

Adenovirus-mediated gene transfer to adult mouse cardiomyocytes is selectively influenced by culture medium

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Abstract

Background As development of cardiac gene therapies progresses, virally mediated genetic manipulations in cultured cardiomyocytes has become an important experimental approach. While adenovirus (Ad)-mediated gene transfer to neonatal and adult rat cardiomyocytes is well established, viral transduction of cultured adult mouse cardiomyocytes (AMCM) has been more difficult. This study was designed to test the hypothesis that culture medium is a critical determinant of efficient gene transfer in AMCM.

Methods AMCM from 8-week-old C57BL/6 mice were cultured in either minimum essential medium (MEM) or medium M199 and then infected with an Ad β -galactosidase and transduction efficiency was quantified by cytochemistry and β -galactosidase activity assay. Coxsackie-adenovirus receptor (CAR) levels and Ad binding were evaluated by immunocytochemistry in M199- vs. MEM-cultured AMCM.

Results Our results demonstrated dramatic differences in efficiency of Ad-mediated gene transfer in AMCM cultured in MEM ($90 \pm 8\%$) vs. M199 ($5 \pm 1.2\%$). This difference was specific to AMCM, and was not observed in a number of other cells including neonatal rat cardiomyocytes. The enhanced transduction in MEM was associated with increased levels of CAR and Ad binding in AMCM.

Conclusions Culture medium has a profound effect on the efficiency of Ad-mediated gene transfer in AMCM, perhaps via differential effects on CAR expression. These findings have important implications for increasing numbers of studies that employ viral gene transfer in adult cardiomyocytes derived from mouse models of cardiac diseases. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords virus; myocytes; cell culture; coxsackie-adenovirus receptor; gene therapy

Introduction

Primary cultured cardiomyocytes are extremely valuable experimental tools for dissecting the mechanisms of cardiac diseases. During the last few years, adenoviral (Ad)-mediated gene transfer to cultured cardiomyocytes has become an increasingly important strategy for unraveling cellular and molecular determinants of cardiac cell function, and for developing cardiac

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gene therapy [1]. Although the methodology for viral transduction of neonatal and adult rat cardiomyocytes has been established for a number of years [2,3], implementation of this experimental approach has been considerably more challenging in mouse cardiomyocytes. In particular, it has been difficult to maintain viable adult mouse cardiomyocytes (AMCM) in culture for adequate amounts of time (at least 24 h) to implement viral-mediated gene manipulations and examine their effects [4]. This is an important hurdle to overcome if full advantage is to be taken of the unique experimental opportunities offered by an ever-growing number of mouse models of human heart diseases.

To our knowledge, only one successful report on the isolation and Ad-mediated gene transfer to AMCM has been published [5]. In that study, it was suggested that successful isolation and culture of AMCM over several days was dependent on the type of culture medium used. However, potential differences in the effects of culture medium on Ad-mediated gene transfer in AMCM were not investigated. Therefore, to confirm and extend the studies of Zhou *et al.* [5], we compared Ad-mediated transduction efficiency in two different culture media commonly used in cardiomyocyte culture, minimal essential medium (MEM) and medium 199 (M199) [2,3,5]. Our results demonstrate dramatic differences in the efficiency of Ad-mediated gene transfer between these two media in AMCM, but not in a number of other cultured cell types including primary rat neonatal cardiomyocytes. Gene transfer efficiency was correlated with levels of coxsackie-adenovirus receptors (CAR) on AMCM, suggesting a potential cell-culture-induced effect on CAR expression. These findings have direct implications for a growing number of studies using Ad-mediated gene delivery in AMCM to unravel mechanisms of cardiac cell function in normal and disease states.

