Journal of Cellular Biochemistry 86:422–431 (2002)

ARTICLES

Analysis of the Energetic State of Heart Cells After Adenovirus-Mediated Expression of hALC-1

Udo B. Zacharzowsky,[1] Gerhard Wolff,[1] Monika Kott,[1] Hannelore Haase,[1] Holger Bartsch,[1] Andreas K. Nuessler,[3] Leonidas G. Baltas,[1] Leonid Karawajew,[2] and Ingo Morano[1,4]*

[1]Max Delbrück Center for Molecular Medicine (MDC), 13122 Berlin, Germany
[2]Robert-Rössle-Clinic, Humboldt-University of Berlin, 13122 Berlin, Germany
[3]Department of General, Visceral, and Transplantation Surgery, Charité, Campus Virchow, Humboldt-University of Berlin, 13353 Berlin, Germany
[4]Department of Physiology, Humboldt-University of Berlin, 13159 Berlin, Germany

Abstract Expression of the human atrial myosin light chain 1 (hALC-1) in the cardiac ventricle in vivo as well as in primary cultivated adult cardiomyocytes caused a pronounced positive inotropic effect. Therefore, it is one of the most promising candidate gene to treat congestive heart failure (CHF). In this work, we investigated, whether hALC-1 expression also modifies the energetic state of cardiomyocytes. Primary cultivated neonatal rat hearts cells (NRHC) were infected with adenoviral vectors (Ad vectors) containing a hALC-1 cDNA (AdCMV.hALC-1) or a control Ad vector. Infection efficiency of NRHC reached 100% at 50 multiplicity of infection (MOI). Interestingly and in contrast to primary cultures of liver cells, there were no cytotoxic side effects or induction of apoptosis up to MOI 50 in Ad vector infected NRHC. NRHC expressed large amounts of hALC-1 upon infection with AdCMV.hALC-1 which could easily been detected by protein staining and Western blot analysis. Analysis of intracellular hALC-1 localization by double-labeling immunofluorescence of AdCMV.hALC-1 infected cardiomyocytes revealed the typical myofibrillar striation pattern, as well as co-localization of hALC-1 with myosin heavy chains. There was no difference in the oxygen consumption between controls and AdCMV.hALC-1 infected NRHC. These data suggest that first: adenoviral vectors could be used as a safe and effective tool for gene transfer to cardiomyocytes, and second: that a positive inotropic effect of hALC-1 is not associated with enhanced oxygen consumption. J. Cell. Biochem. 86: 422–431, 2002. © 2002 Wiley-Liss, Inc.

Key words: adenovirus; gene transfer; human ALC-1; cardiomyocytes

Contractile function of myocytes is triggered by the molecular motor type II myosin which consists of two myosin heavy chains, each associated with one essential and one regulatory myosin light chain [Lowey and Risby, 1971]. The level of gene expression of individual myosin subunits in cardiomyocytes varies in different physiological circumstances and is altered in pathological states [Schiaffino and Reggiani, 1996]. Thus, expression of the human ALC-1 (hALC-1) is tissue specific and developmentally regulated [Barton and Buckingham, 1985]. Embryos express large amounts of hALC-1 both in the whole heart and in skeletal muscle. hALC-1 levels decreased in the ventricle to undetectable levels during early postnatal development but persist throughout the whole life in the atrium as well as in the hypertrophied right ventricle of children with congenital heart diseases [Auckland et al., 1986]. Similarly, the hypertrophied left ventricle of patients with valvular heart diseases and hypertrophic obstructive cardiomyopathy expressed large amounts of hALC-1 [Schaub et al., 1984; Morano et al., 1996; Ritter et al., 1999a]. Surgical intervention and subsequent normalization of the hemodynamic state decrease hALC-1 expression in these patients [Sütsch et al., 1992]. Patients with end stage congestive heart failure (CHF) express no or only small amounts of hALC-1 [Morano et al., 1996].

Grant sponsor: Bundeswirtschaftsministerium (BMWi); Grant number: KF 0056401KUL9.

*Correspondence to: Prof. Dr. Ingo Morano, Max Delbrück Center for Molecular Medicine, Robert-Rössle-Str. 10, 13122 Berlin, Germany. E-mail: imorano@MDC-berlin.de

Received 7 February 2002; Accepted 20 March 2002
DOI 10.1002/jcb.10200

© 2002 Wiley-Liss, Inc.

Recently, the functional role of the hALC-1 in the human heart could be resolved: maximal shortening velocity, rate of tension redevelopment, isometric force generation, and Ca^{2+} sensitivity of force generation rose upon partial replacement of the ventricular myosin light chain 1 by hALC-1 in the ventricle [Morano et al., 1996, 1997]. In addition, there was a significant positive correlation between hALC-1 expression and dP/dt_{max} of patients with hypertrophic obstructive cardiomyopathy (HOCM) in vivo [Ritter et al., 1999b]. Furthermore, transgenic overexpression of the mouse ALC-1 in the mouse ventricle caused a pronounced positive inotropic effect of the whole heart and an enhanced contractile reserve [Fewell, 1998]. These results demonstrate, that expression of ALC-1 in all species investigated increased power output of the sarcomeric motor molecules and improved cardiac contractility. Hence, therapeutic upregulation of hALC-1 represents a reasonable strategy to treat end-stage CHF. One of the most potent vector systems are adenovirus based gene transfer vectors (Ad vector) [Rosenzweig, 1999]. Therefore, we generated an Ad vector carrying the cDNA of the hALC-1 (AdCMV.hALC-1) which was able to induce a pronounced positive inotropic effect of electrically stimulated primary cultivated adult rabbit cardiomyocytes [Kögler et al., 2001].

