt, Germany). RT-PCR of MDR1 and 18S rRNA was performed using the primers MDR1F 5′-TTCGCAACCCCAAGATCCTC-3′, MDR1R 5′-ACAATGGTGGTCCGACCTTT-3′, and the TaqMan probe 5′-6FAM-ATCCAGAGCCACCTGAACCACTGCT XTp, as well as TaqMan Ribosomal RNA Control Reagents (Applied Biosystems) for 18S rRNA and the TaqMan universal PCR mastermix (Applied Biosystems). PCR products were amplified (50C, 2 min; 95C, 10 min; followed by 40 cycles of 95C, 15 sec and 60C, 1 min) and analyzed on a real-time RT-PCR cycler (ABI Prism 7700; Applied Biosystems).

For relative 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 cDNA from P-gp-overexpressing L-MDR1 cells (kindly supplied by Dr. A. Schinkel; The Netherlands Cancer Institute, Amsterdam). The ratio of relative copy numbers of MDR1 divided by those of 18S rRNA thus represents the expression level of P-gp coding MDR1 mRNA.

Conventional RT-PCR was performed as described previously (Westphal et al. 2000). PCR products for MDR1 mRNA (157 bp) and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 110 bp) were separated on a 8% polyacrylamide gel, stained with VistraGreen (Amersham Pharmacia Biotech; Freiburg, Germany) and band intensities were analyzed using the Storm 840 Imager (Molecular Dynamics; Krefeld, Germany). The relative amount of MDR1 mRNA was expressed as the ratio of MDR1 signal to GAPDH signal.

### ISH of MDR1

Non-radioactive ISH was performed in paraffin sections (7 μm) that had been fixed in 4% paraformaldehyde. Sections

were rehydrated and permeabilized by pepsin digestion (750 μg/ml pepsin in 0.2 M HCl, 37C, 30 min) and then post-fixed (paraformaldehyde 4%, 20 min, 4C) and acetylated using 0.25% acetic anhydride in triethanolamine (0.1 M, pH 8.0, 15 min). After dehydration in ethanol (70, 95, 100%), sections were hybridized for 16 hr (56C) in a solution containing 25% formamide, 0.3 M NaCl, 10% dextran sulfate, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 1 × Denhardt's, 0.1 ng/ml herring sperm DNA, 0.5 mg/ml tRNA, 0.1 mg/ml polyuridylic acid, and 125 ng digoxigenin (DIG)-labeled MDR1 cRNA probe (position 471–763 of the human MDR1 cDNA). The corresponding sense cRNA probe served as negative control.

After washing with 50% formamide in 75 mM NaCl, 7.5 mM sodium citrate, pH 7.0, sections were incubated with RNase A followed by additional washing steps and incubation with blocking reagent (Roche; Mannheim, Germany). Bound riboprobe was visualized by incubation with alkaline phosphatase-conjugated anti-DIG antibody (Roche) and subsequent substrate reaction using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride.

### IHC of MDR1

From each heart tissue sample, paraffin sections of 2.0 μm were prepared by standard methods. Staining was performed using the Ventana's NexES IHC Staining System (Ventana Medical Systems; Frankfurt, Germany). For immunostaining, the monoclonal anti-P-gp antibody JSB-1 (mouse, dilution 1:20; Alexis Biochemicals, Grünberg, Germany) was used. The secondary antibody was rabbit anti-mouse diluted 1:100 included in the ABC Detection Kit (Ventana Medical Systems). Renal tissue samples were used as positive controls.

For semi-quantitative evaluation the numbers of specifically stained capillaries in relation to the total numbers of capillaries were determined in DCM, ICM, and NF heart tissue sections.

### Statistical Analysis

The amounts of specific mRNA for human P-gp were compared using the Mann–Whitney U-test; $p<0.05$ was considered significant. Expression in dependence of MDR1 genotype was compared by the Kruskal–Wallis trend test. All data are presented as mean ± SD.

## Results

P-glycoprotein was detected by IHC in all heart tissue samples tested. Immunostained heart tissue sections revealed P-gp localization predominantly in endothelial cells of capillaries and arterioles (Figure 1A). Staining intensity showed wide inter-individual variability. Tissue samples from hearts with dilated cardiomyopathy exhibited less staining compared to ischemic cardiomyopathic or non-failing hearts (Figure 1B; $p<0.05$). Endothelial localization of P-gp in capillaries and arterioles was confirmed by ISH (Figures 1C and 1D).

Real-time RT-PCR detected expression of MDR1-specific mRNA in all heart samples. As calculated by comparison with standard curves (MDR1, $y = -2.1303x + 38.275$; 18S rRNA, $y = -2.434x + 28.009$) generated from LMDR1 total cellular RNA, ratios of MDR1 to 18S rRNA copy numbers were 0.84 ± 0.22 (NF), 1.15 ± 0.62 (ICM), and 0.41 ± 0.13 (DCM). Patients with DCM had significantly lower MDR1 mRNA expression levels compared to non-failing hearts ($p=0.016$; Figure 2). Results were confirmed using conventional RT-PCR. Standardized for the housekeeping gene GAPDH, MDR1 mRNA levels of patients with DCM were significantly lower ($p=0.032$) compared to NF hearts (Figure 3).

MDR1 C3435T genotype prevalence of the samples did not differ significantly from the known MDR1 frequency in whites (Cascorbi et al. 2001). One DCM subject was an MDR1 3435CC genotype carrier and two were 3435CT. Among ICM samples, one was CC, three were CT, and one was TT. Three of the NF samples had the CC and two the CT genotype. MDR1 genotype at the MDR1 exon 26 C3435T locus did not significantly affect P-gp expression or MDR1 mRNA levels, although a tendency to lower expression of P-gp in samples homozygous for the TT genotype was observed [CC 0.79 ± 0.32, $n=5$; CT 0.89 ± 0.59, $n=7$; TT 0.60 ± 0.49, $n=3$; $p=0.39$ (Kruskal–Wallis)].

## Discussion

Here we describe expression and localization of the ABC transporter P-gp in human heart. P-gp was detected at the mRNA and protein levels in all 15 left ventricular samples. Both IHC and ISH localize P-gp expression to cardiac arterioles and capillaries. We observed a wide interindividual variability of P-gp. Various factors may be responsible for this phenomenon. Several mutations were described in the P-gp encoding MDR1 gene, some of which had functional consequences (Hoffmeyer et al. 2000; Cascorbi et al. 2001). For example, the synonymous mutation C3435T in exon 26 resulted in reduced intestinal expression of P-gp which, in turn, increased the bioavailability of digoxin. In our study we did not detect an association of genotype and cardiac P-gp expression. Although a reduced expression in samples containing the TT genotype of the C3435T mutation was observed, this effect was not statistically significant, comparable to the work by Hoffmeyer and co-workers (2000). This observation is supported by recent results in placenta trophoblast, showing decreased P-gp expression in cases carrying G22677A/T which is, in turn, known to be linked to C3435T (Tanabe et al. 2001).

Moreover, the process of disease may affect individual expression of P-gp. In our study we observed lower expression in patients with DCM compared to ICM and NF. This result was consistent at both the

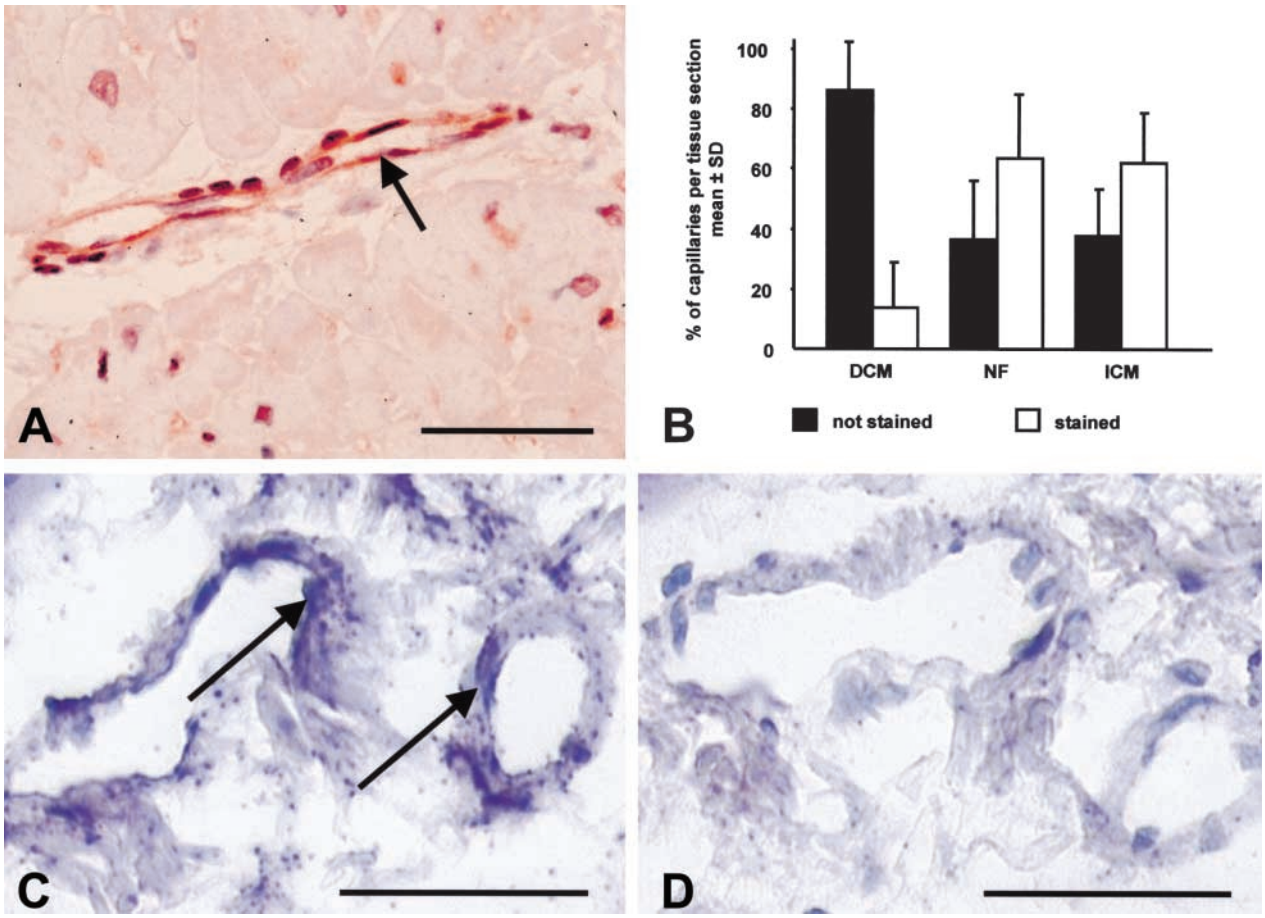
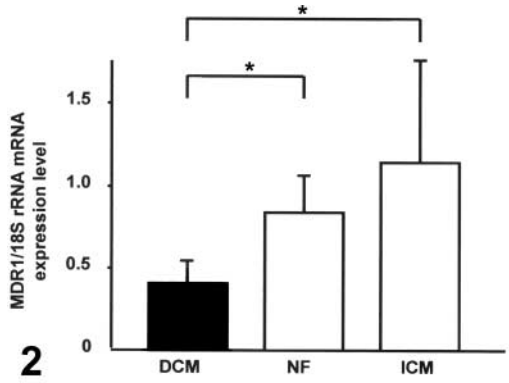
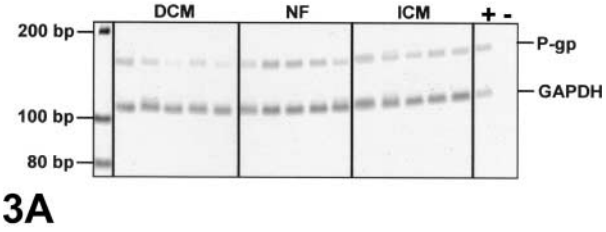



**Figure 1** Immunohistochemistry and ISH of P-gp in paraffin sections from NF. (**A**) P-gp is immunostained in endothelial cells of capillary (arrow); anti-P-gp JSB 1. (**B**) Relation of stained and non-stained capillaries in paraffin sections of NF heart, DCM heart, and ICM heart; $n=5$ for each group. (**C**) Antisense P-gp cRNA probes localize MDR1 mRNA in endothelial cells of capillaries in situ (arrows); methyl green. (**D**) Sense probes served as controls. Bars = 100μm.

protein and mRNA levels and cannot be attributed to drug therapy because there were no differences in drug therapy between DCM and ICM patients. Two different PCR approaches (conventional vs real-time) employing different housekeeping genes confirmed the observation of reduced P-gp in patients with DCM. Heart failure caused by DCM is believed to be mediated by persistent viral infection or autoimmunity, and a variety of antibodies directed against structural components of human heart have been identified. For example anti-β1-autoantibody treatment of isolated cardiomyocytes leads to decreased expression of β-adrenergic receptor protein and mRNA, similar to patients with DCM (Podlowski et al. 1998). Therefore, it appears possible that anti-P-gp antibodies may lead to reduced expression in patients with DCM.

Expression of P-gp in cells of human heart vessels is similar to that of P-gp in brain (Vogelgesang et al. 2001). The functional consequences of P-gp expression in the blood–brain barrier have been addressed in detail [restricted drug entry into the CNS (Schinkel et al. 1996)]. Therefore, we expect P-gp to serve as a functional barrier between blood and cardiac myocytes. High cardiac expression of P-gp can reduce uptake of drugs that are P-gp substrates into heart tissue and may therefore act as an important modulator of drug effects. Therefore, expression of P-gp in human heart can have important therapeutic implications. The cardiac glycoside digoxin has been unequivocally identified as a substrate for P-gp. The positive inotropic effects of digoxin exhibit a substantial inter-individual variability (Dobbs et al. 1987), and cardiac P-gp may be one factor contributing to this effect. Moreover, several β-blocking drugs have been described as P-gp substrates (e.g., talinolol, celiprolol; Karlsson et al. 1993; Westphal et al. 2000). Administration of β-blockers in patients with heart failure has been established as standard therapy and, again, therapeutic effects show a wide inter-individual variability, which may in part be explained by expression of drug transporters in human heart.