Materials and methods

Cell culture

Adult mouse cardiomyocytes (AMCM)

Adult C57BL/6 mice (8 weeks old) were used in these studies. Mice were fed standard chow (Harlan) and water *ad libitum*. All procedures were performed according 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). AMCM were isolated from adult mouse left ventricle according to the protocol of Zhou *et al.* [5] except for the following modifications: (1) The series of three Ca²⁺ 'restoration solutions' (Ca²⁺ solutions I, II, III [5]) contained 0.25, 0.5, and 0.75 mM Ca²⁺, respectively; (2) after the final Ca²⁺ restoration step, AMCM were plated in laminin-coated dishes (GIBCO) in M199 (Sigma M3274) or MEM (Sigma M1018) containing NaHCO₃ (0.75g/l). Both media were supplemented with 2.5% fetal bovine

serum (FBS; GIBCO), 100 U/ml penicillin and 100 µg/ml streptomycin. The AMCM isolation procedure, from perfusion of the heart with collagenase through the Ca²⁺ restoration steps, was routinely completed in 2–3 h. In all experiments outlined below, AMCM were cultured in the respective media containing 2.5% FBS for 2–3 h to allow the cells to attach and to recover from the isolation procedure prior to incubation in FBS-free conditions and experimental treatments. Viable myocytes were defined by their rod-shaped appearance, sarcomeric striations and non-contractile quiescent state [5].

Other cultured cells

A variety of other cell types were compared with AMCM with regard to Ad-mediated gene transfer efficiency in MEM vs. M199. Transformed muscle (C₂C₁₂ [6], H₉C₂ [7]) and non-muscle (Hela [8]) cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Primary cultured non-muscle cells (ferret fibroblasts) were cultured as described previously [9], and primary rat neonatal cardiomyocytes [10] were prepared using a cardiomyocyte isolation system (Worthington Biochemical Corporation, Lakewood, NJ, USA) according to the manufacturer's instructions. All cell types were cultured in MEM or M199 under identical conditions as described above for AMCM and used at 60–80% confluence.

Recombinant adenovirus preparation and transduction

Recombinant E₁-deleted adenoviral vector encoding β-galactosidase (Ad.CMV.LacZ) was amplified in 293 cells and viral stocks were purified through two rounds of CsCl banding as described previously [4,11]. Viral aliquots were stored in 10% glycerol PBS at –80 °C until use. After 2–3 h of culture in FBS-containing medium as described above, AMCM cells were inoculated with Ad.CMV.LacZ at a multiplicity of infection (MOI) of 1–100 plaque-forming units (pfu)/cell in FBS-free MEM or M199 for 1 h. Virus-containing medium was then decanted, cells were washed three times with fresh medium, followed by incubation in FBS-free MEM or M199 for 24 h. The other cell types were infected with Ad.CMV.LacZ (20 pfu/cell) in FBS-free MEM or M199 under identical conditions used for AMCM.

Quantification of β-galactosidase expression

Viral transduction efficiency was evaluated by two different methods. Cytochemical staining for LacZ was performed 24 h post-infection. Cells were fixed in 4% paraformaldehyde for 4 min at room temperature and then stained with X-Gal (1 mg/ml) for 2 h as described previously [4,11]. The percentage of X-Gal-positive cells was determined by counting the number of blue-stained

myocytes relative to all myocytes counted in 5 to 8 randomly selected visual fields for each condition. Only viable, rod-shaped myocytes were counted. To directly measure β -galactosidase enzyme activity, lysates from AMCM and other cultured cells were prepared in 1 \times lysis buffer (Promega, Madison, WI, USA) 24 h post-infection with Ad.CMV.LacZ. Enzyme activity was quantified using the Galacto-Light Plus assay kit (Tropix, Bedford, MA, USA) according to the manufacturer's instructions. Both the cytochemical staining and enzyme activity experiments were performed in triplicate on separate batches of cells.