One major concern in the treatment of CHF is, that the induction of positive inotropic effects increases energy consumption which could have deleterious side-effects on the heart. To test, whether expression of the positive inotropic protein hALC-1 influences the energetic state of cardiomyocytes, we investigated oxygen consumption as a measure of the energetic state of cardiomyocytes. However, those studies cannot be performed with electrically stimulated adult cardiomyocytes, since oxygen is produced at the electrodes during electrolysis. We, therefore, infected NRHC with AdCMV.hALC-1, because cardiomyocytes in NRHC preparations spontaneously pulsate, i.e., without external electrical stimulation. Interestingly, although AdCMV.hALC-1 infected NRHC expressed large amounts of hALC-1, which was co-localized with myosin in the myofibrils, there was no enhanced oxygen consumption between untreated and AdCMV.hALC-1 infected cardiomyocytes.

Furthermore, Ad vector mediated gene transfer itself may induce cytopathic effects and cell cycle dysregulation [Yang et al., 1994; Teodoro and Branton, 1997; Wersto et al., 1998]. Whereas several studies demonstrated the general consequences of Ad vector mediated gene transfer into the cardiovascular system and myocardium, no data about cytopathic effect and cell cycle dysregulation on cardiomyocytes are published. We, therefore, infected cultured NRHC with AdRSV.βgal and analyzed cell cycle distribution and levels of cytopathic marker enzymes. AdRSV.βgal infection of neonatal rat hearts cells (NRHC) resulted in an efficient gene transfer and transgene expression without cell cycle dysregulation and no elevated levels of cytopathic marker enzymes.

RESULTS

Characterization of Primary Cultures of Neonatal Heart Cells

We characterized the cell types present in our primary cultivated NRHC by immunofluorescence labeling at day 2 and 8 of culture (Fig. 1). After 2 days of culture, labeling of the cells with a specific antibody raised against cardiac myosin heavy chain, revealed that $69.0 \pm 9.3\%$ ($n = 10$) of the total population were cardiomyocytes. Double labeling with antibodies raised against cellular fibronectin and smooth-muscle myosin heavy chain identified $19.7 \pm 10.5\%$ ($n = 10$) of the cultivated cells as myofibroblasts and $10.6 \pm 4.5\%$ ($n = 10$) as smooth muscle cells (not shown). Labeling with CD31 antibody revealed that $5.0 \pm 1.3\%$ of the cells were endothelial cells. After 8 days of culture, the number of cardiomyocytes remained constant ($72.0 \pm 16.2\%$; $n = 10$), as well as the number myofibroblasts ($20.0 \pm 3.3\%$; $n = 10$), smooth muscle cells ($8.6 \pm 5.5\%$; $n = 10$), and endothelial cells ($4.5 \pm 1.5\%$; $n = 10$).

Efficiency of Ad Vector Mediated Gene Transfer to NRHC

To analyze the efficiency of Ad vector mediated gene transfer to NRHC, two different constructs containing the cDNAs of either β-galactosidase (AdRSV.β-gal) or hALC-1 (AdCMV.hALC-1) were used. All cell types could be easily infected with both Ad vectors. Analysis of X-Gal staining revealed that with a multiplicity of infection (MOI) of 50 already 100% of the AdRSV.β-gal infected NRHC expressed β-galactosidase as transgene, while

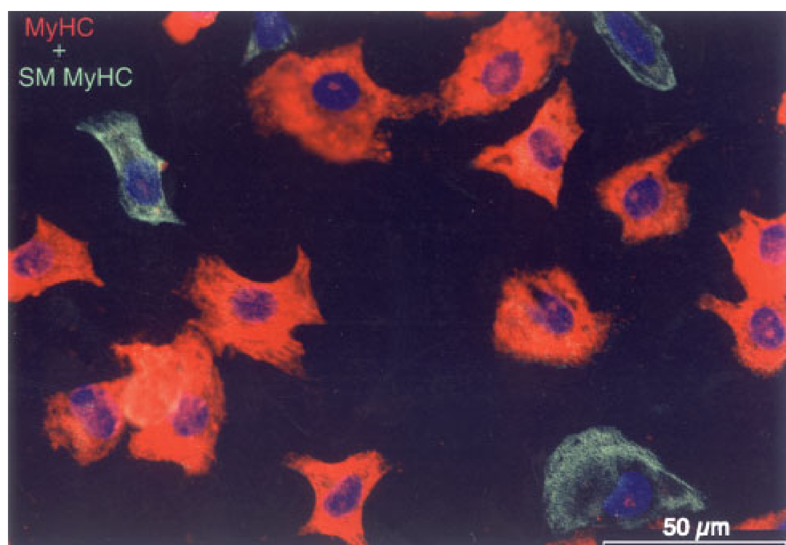


Fig. 1. Double-labeling immunofluorescence of primary cultivated NRHC from ventricular tissue of Sprague–Dawley rats after 2 days of culture. Cells were characterized by incubation with two specific antibodies raised against cardiac myosin heavy chain (red) and smooth muscle myosin heavy chains (green). Bar indicates 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MOI 10 was enough for half-maximal infection efficiency (Fig. 2). Similar results could be observed using the AdCMC.hALC-1 vector (data not shown).

Assessment of Apoptosis and Cell Cycle Distribution by FACS Analysis

The overall cell vitality was analyzed by staining of the nuclei with crystal violet (citric acid). We found no significant difference in the percentage of living cells between mock and Ad vector treated cells (mock treated NRHC: 85.5 ± 7.8%; MOI 50 AdRSV.β-gal infected NRHC 79.0 ± 2.8%). Consistent with the analysis of the overall viability, there was no significant difference in Annexin V-positive staining between mock treated and AdRSV.β-gal treated cardiomyocytes (mock treated cardiomyocytes: 19, 20, and 22%; AdRSV.β-gal infected cardiomyocytes: 17, 20, and 16%). Moreover, the analysis of cell cycle distribution of mock and AdCMV.β-gal treated NRHC did not show any cell cycle dysregulation (Table I) as known from Ad vector infected hepatocytes.