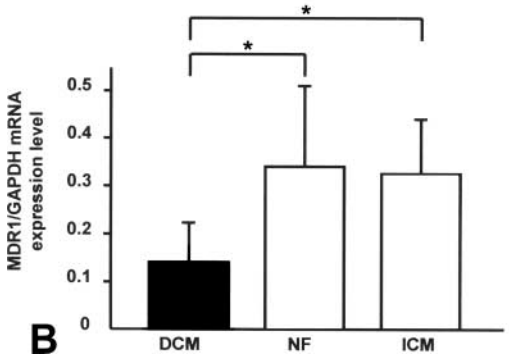




**Figure 2** Analysis of P-gp mRNA expression in human heart tissue by real-time RT-PCR. Relative amount of P-gp mRNA determined as ratio P-gp:18S rRNA. $n=5$ for each group; $*p<0.05$; NF, non-failing heart; DCM, dilated cardiomyopathy; ICM, ischemic cardiomyopathy.

**Figure 3** Analysis of P-gp mRNA expression in human heart tissue by conventional RT-PCR. (**A**) Vistra Green-stained polyacrylamide gel; PCR products of non-failing heart (NF), hearts with dilated (DCM), and ischemic cardiomyopathy (ICM). (**B**) Relative amount of P-gp mRNA was determined as ratio P-gp:GAPDH PCR product. $n=5$ for each group; $*p<0.05$.

Expression of P-gp may also protect heart tissue against cardiac toxicity of certain drugs. The anthracycline doxorubicin is a P-gp substrate [expression of P-gp in cancer cells results in chemoresistance to anthracyclines (Salmon et al. 1989)]. Doxorubicin is known to produce dose-dependent heart failure in patients treated for neoplastic disease (Goorin et al. 1990; Lipshultz et al. 1991) and is known to accumulate in hearts of mice lacking MDR1 (van Asperen et al. 1999). A recent study in children described a reduced ejection fraction after doxorubicin therapy, even with low cumulative doses (Agarwala et al. 2000). The authors observed substantial interindividual variability in cardiac toxicity of doxorubicin, a fact readily explainable by variable expression of P-gp.

In summary, we describe expression of the ABC-transporter P-glycoprotein in human heart. Our findings may have implications for drug therapy (alteration of both effects and toxicity). Moreover, our data point to reduced expression in patients with DCM. In view of recent data indicating cardiac expression of various cytochrome P450 enzymes (Thum and Borlak 2000) the contribution of the human heart to drug disposition should be evaluated.

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# Modulation of multidrug resistance P-glycoprotein 1 (ABCB1) expression in human heart by hereditary polymorphisms

Konrad Meissner[a,b], Gabriele Jedlitschky[a], Henriette Meyer zu Schwabedissen[a], Peter Dazert[a], Lothar Eckel[c], Silke Vogelgesang[d], Rolf W. Warzok[d], Michael Böhm[e], Christian Lehmann[b], Michael Wendt[b], Ingolf Cascorbi[a] and Heyo K. Kroemer[a]

***Objectives*** **Variable expression of the ABC-type multidrug resistance membrane protein P-glycoprotein (P-gp, MDR1, ABCB1) in human heart is a potential modulator of drug effects or drug-induced cardiotoxicity. Expression of P-gp is known to be affected by single nucleotide polymorphisms in the MDR1 gene. Therefore, genotype-dependent expression of P-gp could be an important modulator of action of cardiac drugs.**

***Methods*** **Heart tissue (auriculum) from 51 patients undergoing coronary artery bypass graft surgery was screened for genotype-dependent P-gp expression. P-gp was identified by immunoblotting and localized using immunohistochemistry. MDR1 mRNA was quantified by real-time PCR and immunohistochemistry and related to the MDR1 genotypes G2677T/A (Ala893Ser/Thr) and C3435T.**

***Results*** **MDR1/18S rRNA mRNA copy numbers in heart auriculum were $3.48 \pm 2.25 \times 10^{-6}$ compared to $4.56 \pm 0.58 \times 10^{-6}$ in non-failing ventricular samples studied before. While the exon 26 C3435T genotype did not influence MDR1 mRNA expression, we found significantly elevated MDR1 mRNA expression in 10 patients carrying the exon 21 2677 AT or TT genotype as compared to 12 patients carrying the GG-variant with intermediate MDR1 mRNA expression in 29 heterozygous samples. P-gp was detected in the endothelial wall. Quantitative immunohistochemistry of protein expression, however, did not reveal significant influence of the studied SNPs.**

***Conclusion*** **The present study based on auricular samples suggests that genetic factors play a rather limited role in modulating P-gp expression in human heart. Therefore, the substantial interindividual variability in cardiac P-gp expression is likely related to environmental or disease related factors.** ***Pharmacogenetics*** **14:381–385 © 2004 Lippincott Williams & Wilkins**

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**Keywords: human heart, P-glycoprotein expression, MDR1 genotype, genetic polymorphism**

[a]Peter Holtz Research Center of Pharmacology and Experimental Therapeutics, [b]Department of Anesthesiology and [d]Department of Pathology, Ernst-Moritz-Arndt-University Greifswald, Germany, [c]Department of Cardiothoracic Surgery, Klinikum Karlsburg, Germany and [e]Department of Cardiology, Saarland University, Homburg/Saar, Germany.

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Correspondence and requests for reprints to Heyo K. Kroemer, PhD, Institut für Pharmakologie, Ernst-Moritz-Arndt-Universität Greifswald, Friedrich-Loeffler-Str. 23d, D-17487 Greifswald, Germany.
Tel: +49-3834-865630; fax: +49-3834-865631;
e-mail: kroemer@uni-greifswald.de

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## Introduction

The ATP-dependent efflux pump ABC-type multidrug resistance membrane protein P-glycoprotein (P-gp, MDR1, ABCB1) transports a variety of substrates across membranes. Thereby, P-gp not only confers multidrug resistance to a variety of tumoral tissues, but also contributes to the function of physiological barriers, i.e. in brain and gut. The expression of P-gp in man reveals a wide inter-individual variability, which may in turn lead to variable drug effects. Such variability is of particular relevance if levels of P-gp substrates, i.e. cardiovascular, antiepileptic or human immunodeficiency virus (HIV) drugs, within the target tissues are crucial for intended therapeutic results. Aside from regulation by induction, the influence of genetic variations in the MDR1 gene on P-gp expression has been investigated, unveiling several single nucleotide polymorphisms (SNPs) with potential influence on P-gp function [1,2]. The issue is somewhat controversial since other groups did not identify a significant influence of specific SNPs in exon 21 (G2677T/A, coding for Ala893Ser/Thr) and in exon 26 (C3435T, non-sense) within the MDR1 gene on P-gp expression [3–5]. More recently, clear differences in clinical outcome for anti HIV and antiepileptic drugs have been related to MDR1 genotypes [6,7].

Among P-gp substrates are a number of cardiovascularly

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active compounds (e.g. beta-blockers, cardiac glycosides, doxorubicin). We recently demonstrated expression and localization of P-gp in human heart [8]. The significance of the MDR1 gene polymorphisms for cardiac P-gp expression levels, however, has not been systematically investigated so far. In the present study, we therefore investigated expression levels of MDR1 in human heart by means of real-time PCR, immunoblotting, and immunohistochemistry. Results were compared to recent data from ventricular tissue and correlated to the individual MDR1 genotypes at G2677T/A and C3435T, located in exon 21 and 26, respectively.

## Methods

### Tissue samples

After approval by the local ethics committee and obtained written informed consent, auricular heart tissue samples were taken from 51 patients undergoing coronary artery bypass graft surgery (Caucasians, 39 male, 12 female, 47–80 years old). Five ventricular samples from non-failing hearts (NF) were taken from excised heart left ventricle during orthotopic heart transplantation as described before [8]. Medical therapy of patients consisted of digitalis, diuretics, nitrates and angiotensin-converting enzyme inhibitors. No known pregnane X receptor (PXR) ligands affecting P-gp expression had been administered. Tissue samples were immediately frozen in liquid nitrogen or fixed in 4% paraformaldehyde for subsequent paraffin embedding. The frozen tissue was homogenized using a vibration grinding mill for RNA isolation (Mikro-Dismembrator, Braun, Germany).

### Reverse transcriptase–PCR of MDR1 mRNA

Two hundred ng of total RNA, prepared from 50 mg frozen tissue homogenate (RNEasy mini extraction kit, Qiagen, Hilden, Germany), were reversely transcribed using random hexamers and the TaqMan reverse transcription reagents (Applied Biosystems, Weiterstadt, Germany). PCR of MDR1 and 18S rRNA was performed using the intron-spanning primers MDR1F 5′-TTCGCAACCCCAAGATCCTC-3′, MDR1R 5′-ACAATGGTGGTCCGACCT TT-3′ and the probe 5′-6FAM-ATCCAGAGCCACCTGAACCACTGC T TAMRA-3′ as well as the pre-developed ribosomal RNA control reagents (Applied Biosystems) for 18S rRNA and the universal PCR mastermix (Applied Biosystems). PCR products were amplified (50°C, 2 min; 95°C, 10 min; followed by 40 cycles of 95°C, 15 s and 60°C, 1 min) and analysed on a real-time PCR cycler (ABI Prism 7700, Applied Biosystems). Fluorescence threshold cycles ($C_T$) of each sample were compared to the $C_T$ values of the standardization series, which consisted of the cloned MDR1 PCR-fragment in pGem-Teasy (Promega) resulting in a quantification of mRNA copy numbers. MDR1 mRNA expression levels were normalized with respect to the expression levels of 18S rRNA.

### MDR1 genotype

Genomic DNA was prepared from whole blood using QIAamp whole blood kits (Qiagen) as recommended by the manufacturer. The genotype of each individual at the MDR1 G2677T/A and C3435T loci were determined by a PCR-based restriction fragment length polymorphism (RFLP) assay using *Bsr*I for G2677A, *Ban*I for G2677T, and *Sau3*AI for C3435T according to Cascorbi *et al.* [9].

### Immunohistochemistry

Tissue sections were cut, mounted on slides, dried overnight at 60°C, deparaffinized in xylene, and rehydrated with ethanol. Slides were then treated with 10 mM citrate buffer at pH 6.0. Endogenous peroxidase was blocked with 0.3% $H_2O_2$, and the biotin–streptavidin immunoperoxidase method with the LSAB HRP detection system (DAKO, Hamburg, Germany) was used for staining. For immunohistochemistry the monoclonal anti P-gp JSB-1 was used. This antibody is recommended for immunohistochemistry but not for Western blotting. JSB-1 recognizes a highly conserved cytoplasmic epitope of the human P-gp and cross-reacts with chinese hamster but not with mouse, rat or guinea pig P-gp (JSB-1, mouse, dilution 1:20, Alexis Biochemicals, Grünberg, Germany, and rabbit anti-mouse immunoglobulin G (IgG), dilution 1:100, Ventana Medical Systems, Tucson, AZ, USA).

For morphometric semiquantitative analysis of vascular P-gp expression, we assessed immunostained vessels in five high-magnification fields (0.2 $mm^2$ per field). The number of positive vessels was multiplicated with a score of staining intensity (1 = low, 2 = moderate, 3 = strong), which was consistent in multiple repetitive stainings.

### Immunoblot analysis

For immunoblotting, 100 $\mu$g protein of purified heart membranes [10] were loaded on a 7.5% sodium dodecylsulfate–polyacrylamide gel and blotted onto nitrocellulose. Membranes from MDR1-overexpressing cells (P388 leukemia cells, Alexis) were used as positive control. P-gp was detected using the monoclonal anti-P-gp antibody C219 (Alexis, mouse, dilution 1:500; secondary: horseradish-peroxidase-conjugated goat anti-mouse IgG, 1:2000, Bio-Rad, Munich, Germany) as a 170 kDa-protein in the heart membranes as well as in the control membranes. The C219 antibody recognizes an internal, highly conserved amino acid sequence and is not species specific. It is recommended for Western blots, but is known to cross-react with a ~200 kDa protein which migrates in the same position as myosin [11].

### Statistical analysis

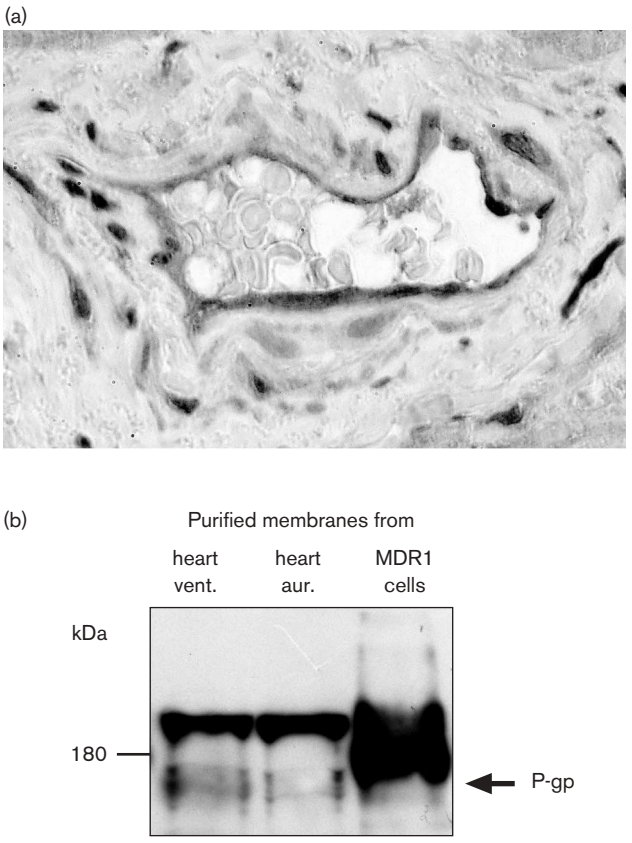
The amounts of specific MDR1 mRNA in relation to MDR1 genotype were compared by Mann–Whitney *U*-test and Kruskal–Wallis trend test using SPSS 11.0 software; $P < 0.05$ was considered significant. All data are presented as mean ± SD as indicated.

## Results

For human heart auriculum, immunohistochemistry localized P-gp predominantly in endothelial cells of capillaries and arteriols (Fig. 1a). P-gp as a 170-kDa-protein in human heart was confirmed by immunoblot analysis using the monoclonal antibody C219 (Fig. 1b).

MDR1 mRNA was detected in all 51 auricular heart tissue samples. MDR1 to 18S rRNA mRNA copy numbers in auricular tissue were $3.5 \pm 2.2 \times 10^{-6}$ compared to $4.6 \pm 0.6 \times 10^{-6}$ in five ventricular samples.

**Fig. 1**




Detection of P-glycoprotein in human heart auriculum. (a) Anti-P-gp JSB1 detects P-gp in endothelial cells of an arteriole, as visible from the staining of the luminal cells adjacent to vascular smooth muscle cells (original magnification ×200). (b) Immunoblot of P-glycoprotein in human heart membranes. Left lanes: purified membranes from human heart ventricular (ventr.) and auricular (aur.) tissue samples, respectively. Right lane: membranes from MDR1-overexpressing leukaemia cells. Anti-Pgp C219 detects P-gp as a double band at about 170 kDa in the heart ventricular as well as auricular membranes. The additional band at about 200 kDa in the heart membranes results from cross-reactivity of this antibody with a protein, which migrates in the same position as myosin [11].