Immunocytochemical detection of CAR levels and adenovirus binding in AMCM

AMCM were cultured for 2–3 h in FBS-containing MEM or M199 as described above except for being plated in laminin-coated chamber slides (Nalge Nunc Interna, Inc., Naperville, IL, USA). After 1 h in FBS-free MEM or M199, AMCM were fixed in 4% paraformaldehyde for 4 min at room temperature, washed three times with PBS and then incubated in 10% horse serum for 2 h at room temperature. CAR expression was detected by incubating with goat anti-human CAR antibody (1 : 300 dilution, Santa Cruz, MA, USA) overnight at 4 °C followed by incubation with rhodamine-conjugated anti-goat IgG (1 : 300 dilution, Santa Cruz, MA, USA) for 1 h at room temperature. The specificity of immunostaining was confirmed by pre-incubating primary CAR antibody with CAR blocking peptide (1 : 5 w/w ratio, Santa Cruz, MA, USA). To determine CAR levels in freshly isolated AMCM, cells were plated in chamber slides immediately following the Ca²⁺ restoration steps and allowed 1 h to attach (in final Ca²⁺ restoration solution) before fixation and staining as described for cultured AMCM.

To detect adenovirus binding/internalization, AMCM were cultured in MEM or M199 as described above and were infected with Ad.CMV.LacZ (20 pfu/cell) for 1 h at 37 °C. Cells were then fixed in 4% paraformaldehyde for 4 min at room temperature and labeled with mouse anti-adenovirus antibody (1 : 40; Lab Vision, CA, USA) by incubating overnight at 4 °C, followed by 1 h incubation with rhodamine-conjugated secondary antibody (1 : 100 dilution, Santa Cruz, MA, USA) at room temperature. Fluorescent images were captured on a Zeiss 510 confocal microscope.

Western blot analysis of CAR expression in AMCM

AMCM were first incubated in MEM or M199 containing 2.5% FBS and then cultured in FBS-free MEM or M199 for 1 h as described above for immunocytochemistry. Cells were then lysed in hypotonic solution containing 5 \times protease inhibitor cocktail (Roche Inc., Indianapolis, IN, USA) by 10 rounds of 5-s sonication. After a quick

spin at 100 g at 4 °C for 7 min to remove cell debris, supernatants were centrifuged at 25 000 g at 4 °C for 2 h. The cell-membrane-containing pellets were dissolved in 1 \times protease inhibitor solution [12], and equal amounts of protein (200 μ g) were resolved by electrophoresis on pre-cast 4–15% gradient SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA). After transfer to cellulose membrane (0.2 μ m; Bio-Rad) and blocking in 3% non-fat dry milk, membranes were incubated in goat anti-human CAR antibody (1 : 200 dilution; Santa Cruz, MA, USA) at room temperature for 1.5 h. After washing, membranes were incubated with HRP-conjugated secondary antibody (1 : 1000; Santa Cruz, MA, USA) for 1.5 h at room temperature. Finally, CAR expression was visualized using an enhanced chemiluminescence system (ECL) (Amersham, Piscataway, NJ, USA).

Statistical analysis

Data are expressed as mean \pm SEM, and were analyzed by Student's *t* test. A probability (*p*) value of <0.05 was considered statistically significant.

Results and discussion

Effect of culture medium on viability and adenovirus transduction efficiency of AMCM

The combination of somatic and germ line transgenesis has become a powerful strategy for dissecting molecular mechanisms of disease. The recent explosion in available mouse models of heart disease, coupled with advances in viral gene delivery systems, provides an unprecedented opportunity to unravel cardiac cell function in health and disease. The methods developed by Zhou and colleagues [5] to isolate and maintain healthy AMCM in culture for several days provide an important tool to help realize these opportunities. Our study was intended to extend this work by establishing culture conditions for optimal Ad-mediated gene delivery in AMCM.