Analysis of Cytopathic Effects After Ad Vector Infection

To assess in more detail potential cytopathic effects of Ad vector infection on cardiomyocytes, we measured levels of glutamic oxaloacetic transaminase (SGOT) and lactate dehydrogenase (LDH) in the medium of AdRSV.βgal infected NRHC and compared them with human primary hepatocytes (HPHC). As expected, HPHC responded to AdRSV.βgal infection with elevated levels of the marker enzymes SGOT

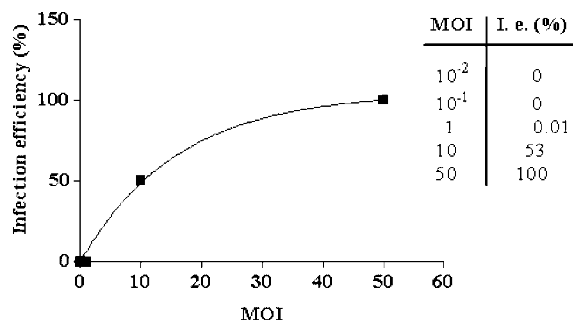


Fig. 2. Infection efficiency of primary cultivated NRHC after infection with AdRSV.β-gal. Infection efficiency is expressed as the fraction of infected cells in percent (%) of the total number of cells; MOI = multiplicity of infection.

TABLE I. Effect of Ad Vector on Cell Cycle Distribution

	Annexin$^-$			Annexin$^+$		
Treatment	G_0/G_1	S	G_2M	G_0/G_1	S	G_2M
Mock infected						
	84	3	13	87	3	10
	85	3	12	88	3	10
	85	3	12	87	3	10
AdCMV.β-gal						
	85	2	12	86	2	11
	86	3	12	85	3	10
	87	2	10	86	3	9

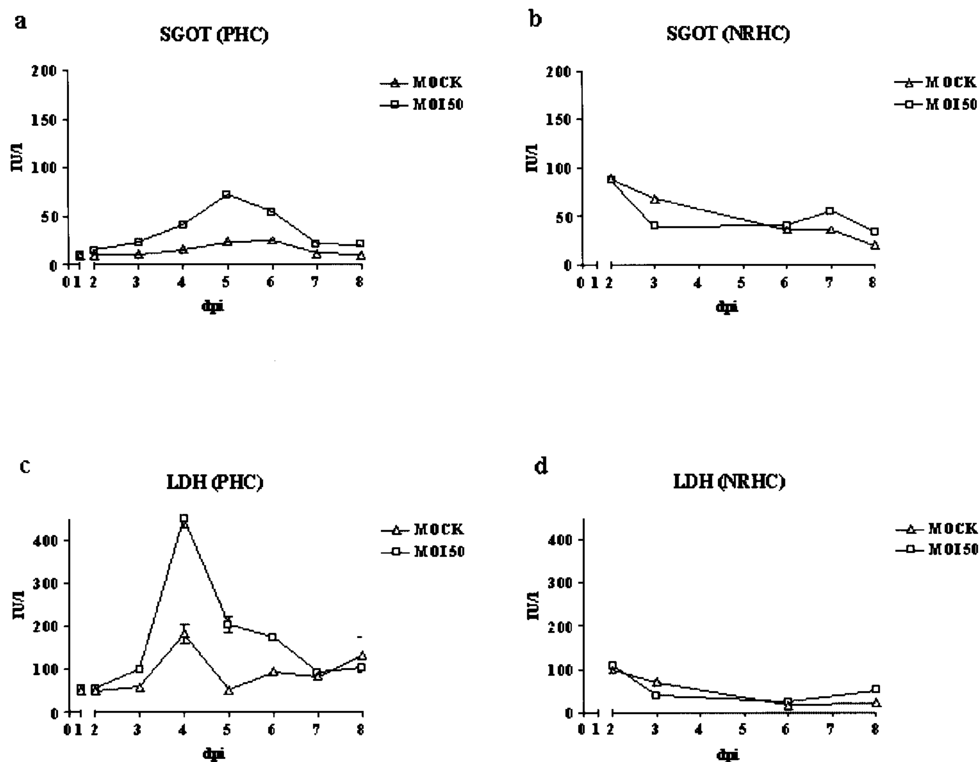


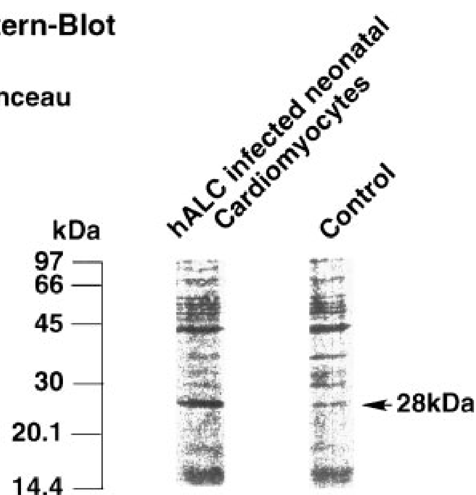
Fig. 3. Characterization of cell integrity of primary cultivated NRHC and primary cultivated human hepatocytes (HPHC). Enzyme levels (IU/l) in the medium were measured during 8 days after infection with AdRSV.β-gal. **a** and **c**: Levels of SGOT. **b** and **d**: Levels of LDH. dpi = days past infection; MOI = multiplicity of infection; MOCK = mock-infected.

and LDH in the medium, leading to a 3-fold increase of SGOT (72 IU/L) as compared to mock infected HPHC (24 IU/L) and to a 8-fold rise of LDH (449 IU/L) as compared to mock infected HPHC (57.5 UI/L) (Fig. 3a,c). In contrast, NRHC did not respond to AdRSV.βgal infection with cytopathic effects (normal levels of SGOT and LDH). Both mock treated and AdRSV.βgal treated groups showed comparable enzyme expression profiles (Fig. 3b,d).