Frequencies of MDR1 SNPs within the studied population were in agreement with published data [9]. We observed no significant influence of the exon 26 C3435T genotype on MDR1 mRNA expression (Fig. 2a). MDR1 mRNA expression was significantly elevated in 10 patients carrying the exon 21 2677 AT or TT genotype as compared to 12 homozygous wild-type individuals (Mann–Whitney *U*-test: $P = 0.01$) with intermediate expression in 29 heterozygous samples (Kruskal–Wallis trend test: $P = 0.02$, Fig. 2b). Haplotype analysis revealed about 80% linkage between the two loci as well as genotype dependence of MDR1 mRNA expression (ANOVA: $P = 0.03$), but did not show significant differences between haplotypes homozygous or heterozygous for both the 2677 and the 3435 SNP (Fig. 2c).

Since the low abundance and the localized expression did not allow for reliable quantitative immunoblot analysis, morphometric analysis of protein expression intensity was performed from quantitative immunohistochemistry. Due to the cross-reactivity of the C219 antibody with myosin [11], the monoclonal antibody JSB-1 was used in immunohistochemistry. The results indicate a pronounced inter-individual variability (81.6 ± 32.5 mean optical density). We did not observe a significant influence of the MDR1 genetic variants on P-gp staining intensity (ANOVA: $P = 0.88$, Fig. 2d).

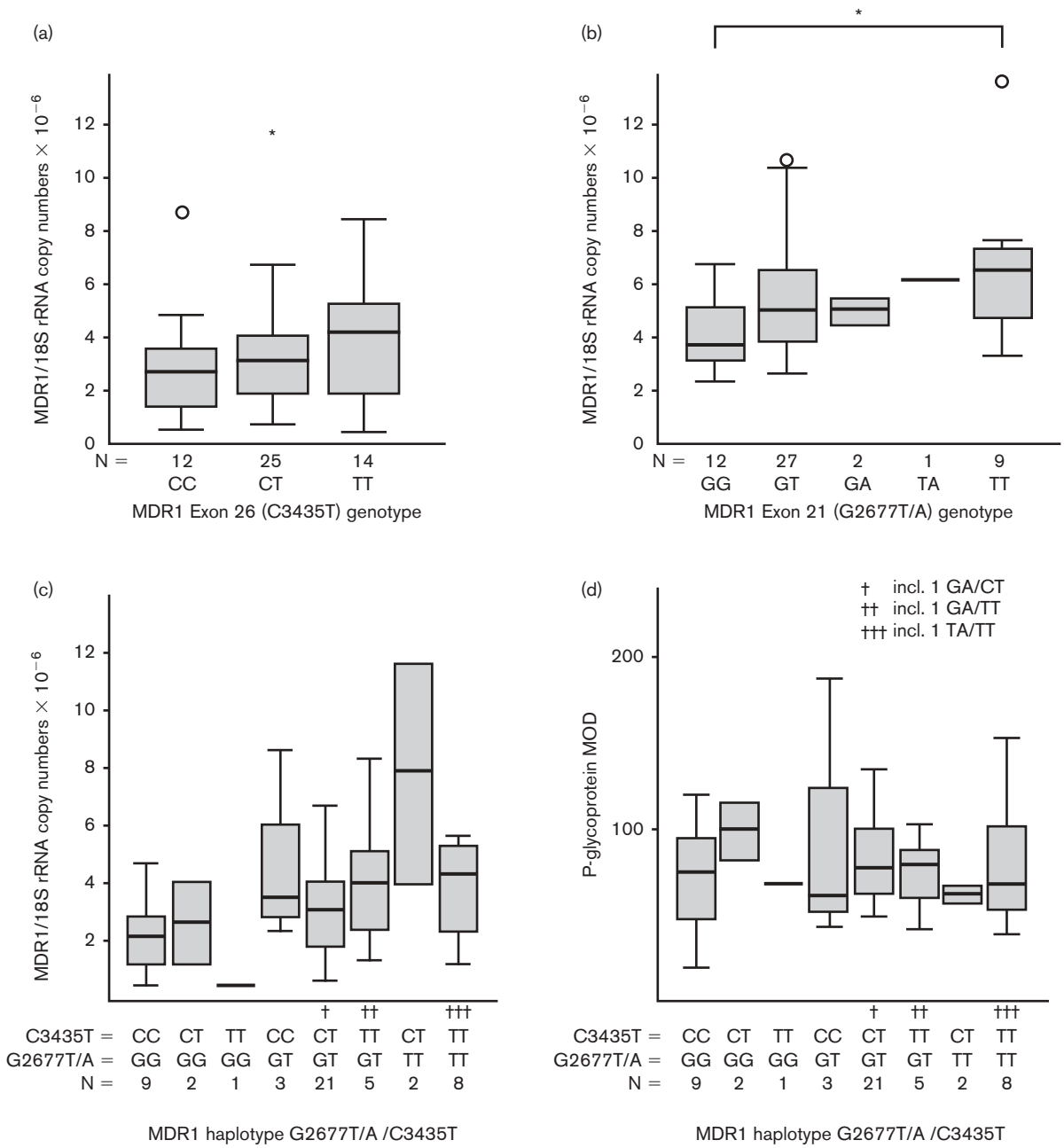
## Discussion

In this paper we describe expression of the ATP-dependent transmembrane transporter P-glycoprotein in human heart auriculum in relation to MDR1 genotypes.

High cardiac expression of P-gp potentially reduces uptake of drugs, which are P-gp substrates, into heart tissue and may therefore modulate drug effects. Thus, expression of P-gp in human heart can have important therapeutic implications not only for cardiac glycosides and several beta-blocking drugs [12,13] but also for anthracyclines, which are known to result in variable and dose-dependent heart failure in patients treated for neoplastic disease [14].

MDR1 mRNA was detected in all 51 auricular samples with a comparable abundance previously reported for ventricular samples. Accordingly, immunohistochemistry localized P-gp expression to arterioles and capillaries as described for ventricular tissue. We observed a wide inter-individual variability of MDR1 mRNA expression and there was no significant correlation between mRNA expression and P-gp staining intensity.

**Fig. 2**




Box plots of MDR1 mRNA expression in human heart auriculum in relation to MDR1 genotype (○, outerlayer; *, extreme). (a) MDR1 mRNA/18S rRNA copy numbers related to the genotype in exon 26 C3435T, (b) related to the genotype in exon 21 G2677T/A (*, $P < 0.05$), and (c) related to the haplotype for both, exon 26 and exon 21. (d) P-gp mean optical density (MOD), determined by means of quantitative immunohistochemistry, related to the haplotype for both, exon 26 and exon 21.

Several polymorphisms with functional consequences were described in the P-gp encoding MDR1 gene [9]. For example, the synonymous mutation C3435T in exon 26 resulted in reduced intestinal expression of P-gp, which in turn increased bioavailability of orally given digoxin [1], whereas rhodamine efflux from CD56 cells was lower in cells displaying the TT genotype in flowcytometry experiments, which is readily explained by decreased P-gp function [15].

In the present study we did not detect a significant association of the SNP in exon 26 (C3435T) to cardiac MDR1 mRNA expression, though a slightly increased MDR1 mRNA expression in auricular samples contain-

ing the 3435 TT genotype was observed (Fig. 2A). In contrast to results from human trophoblast [16], variants at the G2677T/A locus did not correlate with reduced, but with enhanced MDR1 mRNA expression in our experiments (Fig. 2b). Haplotype analysis did not indicate any significant differences between single 2677/3435 genotypes on MDR1 mRNA expression. However, the single individual carrying the 2677GG/3435TT genotype did express a rather low amount of MDR1 mRNA, consistent with data from Johne *et al.* [5], and except for this sample, other genotypes exhibited significantly higher MDR1 mRNA levels than the nine individuals homozygous for both wild-types (2677GG/3435CC, Fig. 2c). Therefore, based on mRNA levels, the influence of genotype on expression appears to be rather modest. It is therefore not surprising, that P-gp as assessed by quantitative immunohistochemistry did not reveal any influence of MDR-1 genotype (Fig. 2d).

In summary, we describe a limited influence of the MDR1 genetic polymorphism on tissue expression levels of the ABC-transporter P-glycoprotein in human heart based on auricular samples. Functional studies are required to assess the clinical implications of MDR1 genotype for cardiac effects and side effects of drugs.

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ARTICLE

# The ATP-binding Cassette Transporter ABCG2 (BCRP), a Marker for Side Population Stem Cells, Is Expressed in Human Heart

Konrad Meissner, Björn Heydrich, Gabriele Jedlitschky, Henriette Meyer zu Schwabedissen, Igor Mosyagin, Peter Dazert, Lothar Eckel, Silke Vogelgesang, Rolf W. Warzok, Michael Böhm, Christian Lehmann, Michael Wendt, Ingolf Cascorbi, and Heyo K. Kroemer

Department of Pharmacology, Peter Holtz Research Center of Pharmacology and Experimental Therapeutics (KM,BH,GJ,HMzS,IM,PD,HKK), Department of Anesthesiology (KM,CL,MW), Department of Pathology (SV,RWW), Ernst-Moritz-Arndt-University Greifswald, Germany; Department of Cardiothoracic Surgery, Klinikum Karlsburg, Germany (LE); Department of Cardiology, Saarland University, Homburg/Saar, Germany (MB); and Medizinische Klinik im Städtischen Krankenhaus (BH), Institut für Pharmakologie (IM,IC), Universitätsklinikum Schleswig-Holstein, Kiel, Germany

**SUMMARY** Efforts to improve severely impaired myocardial function include transplantation of autologous hematopoietic side population (SP) stem cells. The transmembrane ABC-type (ATP binding cassette) half-transporter ABCG2 (BCRP) serves as a marker protein for SP cell selection. We have recently shown that other ABC transport proteins such as ABCB1 and ABCC5 are differentially expressed in normal and diseased human heart. Here we investigated localization and individual ABCG2 expression in 15 ventricular (including 10 cardiomyopathic) and 51 auricular heart tissue samples using immunohistochemistry, confocal laser scanning fluorescence microscopy, and real-time RT-PCR. Individual genotypes were assigned using PCR–restriction fragment length polymorphism (RFLP) analysis and subsequently correlated to ABCG2 mRNA levels. ABCG2 was localized in endothelial cells of capillaries and arterioles of all samples. Ventricular samples from cardiomyopathic hearts exhibited significantly increased levels of ABCG2 mRNA (ABCG2/18S rRNA: $1.08 \pm 0.30 \times 10^{-7}$; $p=0.028$ (dilative cardiomyopathy) and $1.16 \pm 0.46 \times 10^{-7}$; $p=0.009$ (ischemic cardiomyopathy) compared with $0.44 \pm 0.26 \times 10^{-7}$ in nonfailing hearts). The individual haplotypes were not associated with altered mRNA expression. ABCG2 is variably expressed in endothelial cells of human heart, where it may function as a protective barrier against cardiotoxic drugs such as anthracyclines or mitoxantrone. ABCG2 expression is induced in dilative and ischemic cardiomyopathies. **(J Histochem Cytochem 54:215–221, 2006)**

**KEY WORDS**
ABC transporters
human heart
side population stem cells
BCRP
ABCG2 genotype

CELL DEATH AFTER CARDIAC ISCHEMIA or myocardial infarction results in long-lasting functional deficits of the heart. In recent years cellular transplantation or mobilization of endogenous cells has been studied to enable myocardial repair (Davani et al. 2005). Several different cell types have been investigated, among them skeletal myoblasts (Murry et al. 1996), bone marrow stem cells (Orlic et al. 2001), endothelial progenitor cells (Galli et al. 2005), and cardiac stem cells (Messina et al. 2004). Studies of hematopoietic stem cell (HSC) infusions for heart failure treatment have entered the clinical setting (Stamm et al. 2003), though mechanisms of the observed improved cardiac function are not fully understood. In particular, the question of whether these cells transdifferentiate into cardiac myocytes has been a subject of controversy (Alvarez-Dolado et al. 2003; Murry et al. 2004). It is increasingly evident that careful selection and characterization of the HSC used is of paramount importance. One specific population of HSC, the so-called side population (SP) stem cells are being selected for transplantation by their capacity to efflux compounds such as rhodamine 123 or Hoechst 3352 (Bunting 2002). In particular, the transmembrane ABC-type (ATP binding cassette) half-transporter ABCG2 (BCRP) has been identified to be involved in cellular export of Hoechst 3352. Therefore, ABCG2

Correspondence to: Heyo K. Kroemer, PhD, Institut für Pharmakologie, Ernst-Moritz-Arndt-Universität Greifswald, Friedrich-Loeffler-Str. 23d, D-17487 Greifswald, Germany. E-mail: kroemer@uni-greifswald.de

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may now serve as a marker protein for SP cell selection (Zhou et al. 2001).

ABCG2 belongs to the G-branch of the superfamily of ABC transporters. After binding and hydrolyzing ATP, these proteins are capable to transport a variety of xenobiotics and endogenous compounds across cellular membranes, thereby providing both protection against toxins and contributing to cellular signaling. Located at chromosome 4q22, the ABCG2 gene encodes a 655 amino acid polypeptide, which is classified as a half-transporter consisting of six putative transmembrane domains and one nucleotide binding domain. Recently, it has been reported that ABCG2 forms homotetramers to create the functional active protein (Xu et al. 2004). Besides the expression of ABCG2 in human cell lines from breast, colon, ovary, and gastric cancer, the distribution of ABCG2 has been located in human colon, small intestine, hepatic bile canaliculi, mammary gland, and the endothelia of veins and capillaries of heart (Maliepaard et al. 2001) and brain (Zhang et al. 2003). Aside from its expression in HSC and subsequent use as selection marker, ABCG2 has been found recently in mice cardiac progenitor cells, which exhibit stem cell characteristics in the adult heart and are distinct from endothelial cells (Martin et al. 2004).

We have recently described expression and function of other ABC transporters in human heart, namely ABCB1 (P-gp) and ABCC5 (MRP5). Although ABCB1 is thought to be involved in cellular export of drugs and toxins (Meissner et al. 2002, 2004), ABCC5 alters intracellular levels of cGMP (Dazert et al. 2003). In contrast, expression of ABCG2 in human heart has not been studied in detail. In view of the ongoing discussion of ABCG2 as a marker for stem cells, characterization of constitutive expression in human heart is of interest. In the present study, therefore, we assessed expression and localization of ABCG2 in human heart as functions of disease processes and genetic polymorphisms. We describe variable expression of ABCG2 in the endothelial wall of cardiac arterioles and capillaries and a significant increase of ABCG2 in patients with cardiomyopathies.