Initial efforts focused on implementing the methods of Zhou *et al.* [5], including use of the same source of MEM (Sigma M1018), to obtain high yield and good quality AMCM cultures. Our results were successful when the protocol was modified to include: (1) increased Ca²⁺ concentrations in the Ca²⁺ restoration phase (0.25, 0.5, 0.75 vs. 0.125, 0.25, 0.5 mM); and (2) doubling the NaHCO₃ concentration in MEM from 0.375 to 0.75 g/l to maintain the pH of aerated medium at 7.2 (see Materials and methods). With these changes, the vast majority of the myocytes remained healthy and viable, as indicated by retention of the characteristic rod-shaped and striated morphology, for at least 48 h. We routinely isolated 1.5 to 2 million viable cells from one left ventricle of an 8-week-old mouse. Without these modifications to the cell culture

protocol, in our hands, many of the myocytes assumed a spherical shape and died within 24 h.

Since M199 is another very common cell culture medium for cardiomyocytes [2,3,13], we also attempted to culture AMCM in this medium. Interestingly, unlike the report by Zhou *et al.* [5], we found that M199 also produced high yield, viable myocytes in culture for at least 48 h. However, to our surprise, we found dramatically different Ad transduction efficiencies in AMCM cultured in M199 compared with MEM. As seen in representative photomicrographs in Figure 1A, AMCM grown in MEM were effectively transduced with the β -galactosidase transgene, as indicated by uniform, robust X-Gal-positive staining. In contrast, very few M199-cultured AMCM expressed β -galactosidase. Overall, of a total of 2884 M199-cultured myocytes counted over three separate experiments, only $5 \pm 1.2\%$ were X-Gal positive. In contrast, the majority of AMCM cultured in MEM were transduced with β -galactosidase ($90 \pm 8\%$, 1670 myocytes counted, $p < 0.001$ vs. M199). These results were corroborated in studies measuring β -galactosidase activity (Figure 1B). MEM-cultured AMCM showed significantly elevated enzyme activity levels compared with cells cultured in M199. Interestingly, even at viral concentrations as high as 100 MOI, AMCM cultured in M199 were very poorly transduced (data not shown). For MEM-cultured AMCM, we observed maximal Ad-mediated gene

transfer at 20 pfu/cell. Thus, the remainder of our studies were performed at this viral concentration.

Lack of consistency in reagent quality from manufacturers often makes it very challenging to reproduce published data. Initially, we used the same MEM (Sigma M1018) used by Zhou *et al.* [5], in an attempt to reproduce their results. Subsequently, we used different batches of media from Sigma and GIBCO to demonstrate that a lack of Ad-mediated transduction in M199 is not due to problems with a particular batch of this medium from Sigma. We obtained identical medium-specific differences with Ad-mediated transfection efficiency in MEM and M199 using different batches of powder media (MEM, M1018; batches #50k8300 and #50k8301; M199, M3274; batch #40k83062; Sigma) as well as reconstituted liquid medium supplied by the manufacturer (MEM, batches #1088791 and #1113736; M199, batch #114076; GIBCO) (data not shown). Importantly, the same lot of FBS was used in all of our experiments.

Although MEM and M199 are composed of nearly identical elements, there are a number of differences between the two media that could account for our observations with regard to Ad transduction efficiency. For example, MEM contains glutamine (0.292 g/l), while ferric nitrate (0.0072 g/l) is only present in M199. Therefore, to test whether these differences account for the