Analysis of hALC-1 Expression in NRHC After Ad Vector Mediated Gene Transfer

The analysis of hALC-1 expression and localization in NRHC, after Ad vector mediated gene transfer, was performed by Western blot and double-labeling immunofluorescence using specific antibodies raised against cardiac myosin heavy chain and hALC-1. We infected NRHC with 50 MOI of AdCMV.hALC-1. After 24 h of infection, cells were homogenized, subjected to SDS-page, and blotted onto a nitrocellulose membrane. Ponceau staining of the nitrocellulose membrane revealed an increased intensity of a 28 kDa protein band in AdCMV.hALC-1 infected NRHC as compared to control (AdRSV.β-gal) infected NRHC. The ratio between the 28 and 40 kDa actin band was 1.56 and 0.8 in the AdCMV.hALC-1 infected and control infected cells, respectively. As demonstrated by specific antibody binding in ECL, this 28 kDa band contained hALC-1 in the infected but not in the control cells (Fig. 4).

Localization of hALC-1 in the contractile apparatus of cardiomyocytes was analyzed by double labeling-immunofluorescence after infection of NRHC with 50 MOI of AdCMV.hALC-1. After 48 h of Ad vector mediated gene transfer of hALC-1, staining of infected cells with an antibody raised against hALC-1 showed a strong staining preferentially of the myofibrils, leading to a typical striation pattern (Fig. 5). Control infected (AdRSV.βgal) cells remained without signal (data not shown). Moreover, double-labeling immunofluorescence of the infected cells revealed that hALC-1 co-localized



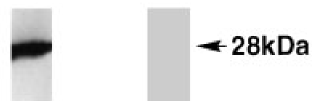


Fig. 4. Western Blot of primary cultivated NRHC 48 h following infection with AdCMV.hALC1 (MOI 50). **a**: Ponceau staining of the nitrocellulose membrane, **b**: ECL signal obtained following reaction with an antibody raised specifically against human ALC-1. Control cultures were infected with AdRSV.β-gal (MOI 50).

in the contractile apparatus with cardiac myosin heavy chains (Fig. 5).

Analysis of Oxygen Consumption of NRHC After Infection With AdCMV.hALC-1

To investigate the effects of hALC-1 on oxygen consumption of cardiomyocytes, we infected NRHC with AdCMV.hALC-1 (MOI 50) at day 2 of culture. Analysis of oxygen consumption was performed 48 h later.

The beating frequency varied between different preparations of NRHC. This provided the opportunity, to investigate the frequency-dependence of oxygen consumption in NRHC. Oxygen consumption rose with increasing beating frequencies. Measurements of oxygen consumption of mock-infected NRHC revealed a significant ($P < 0.001$) linear positive correlation between oxygen consumption and beating frequency (Fig. 6a).

There was also a significant positive linear correlation between oxygen consumption and beating frequency ($P < 0.05$) in NRHC overexpressing hALC-1 upon AdCMV.hALC-1 infection (Fig. 6b). Although there was a tendency to a reduced oxygen consumption, especially at high beating frequencies, oxygen consumption in AdCMV.hALC-1 infected NRHC was not significantly different from mock treated NRHC (Fig. 6b).

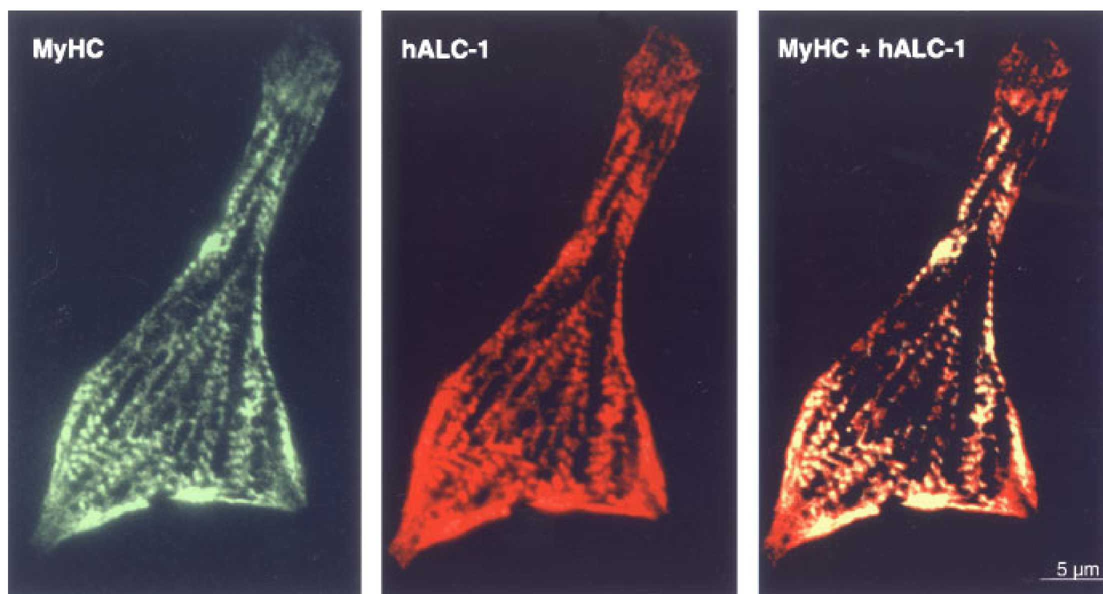


Fig. 5. Double-labeling immunofluorescence micrograph of a primary cultivated neonatal rat cardiomyocyte 48 h after infection with AdCMV.hALC-1 (MOI 50). **a**: Staining with a specific antibody raised against cardiac myosin heavy chains (green), **b**: staining with an antibody raised against human ALC-1 (red), and **c**: merged signals. Bar indicates 5 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