## Materials and Methods

### Tissue Samples

After approval by the local ethics committee and obtained written informed consent, auricular heart tissue samples were taken from 51 patients undergoing coronary artery bypass graft surgery (Caucasians, 39 male, 12 female, 47–80 years of age). The investigation is in line with the principles outlined in the Declaration of Helsinki. Medical therapy of patients consisted of cardiac glycosides, beta blockers, statins, diuretics, nitrates, and angiotensin-converting enzyme inhibitors. Ventricular samples were taken from excised heart left ventricle during orthotopic heart transplantation as described before (Meissner et al. 2002). In brief, samples from five nonfailing hearts were taken from the excised myocardium of potential donors without medical history or diagnostic evidence of heart disease, whereas the valves were taken and used for human homografts. Samples from patients with ischemic or dilative cardiomyopathy ($n=5$, each) were taken from hearts excised because of heart failure. Tissue samples were snap-frozen in liquid nitrogen or fixed in 4% paraformaldehyde. The frozen tissue was homogenized using a vibration grinding mill for RNA isolation (Mikro-Dismembrator; Braun, Germany).

### RT-PCR of ABCG2 mRNA

200 ng of total RNA, prepared from 50 mg frozen tissue homogenate (RNEasy mini extraction kit; Qiagen, Hilden, Germany), were reversely transcribed using random hexamers and the TaqMan reverse transcription reagents (Applied Biosystems; Weiterstadt, Germany). PCR of ABCG2 and 18S rRNA was performed using the intron-spanning primers hABCG2-F TATTACCCAT GACGATGTTA CCAAGTA TTA TATTTACCTG TA, hABCG2-R ATGGTGGCTT ATTCAGCCAG TTCCATGG, and the FAM-marked probe hABCG2-P TTCATGTTAG GATTGAAGCC AAAGGCA-GAT as well as the predeveloped rRNA control reagents (Applied Biosystems) for 18S rRNA and the universal PCR mastermix (Applied Biosystems). PCR products were amplified (50C, 2 min; 95C, 10 min; followed by 40 cycles of 95C, 15 sec, and 60C, 1 min) and analyzed on a real-time PCR cycler (ABI Prism 7700; Applied Biosystems). Fluorescence threshold cycles ($C_T$) of each sample were compared with the $C_T$ values of the standardization series, which consisted of the cloned ABCG2 PCR-fragment in pGem-Teasy (Promega; Mannheim, Germany) resulting in a quantification of mRNA copy numbers. ABCG2 mRNA expression levels were normalized with respect to the expression levels of 18S rRNA.

### ABCG2 Genotype

For studying the influence of single nucleotide polymorphisms (SNPs) on the amount of ABCG2-mRNA in human heart, the 51 auricular heart samples were genotyped for previously described hereditary variants. Genomic DNA (gDNA) was extracted using Qiagen Blood Kit as described by the manufacturer (Qiagen; Frankfurt, Germany). 100 ng of the gDNA were amplified using primers flanking the regions of previously described SNPs. Primers BCRPEx2f GATAAAAACTCTC-CAGATGTCTTGC and BCRPEx2r AGCCAAAACCT-GTGAGGTTCAC were used for amplification of a 287-bp fragment containing 34G>A in exon 2 and BCRPEx5f TGTTGTGATGGGCACTCTGATG and BCRPEx5r ATCA-GAGTCATTTTATCCACAC for a 222-bp fragment containing the 421C>A variant in exon 5. PCR was carried out in a volume of 25 µl containing 200 nM of each primer 10 × PCR buffer, 2 mM $MgCl_2$, 0,8 mM of dNTP-Mix, and 0.5 U TaqPolymerase. PCR products were restricted using sequence-depending endonucleases for 34G>A (BsrI) and 421C>A (HpyCH4 III) and visualized after electrophoretic separation in a 2% agarose gel and staining by ethidium bromide.

### Immunohistochemistry and Immunofluorescence

Tissue samples were fixed in formalin and embedded in paraffin. After cutting, sections were mounted on slides and

dried overnight at 60C. For immunohistochemistry, the sections were deparaffinized using xylene. After rehydratation in decreasing ethanol solutions, the slides were boiled in 10 mM citrate buffer (pH 6.0). The endogenous peroxidase was blocked with 0.3% $H_2O_2$, and the biotin-streptavidin immunoperoxidase method was employed using the LSAB HRP detection system (DAKO; Hamburg, Germany) and the monoclonal anti-BCRP antibody BXP-21 (Alexis Biochemicals; Grünberg, Germany) at a dilution of 1:40. For immunofluorescence, the slides were blocked with 5% fetal calf serum (FCS) in PBS after rinsing with PBS (pH 7.4). The slides were then incubated with the BXP-21 antibody (Alexis) at a dilution of 1:50 overnight in a humidified atmosphere. For double staining of endothelial structures, an anti-PECAM antibody was used at a dilution of 1:100 (anti-PECAM; Sigma, Munich, Germany). After several rinses with PBS, the slides were blocked again with 5% FCS in PBS and then incubated with the respective secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes; Eugene, OR). For staining of the nuclei, DNA was counterstained with TOTO-3 iodide (Molecular Probes) and was added to the mounting medium in a dilution of 1:2000. After several washing steps with PBS, the slides were mounted with anti-fading mounting medium. Fluorescence micrographs were taken with a confocal laser scanning microscope (Chromaphor Analysen-Technik; Duisburg, Germany). Samples were observed with a Nikon inverted microscope and a 100 × oil-immersion objective. A CCD camera and VoxCell scan software from VisiTech International (Sunderland, United Kingdom) were used for analysis.

### Immunoblot Analysis

For immunoblotting, purified heart membranes were prepared by differential centrifugation and purification on a sucrose gradient. 50 μg protein were loaded on a 7.5% SDS gel and blotted onto nitrocellulose as described previously (Dazert et al. 2003). Membrane vesicles from human placenta (50 μg) were used as positive control. The expression of BCRP was detected by chemiluminescence using the monoclonal BXP-21 antibody (diluted 1:1000; Alexis Biochemicals) and a horseradish-peroxidase-conjugated goat-anti-mouse IgG (diluted 1:2000; Bio-Rad, Munich, Germany).

### Data Analysis

Patient data of heart auricular samples were screened for potential influence of the individual's medication, sex, body mass index, blood pressure, and ABCG2 mRNA expression. The amounts of specific ABCG2/18s rRNA mRNA in relation to ABCG2 genotype as well as to cardiomyopathy were compared by Mann-Whitney U-test using SPSS 11.0 software; $p<0.05$ was considered significant. All data are presented as mean ± SD as indicated.

## Results

### Localization of BCRP (ABCG2)

Immunohistochemistry and Immunofluorescence using confocal laser scanning microscopy localized BCRP predominantly in endothelial cells of capillaries and arterioles of human heart tissue for both ventricular and auricular samples. Colocalization experiments displayed simultaneous staining of the endothelial marker PECAM and ABCG2 in the endothelial wall (Figure 1). In contrast to the ABC transport protein ABCC5, the surrounding vascular smooth muscle fibers were devoid of BCRP. However, some staining of the plasma membrane of heart muscle fibers was also detected.

### Immunoblot Analysis of BCRP (ABCG2)

Monoclonal anti-BCRP BXP-21 identified BCRP by Western blotting as a 70-kDa protein in both purified auricular and ventricular heart membranes. Samples from human placenta served as controls (Figure 1C, inset).

### ABCG2 mRNA Expression in Human Heart Samples

In a series of 51 samples from human heart auriculum, ABCG2 mRNA was detected in all samples with substantial interindividual variability (ABCG2/18S mRNA range from 0.3 to $10.3 \times 10^{-7}$ mean value $3.6 \pm 1.7 \times 10^{-7}$ Figure 1C, inset).

For investigating the influence of ischemic and dilative cardiomyopathy on the expression of ABCG2 mRNA, 15 ventricular human heart tissue samples were analyzed by means of real-time RT-PCR and related to 18S rRNA expression. Again, ABCG2 was detectable in all samples. ABCG2/18S rRNA expression in samples from cardiomyopathic hearts was significantly increased compared with that of nonfailing hearts for both dilative (ABCG2/18S rRNA: $1.08 \pm 0.30 \times 10^{-7}$; $p=0.028$) and ischemic cardiomyopathy ($1.16 \pm 0.46 \times 10^{-7}$; $p=0.009$) when compared with nonfailing hearts ($0.44 \pm 0.26 \times 10^{-7}$, Figure 2A).

### Influence of Patient Characteristics and Medication on ABCG2 mRNA Expression

ABCG2 mRNA was not influenced by patients' age, sex, or body mass index. Patient data were screened for common medications such as cardiac glycosides, beta blockers, statins, diuretics, nitrates, and angiotensin-converting enzyme inhibitors, which were rather common and prescribed in varying combinations to all individuals included in the study. None of the drugs or drug combinations had any significant influence on ABCG2 mRNA expression.

### Influence of the Genotype on ABCG2 mRNA Expression

Variant allele frequencies were 6% for the 34G>A and 14% for the 421G>A SNP ($n=51$). None of the SNPs alone or any of the haplotypes was associated

The Journal of Histochemistry & Cytochemistry

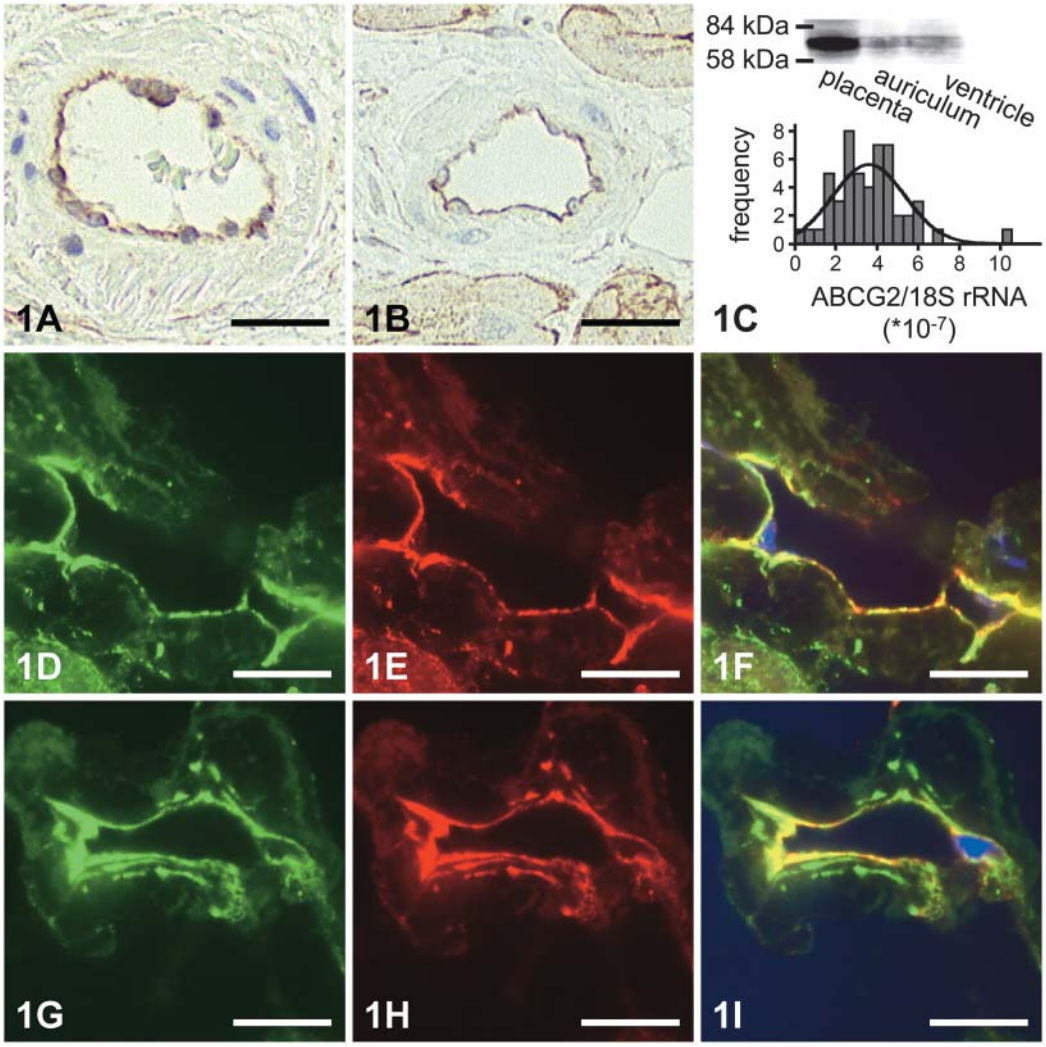


**Figure 1** Detection of BCRP (ABCG2) in human heart (bars: 25 μm). **(A)** Immunohistochemistry of heart auriculum: anti-BCRP BXP-21 detects ABCG2 in endothelial cells of an arteriole, as visible from the brown staining of the luminal cells adjacent to unstained vascular smooth muscle cells. **(B)** Immunohistochemistry of heart ventricle: anti-BCRP BXP-21 detects ABCG2 in endothelial cells of an arteriole, as visible from the brown staining of the luminal cells. The plasma membranes of some cardiomyocytes were stained in addition. **(C)** Frequency distribution of ABCG2/18S mRNA expression in 51 human auricular heart samples. Inset: Immunoblot of BCRP (ABCG2) in human heart membranes. Left lane: purified membranes from placenta (positive control); right lanes: purified membranes from human heart ventricular and auricular tissue samples, respectively. Anti-BCRP BXP-21 detects BCRP as a double band at 70 kDa in ventricular as well as auricular heart membranes. **(D–F)** Colocalization experiment for ABCG2 and the endothelial marker PECAM using confocal laser scanning microscopy of heart auriculum: **(D)** Anti-BCRP BXP-21 detects ABCG2 in endothelial cells of an arteriole, as visible from the green staining of the luminal cells. **(E)** The endothelial marker PECAM detects endothelial cells. **(F)** Merge of **D** and **E**: Endothelial cells appear yellow due to ABCG2 and PECAM overlay. **(G–I)** Colocalization experiment for ABCG2 and PECAM in heart ventricle. **(G)** Anti-BCRP BXP-21 detects ABCG2 in endothelial cells of an arteriole. **(H)** The endothelial marker PECAM detects endothelial cells. **(I)** Merge of **G** and **H**: Endothelial cells are stained by both ABCG2 and PECAM.

with significantly altered ABCG2 mRNA expression (Figure 2B).