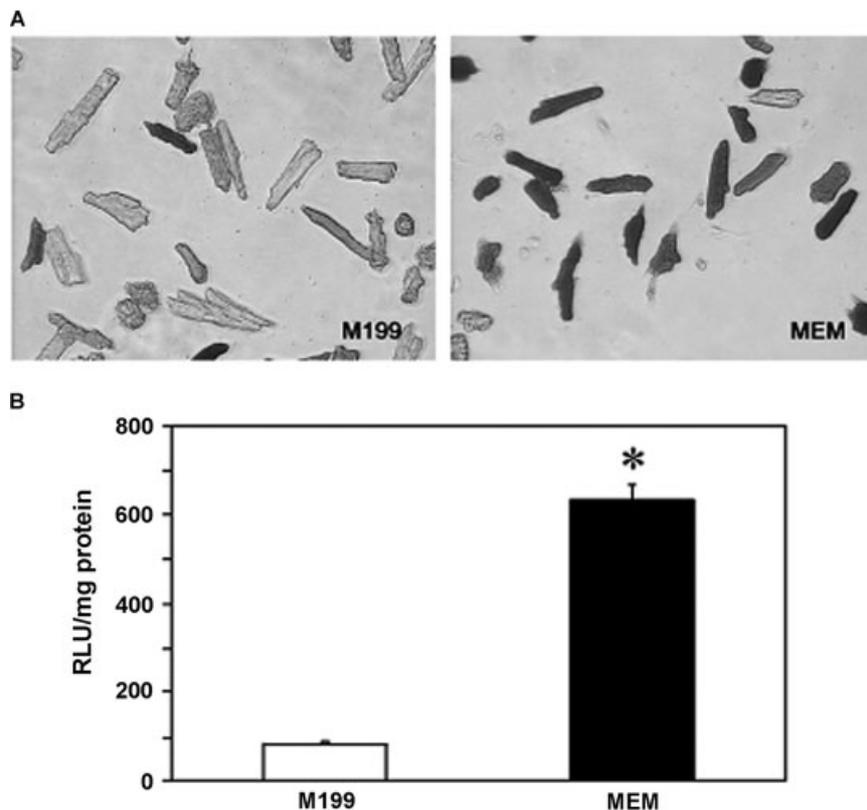


Figure 1. Culture medium markedly affects Ad-mediated gene transfer in AMCM. After 2–3 h in culture in FBS-containing MEM or M199, AMCM were infected with Ad.CMV.LacZ (20 pfu/cell) in FBS-free MEM or M199 for 1 h. After 24 h incubation in virus-free MEM or M199, LacZ expression was determined by cytochemistry (A) and by measuring β -galactosidase activity (B). (A) is representative of six separate experiments carried out on different batches of cells. (B) Values are mean \pm SEM, ($n = 3$). * $p < 0.05$ MEM vs. M199

observed differences in Ad transduction of AMCM, we performed reciprocal complementation experiments, i.e. supplementation of MEM with ferric nitrate and M199 with glutamine. Interestingly, neither of these modifications had any effect on viral transduction efficiency (data not shown), suggesting that these differences alone do not contribute to the differential effect of the two media. However, this does not rule out the possibility that these two factors in combination, or some other factor(s), account for the differences in Ad transduction efficiency. Indeed, a more comprehensive study of the effects of individual components alone or in combination may shed more light on the potential mechanisms.

Effect of culture medium on adenoviral transduction is specific to AMCM

To further test whether the culture-medium-dependent difference in Ad transduction efficiency is a ubiquitous phenomenon or is unique to AMCM, we examined Ad transduction efficiency in a variety of transformed and primary cells cultured in MEM vs. M199. Surprisingly, none of these other cell types, including rat neonatal cardiomyocytes, showed the culture-medium-associated differences in Ad transduction efficiency observed in AMCM (Figure 2). These results are in agreement with published reports where reproducible and robust Ad-mediated gene transfer into many cell types including rat neonatal and adult cardiomyocytes has been observed in cells cultured in several different culture mediums including M199 [2–4,9,11,13–16]. These findings further support the contention that medium-specific differences in Ad transduction in AMCM are specific to AMCM and not due to ubiquitous problems with Ad transduction in M199.

M199 culture medium inhibits binding/internalization of adenovirus to AMCM

Successful Ad transduction is based on efficient viral binding, signaling, internalization, endosomal escaping, intracellular trafficking and nuclear gene transcription and translation [17,18]. To explore whether the differential culture medium effects observed in AMCM were due to differences in adenovirus binding/internalization in these cells, we analyzed viral particle levels with immunocytochemical staining. Since it has been shown that virus binding/internalization is at a maximum by 1 h post-infection [19], we performed immunocytochemistry using anti-Ad antibodies in MEM- and M199-cultured AMCM 1 h after Ad.CMV.LacZ treatment. Indeed, AMCM cultured in MEM showed robust Ad-positive staining 1 h post-infection, whereas there were very few viral particles associated with M199-cultured AMCM (Figure 3, left and middle panels). AMCM cultured in MEM and stained with the secondary antibody only served as a negative control (right panels). Although staining with anti-Ad antibodies cannot discriminate between cell-membrane-associated or internalized virus particles, our data do suggest that M199 inhibits Ad binding and/or internalization in AMCM.