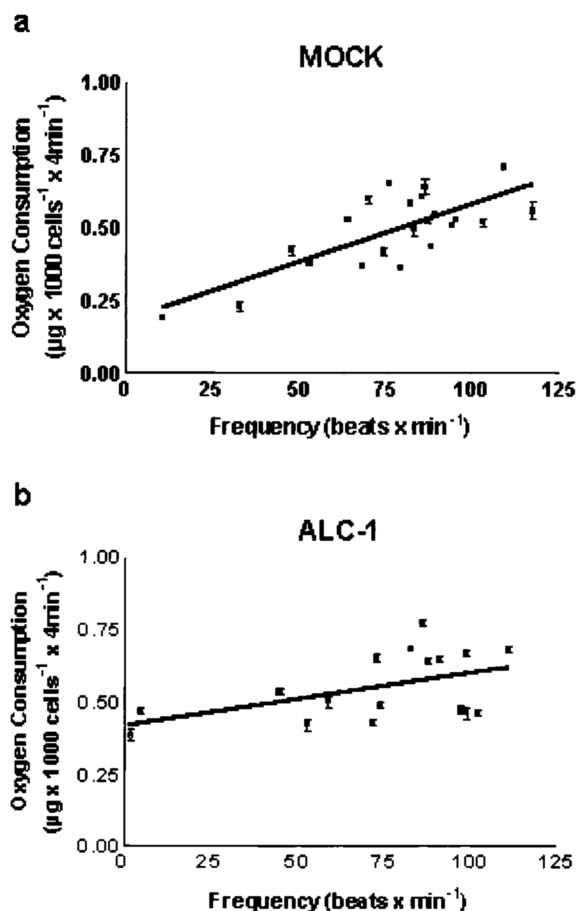


Fig. 6. Characterization of oxygen consumption of 4-day-old cultured neonatal rat heart cells at different beating frequencies 48 h after infection. **a**: Oxygen consumption of 4-day-old mock-infected heart cells ($P < 0.0001$). **b**: Oxygen consumption of heart cells following infection with AdCMV.hALC-1 (MOI 50) ($P < 0.05$).

DISCUSSION

Expression of the atrial myosin light chain 1 (ALC-1) increased cross-bridge cycling kinetics and the inotropic state of the human, mouse, and rat heart [Morano et al., 1996, 1997; Fewell et al., 1998; Baltas et al., 2001]. Furthermore, primary cultivated adult rabbit cardiomyocytes overexpressing the human isoform of ALC-1 (hALC-1) upon infection with an Ad vector containing the hALC-1 cDNA increased their shortening amplitude and calcium sensitivity of contraction [Kögler et al., 2001].

It is well known that induction of a positive inotropic effect, for example, by treatment with conventional drugs like digitalis or catecholamines, also increased oxygen consumption which, in turn, could have deleterious side-effects especially in the failing state of the heart. Therefore, increasing the inotropic state of the heart without elevating oxygen consumption would be a desirable therapeutic goal. An increased inotropic state without additional energy consumption could theoretically derived by assuming a two-state model of the myosin cross-bridge cycle [Brenner, 1988]: force generation (F) depends not only on the apparent kinetic rate constants of cross-bridge attachment (f) and detachment (g), but also on the tension generation of the single myosin motor molecule (F′) which equals:

$$F = F'f/(f + g)$$

Since ATP consumption (ATPase) of the myosin motor molecules equals [Brenner, 1988]:

$$\text{ATPase} = fg/(f + g)$$

ATP consumption is independent from the force generated by the single cross-bridge. Hence, enhanced force generation without elevated ATP, and consequently without elevated oxygen consumption, could indeed be obtained by increased force generation per myosin molecule.

Therapeutic overexpression of hALC-1 may provide such a strategy, i.e., induction of a positive inotropic effect without increasing the oxygen consumption of the heart. In fact, we recently demonstrated that the force generation per cross-sectional area of demembranated (skinned) human heart fibers with hALC-1 increased significantly compared to fibers without hALC-1 [Yang et al., 1994]. In addition, transgenic rat papillary muscles overexpressing hALC-1 revealed an increased force per cross-section as well [Baltas et al., 2001]. These observations provide evidence for an enhanced F′ (force generation per myosin molecule) in the presence of hALC-1. If hALC-1 increases F′, then the positive inotropic effect observed in AdCMV.hALC-1 infected primary cardiomyocytes expressing hALC-1 should not be associated with an enhanced oxygen consumption.

We tested this hypothesis by measurements of oxygen consumption of primary NRHC preparations which expressed hALC-1 upon infection with AdCMV.hALC-1. NRHC were used in these experiments because they spontaneously pulsate, i.e., without electrical stimulation. Deteriorations of oxygen measurements caused by oxygen production on stimulation elec-

trodes could, therefore, be excluded. We found large amounts of hALC-1 expressed 48 h after infection of NRHC with AdCMV.ALC-1. Furthermore, expressed hALC-1 co-localized with myosin heavy chains in the sarcomeres, i.e., where hALC-1 could be functionally effective. It is not possible to perform reliable measurements of contractile parameters of NRHC and, therefore, we have no direct evidence for an inotropic effect of hALC-1 in the cardiomyocytes of NRHC. However, there is a large body of evidence demonstrating the unique positive inotropic effect of hALC-1: expression of hALC-1 induced an enhanced contractility in all animal species (human, rabbit, rat) and muscle preparations (demembranated fibers, papillary muscles, primary cultivated cardiomyocytes) so far investigated [Morano et al., 1996, 1997; Fewell et al., 1998; Baltas et al., 2001; Kögler et al., 2001]. We, therefore, feel justified to suggest that the expression of hALC-1 also induced a positive inotropic effect in cardiomyocytes of NRHC.