## Discussion

ABCG2 has been suggested as a marker for specific selection of SP stem cells (Zhou et al. 2001). Aside from the selection process, such marker proteins are often used to determine the localization of transplanted cells within the heart. ABCG2 expression in SP stem cells has been shown to significantly decrease during cellular differentiation (Martin et al. 2004). In view of the present study indicating significant expression of ABCG2 in normal human heart, ABCG2 appears not to be suitable as a marker protein for intracardiac localization of such cells. We observed a continuous staining of endothelial cells of arterial and capillary vessels both in diseased and normal heart

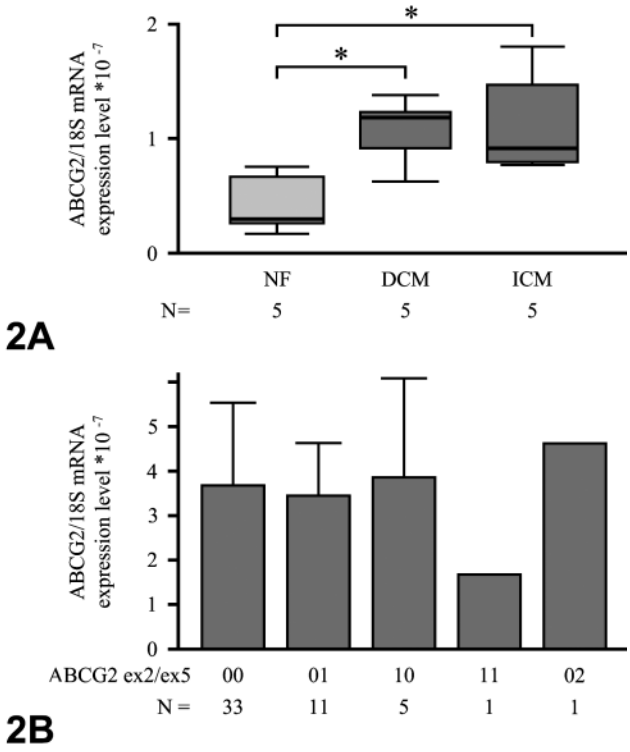


**Figure 2** (**A**) ABCG2 mRNA expression in human heart ventricular samples related to cardiomyopathies. NF: nonfailing heart, DCM: dilative cardiomyopathy, ICM: ischemic cardiomyopathy. ABCG2 mRNA expression is significantly enhanced in both cardiomyopathies compared with nonfailing heart (star: significant difference with $p<0.05$ according to Mann-Whitney U-test). (**B**) ABCG2 mRNA expression levels of human heart auriculum related to the individual haplotype including 34G>A and 421C>A. 00 = 34GG/421CC, 01 = 34GG/421CA, 10 = 34GA/421CC, 11 = 34GA/421CA, 02 = 34GG/421AA (mean values ± SD).

tissue, along with a staining of plasma membranes of some cardiomyocytes.

Localization of ABCG2 in endothelial cells in human heart is similar to that of ABCB1 (Meissner et al. 2002, 2004) but different from ABCC5, which is expressed in cardiac myocytes, smooth muscle cells, and endothelial cells (Dazert et al. 2003). The question arises whether the putative function of ABCG2 in human heart can be assessed based on its endothelial expression. Expression of ABCG2 has been described in the apical membrane of trophoblast cells of placenta, the apical membrane of enterocytes, the apical membrane of the lactiferous duct in mammary glands, in bile canalicular membranes of hepatocytes, and in the blood–brain barrier. Taken together, these sites of expression suggest a major role in detoxification because ABCG2 contributes to transport across the apical membranes of these cells, thereby protecting cells from drug effects and prohibiting entry of compounds into organ systems (Maliepaard et al. 2001).

In line with these findings, expression in endothelial cells in human heart could contribute to reduce or prevent cardiac uptake of ABCG2 substrates. Among the substrates of ABCG2 are a variety of cardiotoxic drugs such as mitoxantrone, topotecan, SN-38, and the HIV-1 nucleoside RT inhibitors ritonavir and saquinavir (Allen et al. 1999; Volk and Schneider 2003; Wang et al. 2003). Mitoxantrone, which, in addition to cancer chemotherapy, was recently introduced to multiple sclerosis therapy, has been described as cardiotoxic (Ghalie et al. 2002). Cardiac uptake of these substrates for ABCG2 can be prevented by endothelial expression, thereby modulating drug effects.

Our data indicate a pronounced interindividual variability of cardiac ABCG2 expression, which can be due to environmental factors (drugs, disease processes) or genetic influence. Aside from its role in drug resistance, regulation of ABCG2 expression is not well understood. We did not find any association between drugs administered and cardiac ABCG2 levels. A recent article indicated that hypoxia increases ABCG2 mRNA expression and thereby cell survival rates in cell culture experiments of hematopoietic stem cells (Krishnamurthy et al. 2004). Our previous work described a significant influence of disease on cardiac expression of ABCB1 (P-gp). Patients with dilative cardiomyopathy had a significantly reduced expression of this protein. In contrast, ABCG2 was upregulated in patients with both dilative and ischemic cardiomyopathy, indicating a differential effect of heart disease on drug transport proteins. In view of the coexpression of ABCG2 and ABCB1 at the apical site of various cells and some overlap in substrates, a combined role of both proteins in elimination of compounds from human heart can be assumed.

Although increased ABCG2 in vitro activity in cancer cell lines has been shown to be influenced by acquired mutations in the ABCG2 gene (Volk and Schneider 2003), the in vivo contribution of hereditary single nucleotide polymorphisms to ABCG2 expression levels is unclear. Among the four identified naturally occurring single nucleotide polymorphisms, 34G>A (V12M) and 421C>A (Q141K) were most common in diverse populations of different ethnic origin in North America (Zamber et al. 2003). Though the study unveiled a wide range of different individual ABCG2 mRNA expression levels, these data did not correlate to either BCRP protein expression or individual ABCG2 genotypes (Zamber et al. 2003). Transfection experiments in LLC-PK1 cells confirmed altered ABCG2 function for two common ABCG2 SNPs, which could in part be attributed to a different localization of ABCG2 within the cells (Mizuarai et al. 2004). However, the impact of SNPs within the coding region of the ABCG2 gene remains to be discussed controversially (Honjo et al. 2002). In contrast to the described inherited mutations,

The Journal of Histochemistry & Cytochemistry

acquired mutations of tumor cell lines were shown to confer resistance to a number of anticancer agents such as anthracyclines (Volk and Schneider 2003), whereas cells transfected with the wild-type were not (Honjo et al. 2001). Explicit search for the SNP in question (482R>T/G) did not unveil its occurrence in a population of 90 DNAs from the single nucleotide polymorphism discovery resource (Honjo et al. 2002).

Given a defense role of ABCG2 in men, influence on its functional state by anticancer drugs such as imatinib or gefitinib (Houghton et al. 2004; Stewart et al. 2004) may unintentionally lead to increased toxic effects of ABCG2 substrates on organs "protected" by ABCG2 expression in capillaries. Furthermore, ABCG2 expression in heart may be inhibited by antiretroviral drugs such as ritonavir or saquinavir (Gupta et al. 2004), thereby potentially damaging the heart, which was reported to be more susceptible to myocardial infarction after HIV therapy (Friis-Moller et al. 2003).

Taken together, our data indicate variable expression of ABCG2 in human heart endothelial cells, which is influenced by cardiomyopathies. Therefore, cardiac ABCG2 expression may be an obstacle for the detection of side population stem cells in stem cell therapy. Our data, based on 51 auricular heart samples, did not indicate a significant influence of the two studied most common SNPs in the ABCG2 gene on ABCG2 mRNA expression. However, a genetic influence on the transporter activity cannot be excluded. Moreover, cardiac ABCG2 expression suggests a protective role against substances entering the heart. Regulation as a consequence of diseases such as cardiomyopathies may alter susceptibility toward these substrates.

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The Journal of Histochemistry & Cytochemistry

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

Peter Dazert,* Konrad Meissner,*† Silke Vogelgesang,‡ Björn Heydrich,* Lothar Eckel,§ Michael Böhm,¶ Rolf Warzok,‡ Reinhold Kerb,‖ Ulrich Brinkmann,‖ Elke Schaeffeler,** Matthias Schwab,** Ingolf Cascorbi,* Gabriele Jedlitschky,* and Heyo K. Kroemer*

*From the Department of Pharmacology,* Peter Holtz Research Center of Pharmacology and Experimental Therapeutics, and the Departments of Pathology‡ and Anesthesiology,† Ernst-Moritz-Arndt-University, Greifswald; the Department of Cardiothoracic Surgery,§ Klinikum Karlsburg, Karlsburg; the Department of Cardiology,¶ Saarland University, Homburg/Saar; the Dr. Margarete Fischer-Bosch-Institut fuer Klinische Pharmakologie,** Stuttgart; and Pharmacogenetics Laboratories,‖ Epidauros Biotechnology, Bernried, Germany*

**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 [$^3$H]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 ± 38% of recombinant MRP5 standard) compared to normal ventricular samples (53 ± 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 μmol/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/ABCC1[6] 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 dysfunctions[17] 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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Address reprint requests to Heyo K. Kroemer, Ph.D., Institut für Pharmakologie, Ernst-Moritz-Arndt-Universität Greifswald, Friedrich-Loeffler-Str. 23d, D-17489 Greifswald, Germany. E-mail: kroemer@uni-greifswald.de.

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′-CACCATCCACGCCTACAATAAA-3′, MRP5R 5′-CACCGCATCGCACACGTA-3′, and the probe MRP5-TM, 5′-6FAM-GCTTGGTTGTCATCCAGCAGCTCCTG XTp (GenBank accession number: NM_005688); SMRP-F, 5′-AGGGCGTACACTCACGTAGCA-3′, SMRP-R 5′-ATGACCCTGGGCTTCGATCT-3′ and the probe SMRP-TM 5′-6FAM-CAGCCACTGAGGCTTCTGAGAGGGACTTTA-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 *AMF*AAAENKVAVKG 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 ± SEM |
|---|---|---|---|---|---|---|
| −1995 | Prom5 | GG | 44.4 | G | 69.4 | 3.1 ± 0.5 |
| | | GA | 50.0 | A | 30.6 | 3.4 ± 0.5 |
| | | AA | 5.6 | | | 5.4 |
| −1826 | Prom5 | TT | 45.0 | T | 70.0 | 3.0 ± 0.4 |
| | | TC | 50.0 | C | 30.0 | 3.4 ± 0.5 |
| | | CC | 5.0 | | | 5.4 |
| −1684 | Prom4 | TT | 80.0 | T | 90.0 | 3.5 ± 0.4 |
| | | TA | 20.0 | A | 10.0 | 2.9 ± 0.6 |
| | | AA | 0.0 | | | — |
| −1210 | Prom3 | CC | 23.8 | C | 54.8 | 3.9 ± 0.5 |
| | | CT | 61.9 | T | 45.2 | 2.9 ± 0.4 |
| | | TT | 14.3 | | | 4.3 ± 0.7 |
| −798 | Prom3 | CC | 81.0 | C | 90.5 | 3.4 ± 0.4 |
| | | CA | 19.0 | A | 9.5 | 2.9 ± 0.6 |
| | | AA | 0.0 | | | — |
| 36156 | Exon8 | AA | 33.3 | A | 64.3 | 3.1 ± 0.5 |
| | | AG | 61.9 | G | 35.7 | 3.2 ± 0.4 |
| | | GG | 4.8 | | | 5.4 |
| 36251 | Intron8 | GG | 85.7 | G | 92.9 | 3.4 ± 0.3 |
| | | GA | 14.3 | A | 7.1 | 3.0 ± 0.8 |
| | | AA | 0.0 | | | — |
| 39270 | Exon9 | TT | 21.1 | T | 55.3 | 3.7 ± 0.7 |
| | | TC | 68.4 | C | 44.8 | 3.0 ± 0.4 |
| | | CC | 10.5 | | | 4.2 ± 1.3 |
| 39557 | Intron9 | AA | 84.2 | A | 92.1 | 3.4 ± 0.4 |
| | | AG | 15.8 | G | 7.9 | 2.4 ± 0.4 |
| | | GG | 0.0 | | | — |
| 39563 | Intron9 | AA | 85.0 | A | 92.5 | 3.3 ± 0.3 |
| | | AC | 15.0 | C | 7.5 | 3.2 ± 1.1 |
| | | CC | 0.0 | | | |
| 39569 | Intron9 | wt/wt | 30.0 | wt | 60.0 | 3.2 ± 0.5 |
| | | wt/del | 60.0 | del | 40.0 | 3.1 ± 0.4 |
| | | del/del | 10.0 | | | 4.2 ± 1.3 |
| 39595 | Intron9 | GG | 85.0 | G | 92.5 | 3.4 ± 0.4 |
| | | GT | 15.0 | T | 7.5 | 2.4 ± 0.4 |
| | | TT | 0.0 | | | — |
| 50138 | Exon12 | TT | 23.5 | T | 55.9 | 3.8 ± 0.6 |
| | | TC | 64.7 | C | 44.1 | 3.1 ± 0.5 |
| | | CC | 11.8 | | | 4.9 ± 0.5 |
| 50423 | Intron12 | AA | 20.0 | A | 53.3 | 4.1 ± 0.7 |
| | | AG | 66.7 | G | 46.7 | 3.2 ± 0.5 |
| | | GG | 13.3 | | | 4.9 ± 0.5 |
| 66276 | Intron19 | GG | 38.1 | G | 66.7 | 3.0 ± 0.4 |
| | | GA | 57.1 | A | 33.3 | 3.4 ± 0.4 |
| | | AA | 4.8 | | | 5.4 |
| 74866 | Intron24 | GG | 38.1 | G | 66.7 | 3.0 ± 0.4 |
| | | GC | 57.1 | C | 33.3 | 3.4 ± 0.4 |
| | | CC | 4.8 | | | 5.4 |
| 75087 | Exon25 | CC | 85.7 | C | 92.9 | 3.3 ± 0.4 |
| | | CT | 14.3 | T | 7.1 | 3.3 ± 0.6 |
| | | TT | 0.0 | | | — |
| 97688 | 3′UTR | CC | 95.2 | C | 97.6 | 3.4 ± 0.3 |
| | | CG | 4.8 | G | 2.4 | 1.6 |
| | | GG | 0.0 | | | — |
| 97827 | 3′UTR | AA | 33.3 | A | 59.5 | 3.1 ± 0.5 |
| | | AG | 52.4 | G | 40.5 | 3.2 ± 0.5 |
| | | GG | 14.3 | | | 4.3 ± 0.7 |
| 97950 | 3′flanking | AA | 85.7 | A | 92.9 | 3.5 ± 0.3 |
| | | AC | 14.3 | C | 7.1 | 2.1 ± 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 (Novocastra, 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 $\mu$mol/L aprotinin, and 1 $\mu$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 Khananshvili and colleagues[32] 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 $\mu$mol/L aprotinin, and 1 $\mu$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 $\mu$g) were denatured and treated with peptide *N*-glycosidase F (PNGaseF) 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-$^3$H]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 $\mu$g protein) were incubated in the presence of 4 mmol/L ATP, 10 mmol/L $MgCl_2$, 10 mmol/L creatine phosphate, 100 $\mu$g/ml creatine kinase, and 4 $\mu$mol/L [$^3$H]cGMP, in incubation buffer containing 250 mmol/L sucrose and 10 mmol/L Tris/HCl, pH 7.4. The final incubation volume was 75 $\mu$l. Aliquots (20 $\mu$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-$\mu$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 [$^3$H]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 $\mu$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 $mm^2$ 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 ± 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 cells[5] used as 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 ± 22% recombinant MRP5 standard, $n = 21$) than the normal ventricular samples (53 ± 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 $\beta$-actin). Because the AMF antiserum de-