Increased viral transduction in MEM correlates with enhanced expression of CAR

To determine the mechanisms of decreased adenovirus binding/internalization in M199 compared with MEM, we next evaluated the expression of Ad-binding receptors in AMCM cultured in the two media. It has been demonstrated that the efficient entry of adenovirus is

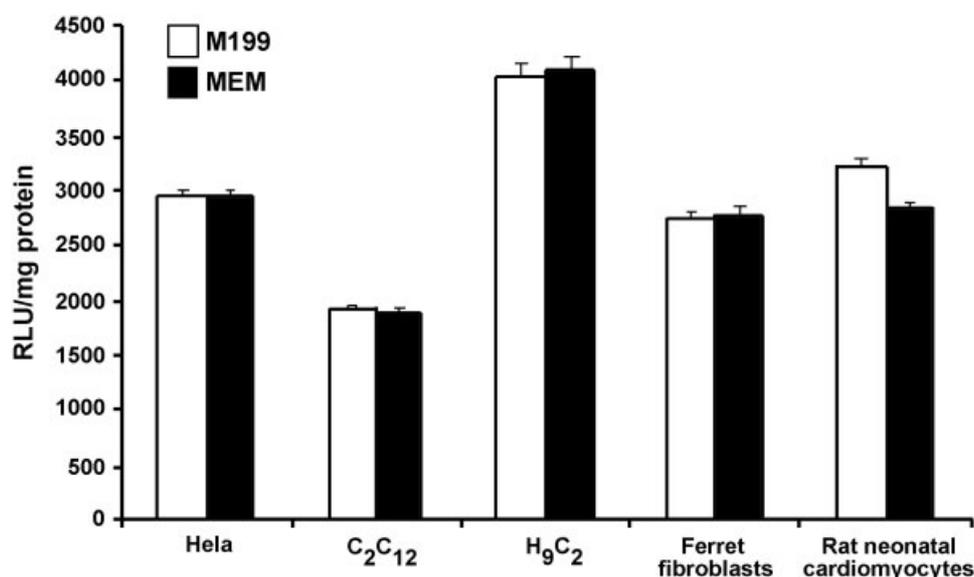


Figure 2. Culture medium has no effect on Ad-mediated transduction in other cell types including rat neonatal cardiomyocytes. Cells were cultured and transduced with Ad.CMV.LacZ (20 pfu/cell) in MEM or M199 as described in Figure 1. Transduction efficiency in different cell types was evaluated by measuring β -galactosidase activity. Data are mean \pm SEM, n = 3

dependent on the high affinity coxsackie-adenovirus receptor (CAR) for adenovirus fiber protein and the low affinity alpha v integrin for adenovirus penton base [18,20,21]. Numerous studies have shown that Ad transduction efficiency is directly correlated with the levels of cell membrane CAR and/or alpha v integrin expression [22–25].

To begin to unravel the mechanism of decreased Ad binding to AMCM in M199 (Figure 3) and decreased Ad transduction in this medium (Figure 1), we compared CAR levels in AMCM cultured in MEM or M199 vs. freshly isolated cells. Interestingly, only very low levels of CAR expression were detected in freshly isolated AMCM, while AMCM cultured in MEM for 3–4 h exhibited dramatically increased CAR levels (Figure 4A). Pre-incubation of MEM-cultured AMCM with CAR-specific blocking peptide completely abolished staining, demonstrating CAR-specific antibody binding (Figure 4A, last column). In contrast to MEM, CAR protein was nearly undetectable in M199-cultured AMCM, with levels comparable to freshly isolated cells (Figure 4A). These effects of culture medium on CAR expression were further confirmed by Western blot analysis of membrane fractions from AMCM cultured in MEM or M199 (Figure 4B). We also performed alpha v integrin immunocytochemistry in AMCM cells cultured in either MEM or M199, but no differences in levels of this protein were observed between the two media (data not shown).