Although levels of O_2 consumption of NRHC rose with increasing beating frequencies (c.f. [Yamada et al., 1985; Riehle and Bereiter, 1994]), oxygen consumption of AdCMV.hALC-1 infected NRHC remained normal at any beating frequency investigated. Rather, there was a tendency to a reduced, albeit not statistically significant, oxygen consumption. A positive inotropic action of hALC-1 in primary cardiomyocytes (c.f. [Kögler et al., 2001]) without enhanced oxygen consumption support the hypothesis of an increased force generation per cross-bridge in the presence of hALC-1 (see above).

Thus, overexpression of hALC-1 could be of primary interest as a novel gene therapeutic approach for the treatment of terminal CHF. Independent of the therapeutic transgene, potential side-effects caused by the gene transfer vectors themselves are a major concern for clinical applications of gene therapy approaches. Application of Ad vectors may cause cytotoxicity by dysregulation of the G_2M phase of the cell cycle of the infected target cell [Wolff et al., 2002]. Thus, liver cells responded to Ad vector infection with dysregulation of the cell cycle, leading to balloning degeneration and apoptosis of the infected cells [Yang et al., 1994; Wersto et al., 1998]. In contrast, we demonstrate herein that cell cycle regulation of cardiomyocytes of NRHC remained normal after Ad vector infection. This observation could be explained by the fact that cardiomyocytes are postmitotic cells, whereas cell types which are still capable of cell division, for example, hepatocytes are vulnerable to cytopathic effects of Ad vector infection [Teodoro and Branton, 1997; Wolff et al., 2002].

Considering the pronounced positive inotropic effect without increasing oxygen consumption, expression of hALC-1 represents a powerful new gene therapeutic approach for the treatment of heart failure. Ad vector mediated gene transfer to cardiomyocytes seems to be appropriate, since it is efficient and without toxic side-effects.

MATERIALS AND METHODS

Preparation and Culture of Neonatal Rat Heart Cells

Primary neonatal heart cell cultures were prepared from ventricular tissue of 1–3-day-old Sprague–Dawley rats (Tierzucht Schönwalde, Germany) as previously described. [Kott et al., 1998] Briefly, the ventricles were removed and were placed into an ice-cold calcium free PBS solution (120 mM NaCl, 4.56 mM KCl, 0.44 mM KH_2PO_4, 0.42 mM Na_2HPO_4, 25 mM $NaHCO_3$, 5.5 mM glucose, 0.5 mg/ml streptomycin, and 5,000 I.E./ml penicillin G, pH 7.5; Biochrom, Germany). They were minced into small pieces of approximately 1 mm^2. Then they were stepwise disaggregated into single cells by incubation in antibiotics-free PBS that was supplemented with 0.12% trypsin (Biochrom, Germany). All cells were pooled and resuspended in cell growth medium SM20-I supplemented with 10% FCS, 2 mM glutamine, 0.02 mg/ml gentamicine (Biochrom, Germany), 2.76 mM hydrocortisone, and 0.002 mM fluorodeoxyuridine (Sigma-Aldrich, Germany). Cardiomyocytes were seeded and cultured in different systems (24-well cell plate with approximate 300,000 cells per well, NUNC, Denmark; 50,000 cardiomyocytes on a cover slight, Menzel, Germany, and 2 million cardiomyocytes in a special cell chamber for oxygen measurements with a surface of ≈ 3.36 cm^2, PTS, Germany, respectively). Daily, cell growth medium was changed in all systems and setups. The human embryonic kidney cells 293 (HEK 293, ATCC, USA), were maintained in DMEM, supplemented with 10% FCS and 2 mM glutamine, and grown at 37°C with 5% CO_2 in a fully humidified atmosphere.

Preparation and Culture of Human Primary Hepatocytes

In accordance with institutional guidelines, human primary hepatocytes (HPHC) were isolated from normal liver wedge resections by collagenase-P (Roche Diagnostics, Grenzau, Germany) digestion as recently described. [Nussler et al., 1992] Briefly, after enzymatic digestion hepatocytes were separated from non-parenchymal cells by differential centrifugation at 50*g* and then passed over a 30% Percoll (Pharmacia, Freiburg, FRG) gradient at a concentration of 10^6 cells/ml Percoll to obtain a highly purified cell population. Hepatocyte purity and viability assessed by microscopy was greater than 95% and viability consistently exceeded 90% by trypan blue exclusion [Dorko et al., 1994]. The primary hepatocytes were cultured in Williams Medium E, supplemented with 10% heat-inactivated (90 min at 70°C) FBS, 1% Penicillin/Streptomycin, 15 mM HEPES Buffer (all Life Technologies, Karlsruhe, FRG), 2.0 μg/ml Insulin, and 0.35 nM Hydrocortisone (both Sigma, Deisenhofen, FRG) at 37°C, 5% CO_2 in a humidified atmosphere.

Construction and Propagation of Adenovirus Vectors

For all experiments, recombinant replication deficient adenovirus vectors of the first generation were used which are based on a deletion of the E1 region of an adenovirus type 5 (Ad vector). The Ad vector AdRSV.β-gal expresses *Escherichia coli* β-galactosidase under the control of the RSV promoter. The Ad vector AdCMV.hALC-1 was constructed by cloning a cDNA of the human essential atrial light chain 1 (hALC-1) into an adenovirus backbone of the AdEasy™ system (Cat. Nr.: AES1000B, QBIOgene, Montreal, Canada) as described by the manufacturer. All vectors were propagated on HEK 293 cells, purified and stored at −80°C, as previously described. The titers of stocks used for these studies measured by plaque assays were 7.7×10^{10} pfu/ml, with a particle/pfu ratio of 30/1. Recombinant adenoviruses were tested for the absence of wild-type virus by polymerase chain reaction of the early transcriptional unit E1 and E1a.