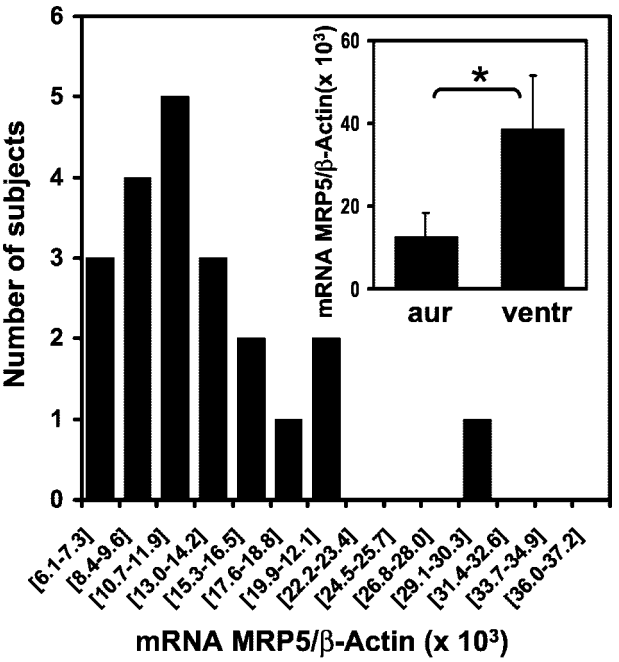


**Figure 1.** Histogram (frequency distribution) of MRP5 mRNA levels in auricular samples of human hearts. MRP5 mRNA levels were determined by real-time PCR and calculated as relative amounts of ratio MRP5/$\beta$-actin. **Inset**: Comparison of the MRP5 mRNA levels in the auricular (aur, $n = 21$) and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

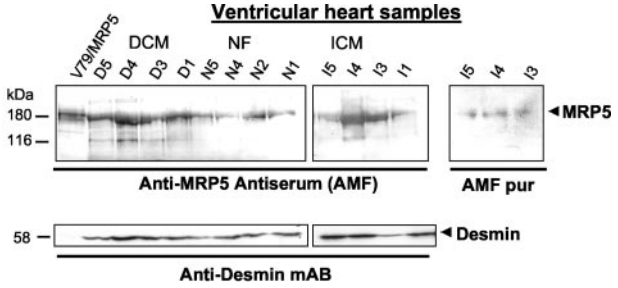


**Figure 2.** Immunoblot analysis of MRP5 in crude membrane fractions prepared from human ventricular heart samples. MRP5 was detected with the anti-MRP5 antiserum AMF (**top left**) and the affinity-purified AMF antibody (AMF pur, **top right**) as a 190-kd protein in membrane fractions (25 $\mu$g of 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 $\mu$g of protein) served as a positive control. **Bottom**: Detection of the muscle cell marker protein desmin on the same blot.

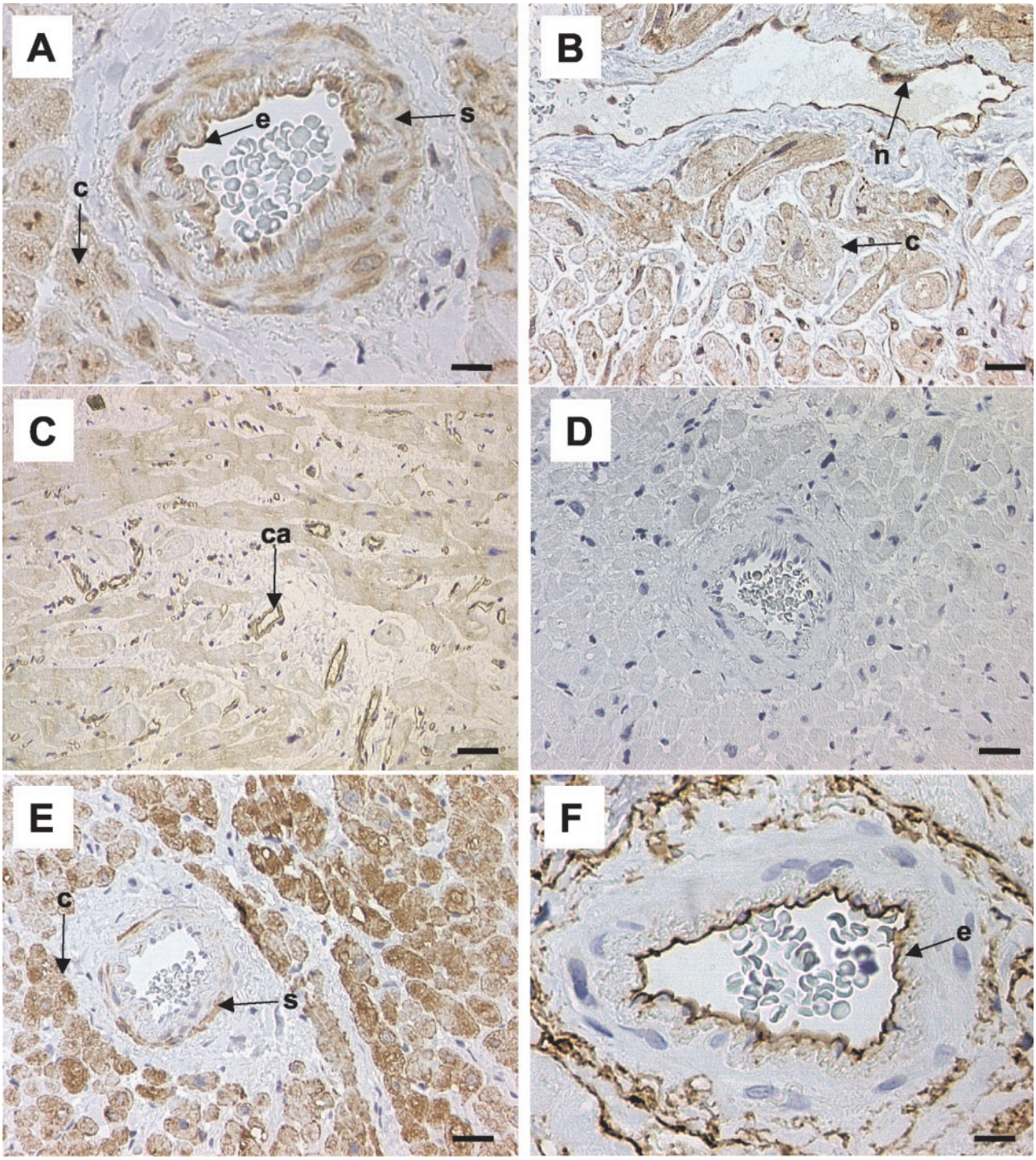


**Figure 3.** Immunohistochemical detection of MRP5 in human heart samples. **A–C:** Detection of MRP5 with the affinity-purified AMF antibody (**brown staining**). **Blue staining**: Counterstaining with hemalaun (nuclear staining). MRP5 staining was observed in endothelial and smooth muscle cells of 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 $\mu$m (**A**, **F**); 50 $\mu$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

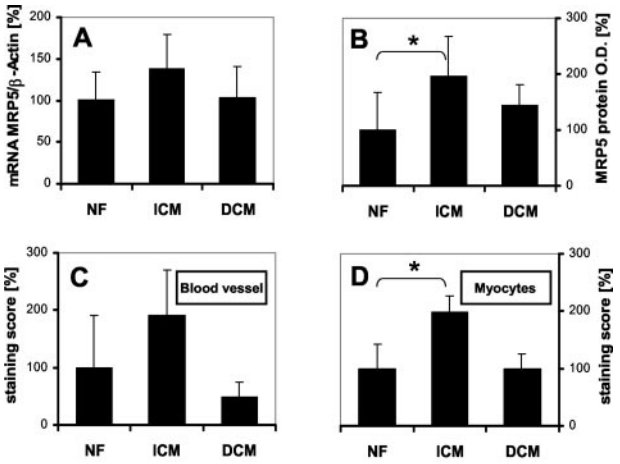


**Figure 4.** Relative MRP5 levels in ischemic (ICM) and dilated (DCM) cardiomyopathy compared to nonfailing hearts (NF). MRP5 mRNA levels (**A**) as well as protein levels detected by immunoblotting (**B**) or by immunohistochemistry (**C**, **D**) were quantified as described in Materials and Methods. 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 ± SD ($n = 5$ for each group; *, significant difference at $P < 0.05$). OD, optical density.

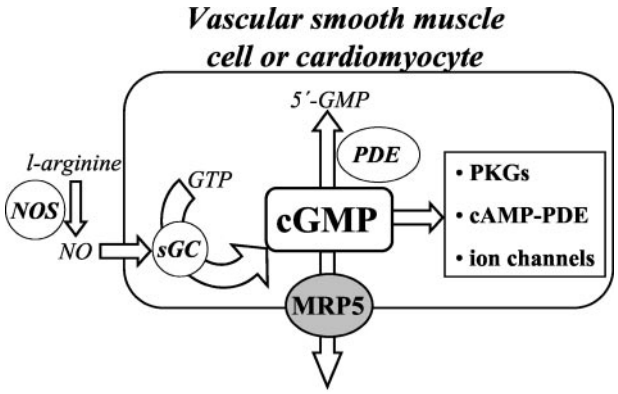


**Figure 6.** Possible involvement of MRP5 in the regulation of the function of cardiovascular smooth muscle cells and cardiomyocytes via the NO/cGMP-signaling pathway. NO is formed by nitric oxide synthases in cardiovascular endothelial cells, eg, in response to an ischemic stimulus, and diffuses to 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.

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).

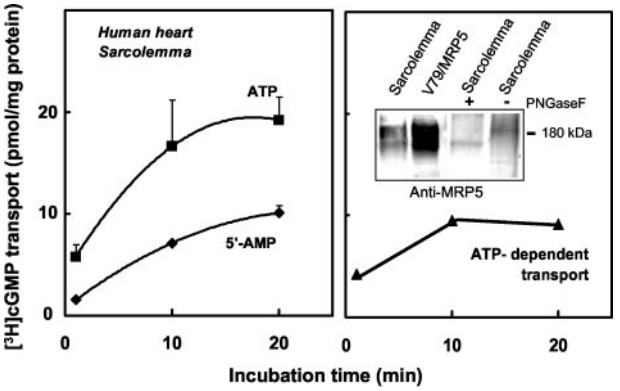


**Figure 5.** Transport of cGMP into sarcolemma vesicles from human heart. Membrane vesicles (140 μg of protein) were incubated with [$^3$H]cGMP (4 μmol/L) in the presence of 4 mmol/L ATP (■) or 4 mmol/L 5′-AMP (♦) (**left**) and the vesicle-associated radioactivity was determined as described in Materials and Methods (mean values ± 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 μg of total protein) and V79/MRP5 membrane vesicles (20 μg 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.

## *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 ± 38% of recombinant MRP5 standard) compared to the DCM (76 ± 20% of recombinant standard) and normal (NF) samples (53 ± 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 μmol/L, was 9.1 ± 1.1 pmol × mg protein$^{-1}$ at 20 minutes (mean value C SD, $n = 3$).

To determine whether the ATP-dependent uptake of [$^3$H]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 ± 1.7% (mean ± 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 [$^3$H]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 ± 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 colleagues[36] 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 colleagues[25] 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 before[30] 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 $Ca^{2+}$ channels[42] 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-$\kappa$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 colleagues[52] 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 line[27] 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 tissues[33] 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 MRP5[5] 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 pump[24] 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 monooxygenases[56] as well as of the ABC transporter MDR1/P-glycoprotein[26] 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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# SEPSIS AFFECTS CARDIAC EXPRESSION OF MULTIDRUG RESISTANCE PROTEIN 5 (MRP5, ABCC5), AN ABC-TYPE CGMP EXPORT PUMP

**Konrad Meissner,*† Wolfram Kessler,‡ Henriette E. U. Meyer zu Schwabedissen,† Katharina Schuster,† Kay Saalfeld,† Markus Grube,† Anna Buck,† Gabriele Jedlitschky,† Stefan Maier,‡ Tobias Traeger,‡ Jörg Mostertz,§ Georg Homuth,§ Claus-Dieter Heidecke,‡ Christian Lehmann,* and Heyo K. Kroemer†**

**Klinik für Anästhesiologie und Intensivmedizin, †Institut für Pharmakologie, ‡Klinik für Chirurgie, and §Zentrum für Funktionelle Genomforschung, Ernst-Moritz-Arndt-Universität Greifswald, Greifswald, Germany*

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ABSTRACT—One of the clinical characteristics associated with septic shock is heart failure. Several lines of evidence indicate that functional consequences of heart failure in septic shock are linked to the activated NO–cyclic guanosine monophosphate (NO-cGMP) pathway. We have previously shown that the high-affinity cGMP export transporter, multidrug resistance protein 5 (MRP5), is expressed in the heart, which modulates intracellular concentrations and, hence, the effects of cGMP. Thus, modified expression of cardiac MRP5 in septic shock can alter cGMP concentrations and contribute to the development of heart failure. We therefore investigated MRP5 expression in the heart using two established murine models of septic shock (intraperitoneal LPS injection and surgical implantation of a stent into the ascending colon, resulting in a multibacterial peritonitis [CASP, colon ascendens stent peritonitis] in C57BL/6N mice, respectively; n = 38). Cardiac MRP5 was assessed by quantitative polymerase chain reaction and immunofluorescence. The protein was localized in the endothelial wall, smooth muscle, and cardiac myocytes. MRP5 mRNA expression was significantly reduced compared with controls both in the LPS ($31.9 \pm 16.8 \times 10^{-4}$ vs. $54.1 \pm 14.8 \times 10^{-4}$, $P = 0.025$) and CASP model ($18.3 \pm 9.4 \times 10^{-4}$ vs. $42.8 \pm 12.1 \times 10^{-4}$, $P = 0.009$; MRP5/glyceraldehyde 3-phosphate dehydrogenase copy numbers, respectively). In parallel, IL-6 plasma levels were significantly increased in both models. Incubation of cultured murine cardiomyocytes (HL1) with 5 ng/mL IL-6 resulted in decreased expression of MRP5 (54% of control), as did incubation of the cells with serum from septic mice (LPS serum, 22% of control; CASP serum, 11% of control). In conclusion, cardiac expression of the cGMP export transporter MRP5 is decreased in two murine models of septic shock, most likely by a transcriptional mechanism. Reduced cGMP export as a consequence of decreased MRP5 expression can attenuate heart failure in sepsis.