The mechanisms of medium-specific regulation of CAR expression in AMCM are currently unknown but are the subject of ongoing investigations. Our findings that freshly isolated AMCM exhibit very low levels of CAR expression suggest that culture of these cells in MEM induces CAR expression and/or translocation to the membrane, while culture in M199 fails to do so. However, this conclusion

is difficult to reconcile with recent evidence of CAR expression in the adult murine heart *in vivo* [26], and our recent studies showing efficient adenoviral transduction of mouse hearts *in vivo* [27]. Our findings that freshly isolated cells contain very little CAR are surprising in light of these studies, and lead us to suggest that perhaps the cell isolation procedure itself initiates a protein degradation and/or sequestration mechanism that causes a loss of CAR receptors in freshly isolated AMCM. This may in turn be differentially modulated by MEM and M199, such that MEM causes 'restoration' of CAR levels and/or their localization to the membrane, whereas M199 does not. Interestingly, Ito *et al.* [28] have shown that culture medium can affect CAR expression in rat cardiomyocytes. Clearly, more studies will be required to sort out the precise mechanisms of medium-specific regulation of CAR levels in AMCM.

In summary, our results demonstrate that Ad-mediated gene transfer to AMCM is highly dependent on the culture medium. AMCM cultured in M199 were difficult to transduce (<5% transduction) even at MOI as high as 100 pfu/cell, while cells cultured in MEM could be effectively transduced (>90% transduction) at a concentration of 20 pfu/cell. These differences in viral transduction efficiency in the two different media correlated with the expression of CAR in the cell membrane. To the best of our knowledge, this is the first report demonstrating such remarkable culture-medium-dependent differences in Ad-mediated gene transfer and in CAR expression levels. These results have important implications for future studies that may utilize Ad-mediated gene transfer into adult cardiomyocytes isolated from a growing number of mouse models of human cardiac diseases.