Infection Procedure

Cultured cardiomyocytes were infected as previously described [Wolff et al., 1998]. Briefly, 2-day-old cardiomyocyte cultures were washed twice with FCS-free culture medium and transferred into OPTIMEM 1 (Gibco BRL). Infections with different amounts of AdRSV.β-gal and AdRSV-hAAT.2 (MOI 0.01-50) were performed in the same medium for 90 min on a rocking platform in a CO_2-incubator and were terminated by addition of FCS. Subsequently, cells were incubated in SM20-I for 24 h.

Analysis of Glutamic Oxaloacetic Transaminase and Lactate Dehydrogenase

To measure concentrations of the enzymes glutamic oxaloacetic transaminase (SGOT) and lactate dehydrogenase (LDH), the supernating medium (1 ml) of infected or mock treated cell cultures was harvested and analyzed in a laboratory system as described by (Synchron CX5, Beckmann Coulter, USA) and according to published procedures [Amador et al., 1963].

Assessment of Apoptosis and Cell Cycle Distribution by FACS Analysis

For experiments using FACS analysis, 8×10^5 cells were seeded in triplicates in 10-cm dishes and cultured overnight to allow adherence. Next morning, cells were infected with Ad vectors at the indicated MOI as described [Wolff et al., 1998]. Cells were harvested by trypsinization at the indicated time points. For detection of apoptosis, cells were stained with FITC-conjugated Annexin V and propidium iodide (PI) using the Annexin V kit (Immunotech, Marseille, France) as indicated by the manufacturer. Samples were subsequently analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA) for the presence of viable (FITC-negative and PI-negative) and apoptotic (FITC-positive) cells which include both, primary (PI-negative) and secondary (PI-positive) apoptotic subpopulations. For analysis of cell cycle distribution, FITC-Annexin V-stained samples were fixed and permeabilized. Briefly, following two washes with PBS, the cell pellet was fixed with ice-cold 70% ethanol for 1 h at 4°C. After two additional washes, cells were resuspended in 0.2 mL PBS containing 1.0 mg/ml PI, pH 7.5, and 0.5 mg/ml RNase (type I-A; Boehringer Mannheim, Mannheim, Germany) at room temperature in the dark for 30 min and subsequently analyzed by flow cytometry. Cell cycle analysis was performed using either CellQuest software (Becton Dickinson, San Jose, CA) or ModFit LT (Verity, Topsham,

ME). Cell aggregates were excluded from the analysis by using the Doublet Discriminating Module (Becton Dickinson).

Western Blot

Expression of hALC-1 in cultured cardiomyocytes was analyzed by SDS–PAGE 5% seperation gel, both containing 25% glycerol. Gels were run overnight at a constant 5 mA and 15°C, and processed for Western blot. Proteins were transferred onto a nitrocellulose membrane which was blocked with 3% ovoalbumin and incubated with a peptide directed antibody raised against the amino acid sequence 29 to 43 (PAPEAPKEPAFDPKS) of hALC-1. Membranes were then incubated with the secondary peroxidase-conjugated antibody. Proteins were visualized by enhanced chemoluminescence (ECL; Amersham) and exposure to X-ray film.

Immunofluorescence

Cells were fixed with methanol/acetone (50/50 v/v) at −20°C for 5 min and incubated with antibodies raised against hALC-1 (see above) and cardiac myosin heavy chain (monoclonal antibody 2C9 which recognized both alpha- and beta-myosin heavy chains; gift from D. Mornet, Montpellier). Secondary antibodies were conjugated with DTAF (anti-rabbit) or Cy3 (anti-mouse). Fluorescence was detected using an Axioplan fluorescence microscope and a MC100 automatic camera (Zeiss) using Tmax400 films.

Measurement of Oxygen Consumption

Measurements of oxygen (O_2) consumption of neonatal rat cardiomyocytes was performed in a commercially available integrated sensor system (PCM®, PTS, Germany). The PCM® system was mounted on the stage of an inverted microscope (Nikon Diaphot 300, Japan) for determination of beating rate of cardiomyocytes during oxygen consumption measurements. At the beginning of each experiment, the cell chamber (150–200 μL volume) with the neonatal rat cardiomyocyte monolayer was closed airtight and perfused with serum-free medium, SM-20-I, supplemented with 100 μM dexamethasone, 200 μM glutamine, 0.5 mg/ml insulin, 0.5 μg/ml transferrin, 10 mg/ml gentamicine (Biochrom, Germany), and 0.2 μM fluorodeoxyuridine (Sigma-Aldrich, Germany) with a peristaltic pump (LKB, Sweden). The pump provides a constant flow (72 μl/min) of culture medium in the system. The flow of medium was controlled by a computer managed 3-directional multitube valve. The medium was flowed alternately through the cell chamber (5 min) or through a bypass (4 min) to a miniature oxygen sensor. O_2 consumption was calculated as the difference between O_2 concentration of the medium in the cell chamber and O_2 concentration in the fresh culture medium of the bypass. Computer controlled electronic interfaces provided on-line data acquisition of oxygen concentration and temperature, which was held at 37°C. After each experiment, the cells were lyged, their nuclei were stained with crystal violet/citric acid, and the total cell number was counted in a Fuchs-Rosenthal chamber under a microscope.

ACKNOWLEDGMENTS

The authors thank K. Jäger, P. Pierschalek, and K. Wendt as well as D. Balzereit for expert technical assistance. Furthermore, the authors thank Dr. Mark Kay (Stanford University, Stanford, USA) for generously providing AdRSV. β-gal and Dr. A. Schumacher for critically reading the manuscript. The work was partly funded by the "Bundeswirtschaftsministerium (BMWi)" grant number KF 0056401KUL9.