KEYWORDS—Multidrug resistance, MRP5, cGMP transport, LPS shock, CASP model

## INTRODUCTION

Sepsis remains to be a major cause of death in intensive care patients (1). One example of organ dysfunction in sepsis and septic shock is heart failure caused by impaired cardiac contractility (2). In addition to apoptosis of cardiomyocytes secondary to endotoxin challenge (3, 4), the intracellular level of 3′,5′-cyclic guanosine monophosphate (cGMP) does have profound impact on the function of contractile cells such as cardiac myocytes as well as vascular smooth muscle cells (5, 6). The identified mechanisms leading to myocardial depression in sepsis and septic shock include an activated NO-cGMP pathway (7). Therefore, the mechanisms of cGMP homeostasis have been under investigation to explain physiologic processes as well as to identify potential therapeutic targets. High cGMP levels because of phosphodiesterase 5 (PDE5) down-regulation were found to impair cardiac contractility in heart failure (7). Moreover, the increase in intracellular cGMP in experimental sepsis could be also linked to increased cGMP synthesis via the NO-cGMP pathway, which was stimulated by the addition of proinflammatory cytokines (5). The regulation of PDE5 is in the view of current studies, because it may be a key instrument for preconditioning of cardiomyocytes (8, 9).

However, if the resulting intracellular cGMP levels are of crucial importance, active transport of cGMP by members of the ABCC branch of ATP-binding cassette (ABC) transporter superfamily represents an alternative way of cGMP elimination from cells. The ATP-dependent transport of substrates across membranes by this group of membrane-bound proteins has been initially described as a mechanism for multidrug resistance in cancer tissues and cells (10). Meanwhile, a wide range of physiological functions could be attributed to ABC proteins in organs with barrier functions such as the gut, liver, brain, and placenta (11). Moreover, endogenous compounds have been identified as high-affinity substrates of ABC transport proteins, such as cGMP for the multidrug resistance proteins 4, 5, and 8 (MRP4, MRP5, MRP8, ABCC4, ABCC5, ABCC11) (12). We recently identified MRP5 in human heart within cardiomyocytes as well as vascular smooth muscle cells (13). In addition to its localization, we demonstrated regulatory influence on MRP5 expression by ischemic cardiomyopathy. We concluded that there might be a

Address reprint requests to Heyo K. Kroemer, PhD, Institut für Pharmakologie, Universitätsklinikum Greifswald AöR, Fr.-Loeffler-Str. 23d, D-17475 Greifswald, Germany. E-mail: kroemer@uni-greifswald.de.

Present address: Department of Anesthesiology, Washington University in St. Louis, Barnes-Jewish Hospital, St. Louis, Missouri (Meissner); Department of Anesthesia, Dalhousie University, Halifax, Canada (Lehmann).

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substantial variability of substrate concentrations because of altered expression of MRP5. Given regulatory influence on MRP5 expression by cardiomyopathy, we ought to identify regulatory processes in situations of cGMP excess under septic conditions leading to heart failure.

The present study therefore investigated the influence of two established models of septic shock, namely, the LPS and the colon ascendens stent peritonitis (CASP) shock models, on cardiac MRP5 expression by means of quantitative polymerase chain reaction (PCR), immunoblotting, and immunofluorescence. For quality control, both models were analyzed for cytokine serum levels of IL-6, TNF-$\alpha$, and interferon $\gamma$ (IFN-$\gamma$) (14). Cardiomyocyte-derived mice HL1 cells (15) were exposed to pooled serum of septic mice, LPS, and the proinflammatory cytokines IL-6, IFN-$\gamma$, and TNF-$\alpha$ to study the effects of septic conditions on MRP5 *in vitro* and to further elucidate the underlying mechanisms.

## MATERIALS AND METHODS

### Animal samples

After approval by the local standing committee on animal research, 8- to 12-week-old female C57BL/6N mice (n = 38), weighing between 20 and 25 g (Charles River, Sulzfeld, Germany) were kept in a conventional animal facility at least 2 weeks ahead of the experiments. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All mice were anesthetized using 17 $\mu$L/g 2,2,2 tribromethanol and 2-methyl-2-butanol 2.5% (Avertin; Sigma-Aldrich, St. Louis, Mo) in phosphate-buffered saline (PBS) intraperitoneally. In a first set of experiments, hearts were excised, and blood samples were obtained after 5, 10, 15, and 20 h to identify the time course of MRP5 mRNA as well as cytokine expression. The animals were randomly distributed to the five study groups, and hearts were excised 10 h after injection or surgery, respectively. Groups consisted of five to nine animals. In a subseries of experiments, the left ventricular heart tissue of three additional animals per group was exclusively screened for RNA integrity.

### LPS and CASP shock models

For the LPS experiments, 8 $\mu$g/g LPS (L5886; Sigma-Aldrich) in PBS (containing 0.05% hydroxylamine) was injected intraperitoneally. Native as well as PBS-injected mice served as controls. For the multibacterial CASP experiments, a 16-gauge stent (Venflon; BOC, Ohmeda AB, Sweden) was surgically inserted into the ascending colon as described before (14). In brief, after a 1-cm midline incision, the ascending colon was exposed approximately 10 mm from the ileocecal valve, and the prepared catheter was stitched through the antimesenteric wall into its lumen and fixed with two stitches (7/0 Ethilon thread; Ethicon, Norderstedt, Germany). Consecutively, the inner needle of the stent was removed, and the stent was cut at the prepared site. To ensure proper intraluminal positioning of the stent, stool was milked from the cecum into the ascending colon and the stent until a small drop of stool appeared before closure of the abdominal walls (two layers, muscle and skin; 5/0 Ethilon thread). For control purposes, sham operations without puncturing the colonic wall were performed. Native animals served as controls for both shock models. The LPS dose and the stent diameter as well as the animals' sex were chosen to result in severe shock, which could be survived for 10 h to allow for mRNA studies. According to animal care guidelines, a score for the assessment of sepsis severity was used (16). The scores were based on clinical symptoms such as fur appearance, breathing, behavior, and activity. Scoring points were assigned to each item and then added up. Lethal outcome was assumed in all the animals of the two shock groups by mice revealing a score of more than seven points on a scale ranging from 0 to 12. The clinical status of the animals was completed by the analysis of the inflammatory markers IL-6 and monocyte chemaattractant protein-1, which were dramatically elevated in all animals enrolled in either one of the two shock groups.

### Cytokine analysis

After the animals were killed, blood was collected using heparinized syringes. Plasma was removed after centrifugation of the blood samples. The serum levels of IL-6, IFN-$\gamma$, and TNF-$\alpha$ were determined using a cytometric bead array detection kit according to manufacturer's instructions (CBA mouse inflammation kit; BD bioscience, Heidelberg, Germany).

### Confocal laser scanning microscopy

Tissue sections from the left ventricle were embedded in cryostat medium (Tissue-Tek; Sakura, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen. Sections of 5-$\mu$m thickness were dried at 60°C and incubated with the diluted antimouse MRP5 AMF antibody (1:100) in a humidified atmosphere at 4°C overnight. The unbound antibody was washed off by several washing steps with PBS. For double staining of endothelial structures, an anti-PECAM antibody was used at a dilution of 1:100 (anti-PECAM; Sigma, Munich, Germany). After several washing steps with PBS, the slides were blocked again with 5% fetal calf serum in PBS and incubated with the respective secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes, Eugene, Ore) for 1.5 h at room temperature. Then, the slides were washed and mounted in antifading mounting medium (DAKO, Hamburg, Germany) containing Toto 3-iodide dye (1:1,000) for DNA staining. Fluorescence micrographs were taken with a confocal laser scanning microscope (Chromaphor, Duisburg, Germany) using excitation wavelengths of 488, 568, and 647 nm. Samples were observed with a Nikon inverted microscope and a 100 × oil-immersion objective. A CCD camera and VoxCell scan software from VisiTech International (Sunderland, United Kingdom) were used for analysis.

### Cardiomyocyte culture

Immortalized mice heart muscle cells (HL1) were exposed for 24 h to pooled serum of septic mice (10% solution), PBS, and LPS (1 $\mu$g/mL) to determine the effects on heart muscle cell MRP5 mRNA expression. Moreover, 5 ng/mL IL-6, IFN-$\gamma$, and TNF-$\alpha$ were used to determine the individual effects on MRP5 expression. Untreated HL1 cells served as controls.

### RNA isolation and integrity analysis

The left ventricular heart samples were immediately frozen in liquid nitrogen after excision. After homogenization using a dismembrator (Braun, Melsungen, Germany), total RNA was isolated from 60-mg tissue powder using a total RNA isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After elution, RNA concentration and quality were assessed by absorbance measurement using a spectrophotometer (NanoDrop Technologies, Wilmington, Del). Calculation of the 260/280- and 260/230-nm ratios was performed for purity and extraction performance. Integrity of the prepared RNA was controlled by denaturing MOPS-agarose electrophoresis. In a subseries of experiments, RNA was isolated using the modified Trizol extraction method (17) to study the putative influence of the sepsis models on RNA integrity, which was analyzed using the lab-on-chip capillary electrophoresis technology (Bioanalyzer 2100; Agilent Technologies, Waldbronn, Germany) in combination with the software-based RNA integrity number classification algorithm (18).

### Isolation of RNA from cultured cells

After incubation with 250 $\mu$L of guanidine thiocyanate–containing buffer PeqGold RNApure (Peqlab, Erlangen, Germany), the cell lysate was collected. Chloroform, 100 $\mu$L, was added to the lysate, mixed, and incubated for 5 min at room temperature. After centrifugation for 15 min at room temperature at maximum speed, the clear supernatant was collected, and RNA was precipitated using 250 $\mu$L isopropanol. After incubation for 15 min at room temperature, the RNA was pelleted by centrifugation at maximum speed (10 min at room temperature). The RNA was washed twice with 75% ethanol, dried, and dissolved with 50 $\mu$L RNase-free water. The amount of RNA was determined as described above.

### Cell lysis and preparation of protein extracts from cells

Before harvesting by scraping off the dishes, cells were washed three times with prewarmed PBS. The detached cells were collected with 750 $\mu$L PBS and pelleted by centrifugation. The cells were resuspended in 5 mM Tris/HCl, pH 7.4, supplemented with protease inhibitors (1 mg/L aprotinin, 0.5 mg/L leupeptin, and 100 $\mu$M phenylmethylsulphonyl fluoride). To support cells lysis, the lysates were shock frosted in liquid nitrogen followed by rapidly thawing at 37°C four times. Thereafter, crude membranes were isolated by centrifugation for 45 min at 100,000 *g* and 4°C. The supernatant was collected, and the pellet was resolved in 5 mM Tris/HCl. Protein concentration was measured according to the bicinchoninic acid method. The separated crude membrane fractions were stored at −80°C.

### Real-time reverse transcriptase–PCR

Total RNA was reverse transcribed in a 25-$\mu$L reaction volume containing 500 ng RNA using the TaqMan Reverse Transcription Kit (Applied Biosystems, Weiterstadt, Germany) as described in the manufacturers' instructions. The amounts of MRP5 and (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) mRNA were measured by TaqMan quantitative real-time PCR with an ABI PRISM 7700 sequence detection system (Applied Biosystems). The sequence of primers and probe used for MRP5 mRNA analysis were as follows: forward primer,

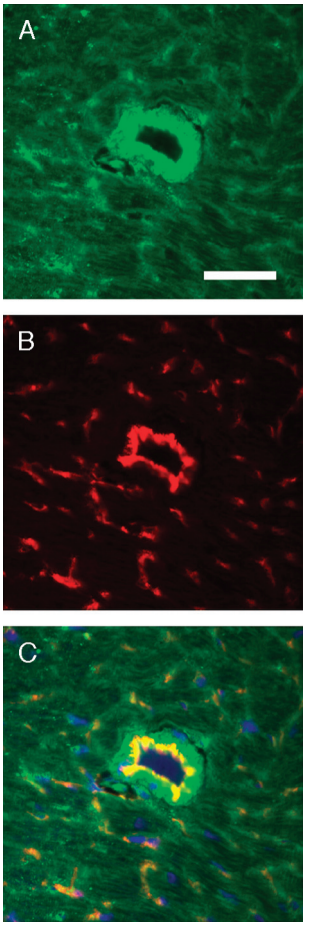


FIG. 1. **Confocal laser scanning microscopy of MRP5.** Anti-MRP5 AMF (green) was stained predominantly in the endothelial and vascular smooth muscle cells of a capillary and also within the surrounding cardiomyocytes (A–C). Anti-PECAM (red) identified endothelial tissue and was costained with MRP5 (yellow). Core staining, TOTO; bar length, 50 $\mu$m.

5′AAATGTATGCCTGGGTCAAAGC; reverse primer, 5′TGGCGATCACTAC-CACAATAGG; and FAM-labeled TaqMan probe, 5′AAAAAATCCGAGAGGA-GGAACGTCGGATA. GAPDH mRNA was analyzed using the TaqMan Rodent GAPDH Control Reagents (Applied Biosystems). Threshold cycles ($C_T$) of each sample and gene were compared with the $C_T$ values of the standardization series, which consisted of the cloned PCR fragment of mMRP5 or GAPDH (both in the pGem-Teasy cloning vector; Promega, Mannheim, Germany). Thereby start copy numbers for each probe and gene could be calculated.