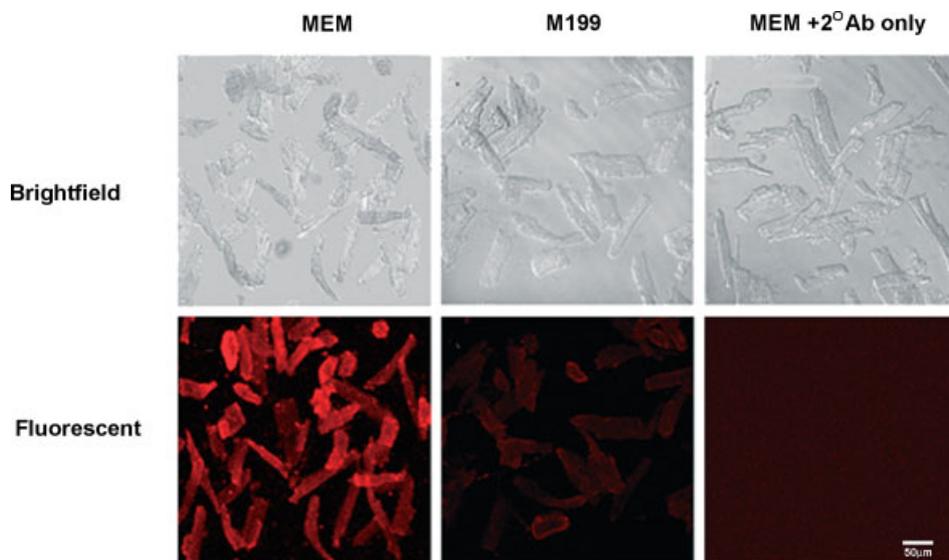


Figure 3. Culture in MEM increases Ad association with AMCM. Cells were cultured and transduced with Ad.CMV.LacZ (20 pfu/cell) in MEM or M199 as described in Figure 1. Viral particle levels in cells cultured in the two media were analyzed by immunocytochemistry using anti-Ad antibodies. Representative bright field and corresponding fluorescent images are shown for AMCM cultured in MEM and M199. Cells cultured in MEM but incubated with secondary (2°) antibody only served as a control for non-specific staining. Data are representative of two separate experiments carried out on two different batches of cells

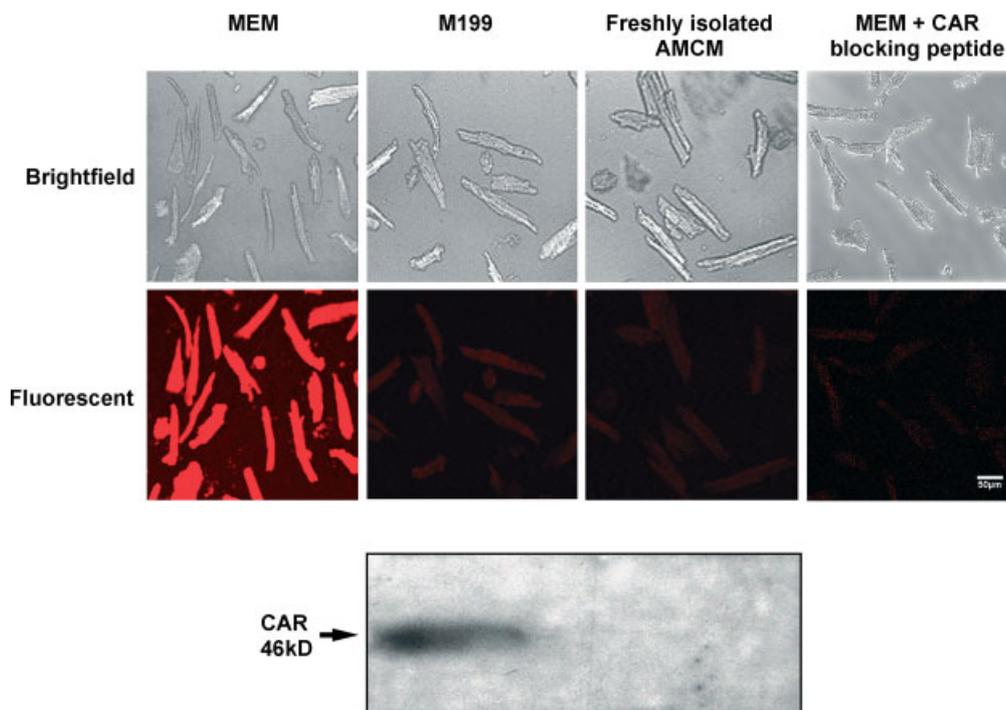


Figure 4. Culture of AMCM in MEM increases coxsackie-adenovirus receptor (CAR) expression. CAR expression in AMCM was evaluated by immunocytochemistry (A) and Western blot analysis (B) using anti-CAR antibodies. For immunocytochemistry, cells were fixed either 1 h following plating onto laminin-coated chamber slides (freshly isolated AMCM) or after culture in FBS-containing MEM or M199 for 2–3 h followed by 1 h in FBS-free conditions. Western analyses were carried out on membrane fractions from AMCM cultured in MEM or M199 as described for immunocytochemistry. Representative bright field and corresponding fluorescent images are shown for each treatment group in (A). Cells cultured in MEM and pre-incubated with CAR-specific blocking peptide served as a control for non-specific staining. A typical Western blot is shown in (B). Data are representative of three experiments for both immunocytochemistry and Western blot analysis carried out on three different batches of cells

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