REFERENCES

Amador E, Dorfman LE, Wacker WEC. 1963. Serum Lactate Dehydrogenase Activity: An Analytical Assessment of Current Assays. Clin Chem 9:391–399.

Auckland LM, Lambert SJ, Cummins P. 1986. Cardiac myosin light and heavy chain isotypes in Tetralogy of Fallot. Cardiovasc Res 20:828–863.

Baltas LG, et al. 2001. Increased contractility and hypertrophy of the heart of transgenic rats harbouring the human atrial myosin light chain 1 (hALC-1). Pflügers Arch 441(6):R 253 (abstract).

Barton P, Buckingham ME. 1985. The myosin alkali light chain proteins and their genes. Biochem J 231:249–261.

Brenner B. 1988. Effect of Ca^{2+} on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: Implications for regulation of muscle contraction. Proc Natl Acad Sci USA 85:3265–3269.

Dorko K, Freeswick PD, Bartoli F, Cicalese L, Bardsley BA, Tzakis A, Nussler AK. 1994. A new technique for isolating and culturing human hepatocytes from whole or split livers not used for transplantation. Cell Transplant 5:387–395.

Fewell JG, Hewett T, Sanbe A, Klevitsky R, Hayes E, Warshaw D, Maughan J, Robbins J. 1998. Functional significance of cardiac essential light chain isoform switching in transgenic mice. J Clin Invest 101:2630–2639.

Kögler H, Hasenfuss G, Morano I. 2001. Overexpression of the human atrial myosin light chain 1 increases

contractile function in rabbit isolated ventricular cardiomyocytes. Eur Heart J 22 (Suppl):486 (abstract).
Kott M, Haberland A, Zaitsev S, Buchberger B, Morano I, Bottger M. 1998. A new efficient method for transfection of neonatal cardiomyocytes using histone H1 in combination with DOSPER liposomal transfection reagent. Somat Cell Mol Genet 24:257–261.
Lowey S, Risby D. 1971. Light chains from fast and slow muscle myosins. Nature 234:81–85.
Morano M, Zacharzowski U, Maier M, Lange PE, Alexi-Meskishviliv , Haase H, Morano I. 1996. Regulation of human heart contractility by essential myosin light chain isoforms. J Clin Invest 98:467–473.
Morano I, Hädicke K, Haase H, Böhm M, Erdmann E, Schaub MC. 1997. Changes in essential myosin light chain isoform expression provide a molecular basis for isometric force regulation in the failing human heart. J Mol Cell Cardiol 29:1177–1187.
Nussler AK, Di Silvio M, Billiar TR, Hoffman RA, Geller DA, Selby R, Madariaga J, Simmons RL. 1992. Stimulation of the nitric oxide synthase pathway in human hepatocytes by cytokines and endotoxin. J Exp Med 176:261–264.
Riehle M, Bereiter HJ. 1994. Ouabain and digitoxin as modulators of chick embryo cardiomyocyte energy metabolism. Arzneimittelforschung 44:943–947.
Ritter O, Haase H, Schulte HD, Lange PE, Morano I. 1999a. Remodeling of the hypertrophied human myocardium by cardiac bHLH transcription factors. J Cell Biochem 74:551–561.
Ritter O, Luther HP, Haase H, Baltas LG, Baumann G, Schulte HD, Morano I. 1999b. Expression of atrial myosin light chains but not α-myosin heavy chains is correlated in vivo with increased ventricular function in patients with hypertroph obstructive cardiomyopathie. J Mol Med 77:677–685.
Rosenzweig A. 1999. Vectors for gene therapy. In: Dracopoli NC, Haines JL, Korf BR, Moir DT, Morton CC, Seidman CE, Seidman JG, Smith DR, editors. Current protocols in human genetics. New York: John Wiley & Sons, Inc. p 12.10.11–12.18.19.
Schaub MC, Tuchschmidt CR, Srihari R, Hirzel HO. 1984. Myosin isoenzymes in human hypertrophic hearts. Shift of atria myosin heavy chains and ventricular light chains. Eur Heart J 5:85–93.
Sütsch G, Brunner UT, von Schulthess C, Hirzel HO, Hess OM, Turina M, Krayenbuehl HP, Schaub MC. 1992. Hemodynamic performance and myosin light chain 1 expression in the hypertrophied left ventricle in aortic valve disease before and after valve replacement. Circ Res 70:1035–1043.
Schiaffino S, Reggiani C. 1996. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. Physiol Rev 76:371–423.
Teodoro JG, Branton PE. 1997. Regulation of apoptosis by viral gene products. J Virol 71:1739–1746.
Wersto RP, Rosenthal ER, Seth PK, Eissa NT, Donahue RE. 1998. Recombinant, replication-defective adenovirus gene transfer vectors induce cell cycle dysregulation and inappropriate expression of cyclin proteins. J Virol 72: 9491–9502.
Wolff G, Schumacher A, Nuessler AK, Ruppert V, Karawajew L, Wehnes E, Neuhans P, Dörken B. 2002. Coexpression of $p21^{WAF1/CIP1}$ in adenovirus vector transfected human primary hepatocytes prevents apoptosis resulting in improved transgene expression. In press.
Wolff G, Korner IJ, Schumacher A, Arnold W, Dorken B, Mapara MY. 1998. Ex vivo breast cancer cell purging by adenovims-mediated cytosine deaminase gene transfer and short-term incubation with g-fluorocytosine completely prevents tumor growth after transplantation. Hum Gene Ther 9:2277–2284.
Yamada T, Yang JJ, Ricchiuti NV, Seraydarian MW. 1985. Oxygen consumption of mammalian myocardial cells in culture: Measurements in beating cells attached to the substrate of the culture dish. Anal Biochem 145:302–307.
Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E, Wilson JM. 1994. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. Proc Natl Acad Sci USA 91:4407–4411.