### *Western blot analysis*

Protein extracts (membrane fraction) were incubated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer at 95°C for 10 min. The proteins were separated on a 10% acrylamide gel followed by electrotransfer onto a nitrocellulose membrane using a tank blotting system. Transfer of the proteins was controlled by Ponceau-S staining (Sigma). Thereafter, the membranes were incubated with 10% milk powder in Tris-buffered saline (0.5 M Tris, 1.5 M NaCl) supplemented with 0.1% Tween for blocking unspecific antibody binding. Incubation with the primary antibody was performed overnight at 4°C. The following antibodies were used: AMF antibody (generated against the C-terminal sequence of MRP5 at the Deutsches Krebsforschungszentrum, Heidelberg, and affinity purified as described previously (12, 13, 19)) diluted 1:1,000 for detection of MRP5 and mouse monoclonal antidesmin, clone DE-U-10 (Sigma), diluted 1:500 for the detection of desmin. Specific antibody binding was detected using ECL Plus Western blot detection reagent (Applied Biosystems). Luminescence was detected using an x-ray film, and densitometric evaluation was performed using the program ImageQuant (Molecular Dynamics, Piscataway, NJ).

### *Dual-Luciferase Reportergene Assay for the human ABCC5 promoter region*

A 4.2-kb fragment of the 5′ upstream regulatory region of the ABCC5 gene was amplified and cloned into the pGL3-Basic firefly luciferase reporter vector (Promega), and the correct sequence was analyzed by cycle sequencing. For transfection, HeLa cells were cultured in RPMI 1640 medium (10% fetal calf serum, 2 mM L-glutamine) and seeded in 96-well plates at a density of $5 \times 10^4$ cells per well. Then, cells were transfected with reporter constructs via FuGene Transfection Reagent (Roche, Mannheim, Germany) and treated for 24 h with 10 ng/mL IL-6, IL-6 + soluble IL-6 receptor, and PBS as solvent control, respectively. Each experiment was carried out by cotransfection of the pRL-CMV renilla luciferase vector (Promega), which was used for normalization. After lysis of the cells, luminescent signals generated by the Dual-Luciferase Reporter Assay System (Promega) were detected using a Victor$^2$ 1420 Multilabel Counter (Wallac, Boston, Mass). Data were calculated as ratio of firefly/renilla luminescence (control = 1).

### *Statistical analysis*

MRP5 mRNA levels of heart samples and HL1 cells were screened for potential influence of the respective shock model after 10 h. The amounts of specific MRP5/GAPDH mRNA in relation to shock type were compared using Mann-Whitney $U$ test for 2 samples and one-way ANOVA with Scheffé post hoc analysis for multiple comparisons using SPSS 11.0 software. $P < 0.05$ was considered significant. All data are presented as mean ± SD.

## RESULTS

MRP5 was found on mRNA as well as protein levels in all the hearts tested. MRP5 could be localized predominantly in endothelial cells, vascular smooth muscle cells, and cardiomyocytes, thus being similarly localized in mice and humans (13) (Fig. 1). MRP5 mRNA levels within the mouse heart tissue samples and cell culture experiments varied significantly according to the experimental intervention. The influence on both the MRP5 mRNA and the expression of proinflammatory cytokines were attenuated 10 h after initiation of the shock model (data not shown).

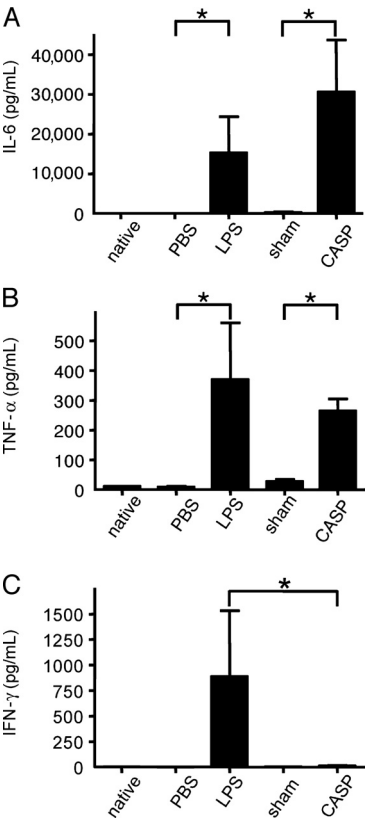


FIG. 2. **Cytometric bead array of proinflammatory cytokines in serum of mice.** IL-6 and TNF-$\alpha$ are both significantly increased in the LPS and the CASP model compared with its respective control and native animals (A and B), whereas IFN-$\gamma$ levels are elevated only in LPS-treated animals (C) (mean ± SD). Asterisk significant difference compared to control (A,B) or between the two applied shock models (C).

### *Inflammatory cytokine levels*

The intensity of the two shock models that were used could be evaluated by means of the serum levels of the inflammatory cytokines IL-6, TNF-$\alpha$, and IFN-$\gamma$. Neither the native animals nor the respective controls (PBS-injected and sham-operated animals) displayed remarkable serum levels of these cytokines, whereas cytokine levels were significantly elevated in both shock models with a peak after 10 h (Fig. 2). Whereas IL-6 and TNF-$\alpha$ levels reached similar levels in both models, IFN-$\gamma$ did only peak in the LPS experiments (Fig. 2C).

### *MRP5 mRNA expression in heart tissue*

In case of the RNA samples, which were analyzed to check for putative effects of the sepsis models on RNA integrity, the calculated RNA integrity numbers were between 8.3 and 8.9.

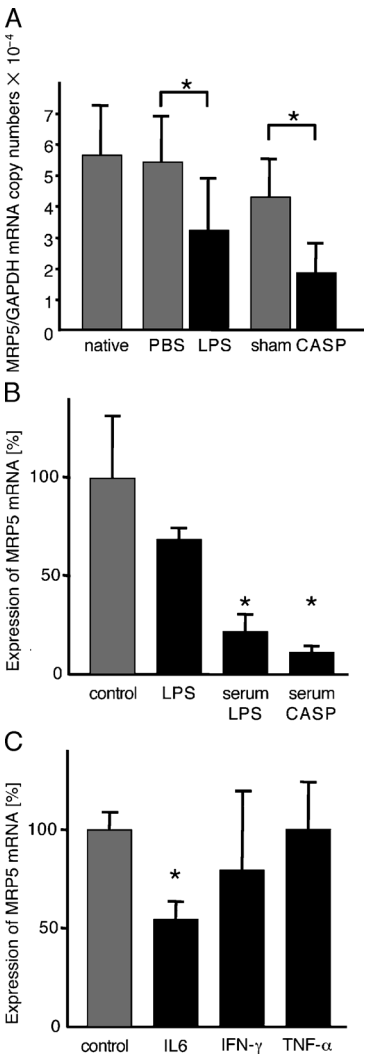


FIG. 3. **MRP5 mRNA expression.** MRP5 mRNA expression in heart tissue samples is significantly decreased in both the LPS and the CASP shock model compared with the respective controls as well as native animals (A). Concomitantly, exposure to pooled serum of septic animals down-regulates MRP5 mRNA expression of HL1 mouse cardiomyocytes compared with untreated cells and plain LPS (B). IL-6 was found to decrease MRP5 mRNA in HL1 cells (C), whereas IFN-$\gamma$ and TNF-$\alpha$ did not remarkably influence MRP5 expression in HL1 cells (mean ± SD). Asterisk significant difference compared to control (A,B) or between the two applied shock models (C).

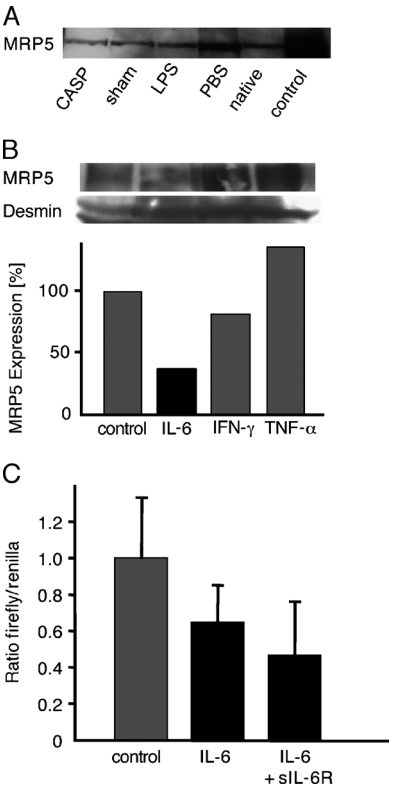


FIG. 4. **MRP5 regulation.** MRP5 is expressed in all heart tissue samples (control: transfected cells) and HL1 cell culture experiments as shown in Western blots (A, B). IL-6 exerts a depressing regulatory effect on HL1 MRP5 expression (B) as well as on MRP5 transcription as shown in a decreased firefly/renilla ratio in Dual-Luciferase Reportergene Assay for the human ABCC5 promotor region with and without the soluble IL-6 receptor component (C) (mean ± SD).

This indicated a high level of integrity of the total RNA for all tissue preparations.

Cardiac MRP5/GAPDH mRNA was $56.3 \pm 16.1 \times 10^{-4}$ (n = 9) for native controls and $54.1 \pm 14.8 \times 10^{-4}$ for PBS controls (n = 6), compared with $31.9 \pm 16.8 \times 10^{-4}$ in the LPS experiments (n = 9, $P = 0.025$ compared with PBS). Within the sham-operated group (n = 5), MRP5/GAPDH mRNA copy numbers were $42.8 \pm 12.1 \times 10^{-4}$ compared with $18.3 \pm 9.4 \times 10^{-4}$ in the animals of the CASP group (n = 9, $P = 0.009$) (Fig. 3A).

### *MRP5 mRNA expression in HL1 cells*

MRP5/GAPDH mRNA levels in HL1 cells exposed for 24 h to serum of septic mice were decreased to 21.6% ± 8.6% (LPS group, $P = 0.003$) and 11.3% ± 2.7% (CASP group, $P = 0.002$) as compared with the untreated control, whereas HL1 incubation with LPS alone reduced MRP5 expression to only 68.9% ± 5.5% of control ($P = 0.241$) (Fig. 3B). In addition, HL1 cells were directly incubated with different cytokines (5 ng/mL each, for 24 h). Thereby MRP5/GAPDH mRNA levels of HL1 cells were decreased to 54.4% ± 12.8% of control ($P = 0.025$) after IL-6 incubation, whereas IFN-$\gamma$ and TNF-$\alpha$ did not significantly influence expression of MRP5 mRNA.

### *MRP5 protein expression*

MRP5 could be found in all hearts and HL1 cell cultures tested (Fig. 4, A and B). In the cell culture experiments using

proinflammatory cytokines, MRP5 expression in relation to desmin, a class 3 intermediary filament protein ubiquitously present in muscle cells, was decreased after exposure to IL-6 as compared with untreated controls (Fig. 4B).

### *Dual-Luciferase Reportergene Assay for the human ABCC5 promotor region*

The ratio firefly/renilla as a measure of transcriptional regulation was decreased after exposure to IL-6 (65.0%) and IL-6 in conjunction with the soluble IL-6 receptor (46.7%) (Fig. 4C). Because HeLa cells do not display IL-6 receptors, the increased effect of IL-6 with the IL-6 receptor points to a pronounced regulatory effect of IL-6 on expression of the human ABCC5 gene, which codes for MRP5.

## DISCUSSION

The present study investigates the expression and regulation of the cardiac ABC-type cGMP transport protein MRP5 in response to septic stimuli. MRP5, which is expressed in vascular smooth muscle cells as well as cardiomyocytes in the mouse heart, was down-regulated by both the LPS and multibacterial (CASP) shock models. This observation could be verified by cell culture experiments using murine HL1 cardiac muscle cells and serum of septic mice as well as the proinflammatory cytokine IL-6, whereas other cytokines, such as TNF-$\alpha$ and IFN-$\gamma$, did not influence MRP5 expression. These results are in line with findings showing a down-regulation of MRP5 after LPS challenge in rat liver (20), but in contrast to experiments demonstrating hepatic MRP5 up-regulation after LPS treatment of rats (21). Earlier findings of hepatic MRP2 down-regulation (22), which was also found in hepatocyte culture, might in part explain cholestasis after cecal ligation-and-puncture experiments in mice (23).

The quantitative relevance of MRP5 contribution to intracellular cGMP homeostasis is still unclear. Current projects focusing on the intracellular cGMP level as a key target for cell relaxation mostly cover cGMP hydrolysis by phosphodiesterases, especially PDE5 (9). However, the inhibition of PDE5 did increase not only intracellular but also extracellular cGMP levels in cell culture–based transport experiments, which led to the assumption of relevant cGMP outward transport, which was inhibited by PDE5 blockade (12). As for vascular contractility, it has been shown that functional knockdown of MRP5 in pial arterioles did in fact result in enhanced relaxation (24). These findings suggest that potential regulation of cardiac MRP5 may have functional relevance and, if so, which stimuli may contribute to MRP5 regulation. It is notable that LPS-treated dogs demonstrate a rapid increase of the proinflammatory cytokines IL-6 and TNF-$\alpha$, which is followed by a delayed increase of serum NO levels, suggesting a possible regulatory involvement of these cytokines (25). To address this issue with respect to MRP5 regulation, HL1 cells were incubated with IL-6, IFN-$\gamma$, and TNF-$\alpha$ as well as with serum from septic animals of both experiments and pure LPS. MRP5 down-regulation, which could be found in experiments from both shock models, was more pronounced in the CASP-serum–incubated cells than in cells incubated with serum from LPS-injected mice. Incubation with the pure cytokines revealed a suppressive effect on MRP5 mRNA, which was most pronounced for IL-6. Although quantitative conclusions cannot be drawn from these experiments, because effects in cultured cells always depend on the receptor status of the respective cell line, there is evidence for the three candidates to be involved in MRP5 down-regulation. LPS-induced cardiac impairment has been related to TNF-$\alpha$ in various studies on a functional and substructural level (26–28). TNF-$\alpha$–induced caspase activation contributes to the functional deficit (3). Consequently, caspase inhibition prevented cardiac dysfunction and apoptosis in a rat model of sepsis (29), although clinical studies did not confirm laboratory results. However, in a murine model of septic shock, iNOS inhibition results in improved cardiac responsiveness to catecholamines (30).

In brief, the present article shows that there is evidence for MRP5 down-regulation in heart smooth muscle cells and cardiomyocytes by experimental LPS endotoxemia and multibacterial peritoneal shock. Given the capability of MRP5 to reduce intracellular cGMP levels, this may potentially contribute to impaired cardiac function in septic heart failure caused by elevated intracellular cGMP levels.

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