Antimyogenic Effect of SARS-CoV Spike Protein in C2C12 Myoblasts

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Abstract: C2C12 myoblasts serve as well-established model system to study myogenesis, as they fuse to form multinucleated myotubes. Severe acute respiratory syndrome coronavirus (SARS–CoV) spike (S) protein plays a crucial role in viral entry. Exogenous expression of S protein in C2C12 myoblasts inhibits the formation of myotubes. Global changes in gene expression were studied in C2C12 cells expressing S protein using oligonucleotide microarray analysis. The expression profile showed that, most of the myogenic marker genes were downregulated. Next, we used RT-PCR analysis to reexamine some downregulated and upregulated genes. To further study the antimyogenic effects induced by the S protein, we introduced antisense Plf (proliferin), an upregulated gene, into the antimyogenic cells. Antisense Ace2 (angiotensin-converting enzyme 2), the cellular receptor of S protein, was also introduced into C2C12 myoblasts. Results indicated that antimyogenic effect induced by S protein was partially rescued in cells expressing antisense Plf, while C2C12 cells expressing antisense Ace2 showed upregulation of Plf.

Keywords: Ace2, microarray, myogenesis, proliferin.

INTRODUCTION

Highly contagious SARS–CoV affected around 30 countries with more than 8000 cases of infection resulting in 800 fatalities (http://www.who.int/csr/sars/country/en/). Spike protein (S) of this virus was responsible for virus binding, fusion and entry, hence became a main focus of study as a major inducer of neutralizing antibodies against the disease. Functional studies in S protein were mainly concentrated on the interaction with ACE2 (angiotensin-converting enzyme 2) receptor as a preliminary requirement for cell-viral membrane fusion. It has been demonstrated that S protein decreases surface level expression of human and mouse over-expressed Ace2 in 293 cells and deteriorated acute lung failure upon in vivo injections [1].

Myogenesis is an important process during embryonic development and muscle regeneration. Murine skeletal muscle cells (C2C12 myoblasts), are known to have pluripotent mesenchymal character, which allows them to differentiate into adipocyte [2] and osteoblast [3] lineages. C2C12 myoblasts are extensively used to study differentiation of skeletal myogenesis [4]. We have previously established a method for quantifying the cell-cell fusion mediated by S-ACE2 interaction [5], C2C12 cell line stably expressing S (C2C12-SG) was used to confirm the process. The key stage of myogenesis involves the fusion of myoblasts to form myotubes. Hence, we explored the effects of S protein on cell-cell fusion during myogenesis. Muscle weakness and elevated creatine kinase levels were shown to be associated with SARS [6]. Few cases of rhabdomyolysis associated with probable SARS cases were reported as well [7]. However, the nature and cause of myopathy in association with SARS remains unknown. In this paper, we show that the S protein inhibits myogenesis by upregulating Plf (proliferin). ACE2 is known to balance the role of angiotensin-converting enzyme (ACE) in renin-angiotensin system (RAS) in conversion of angiotensin II (AngII) to Ang 1-7, a vasodilator and helps in decreasing blood pressure [8]. The existence of RAS constituents in skeletal muscle, including ACE, Ang II receptor, and local de novo Ang II production [9] had been reported, suggesting existence of RAS in myogenesis. Therefore, in this study we were interested in understanding the antimyogenic effect of S protein and additional factors required to facilitate the effect as well. We were also interested in understanding whether the antimyogenic effect of S protein was responsible for downregulation of Ace2 in C2C12 cells.

MATERIALS AND METHODOLOGY

Cell Culture

C2C12 cells were purchased from ATCC (CRL 1772; Bethesda, MD), and cultured in a humidified incubator at 37 °C with 5 % CO2 in DMEM (growth medium, GM) containing 1 g L−1 glucose, 1X Pen/Strep (0.1 mg ml−1 streptomycin, 100 U ml−1 penicillin, Sigma) and 10 % FBS (HyClone). C2C12-SG and C2C12-E2G were selected using 1 mg ml−1 G418 (Gibco BRL) while C2C12-AE2CER cells were selected using 0.5 mg ml−1 of Hygromycin B (Roche). C2C12-SG-MyoD, C2C12-SG-PLFR and C2C12-SG-ZAKI-4R cells were grown in medium containing both the antibiotics. To induce differentiation, nearly confluent C2C12 cells were incubated in DMEM containing 10 % heat-inactivated horse

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serum (Invitrogen) and 1X Pen/Strep (differentiation medium, DM) for 7 days unless addressed specifically.

Analysis of Gene Expression by Oligonucleotide Microarray and RT-PCR

For microarray expression analysis, total RNA was extracted from C2C12 and C2C12-SG cells cultured in GM or DM using the RNeasy Kit (Qiagen). RNA samples (10 µg each) were processed, labeled and hybridized to Affymetrix MOE 430A GeneChips according to standard protocols available from Affymetrix. For RT-PCR analysis, total RNA was extracted from C2C12 myoblasts and derivative cell lines using Trizol reagent (Gibco BRL) following manufacturer's protocol. 1 µg of total RNA from each sample was reverse transcribed using Superscript II RNase H - reverse transcriptase (Invitrogen). 1 µl of the product was then used as the template for PCR amplification using specific primers listed in Supplementary Data (SD) 1 Table 1. PCR was performed using HotStar Taq DNA polymerase (Qiagen) under following conditions: 95 ºC for 2 min, followed by 33 cycles of 95 ºC for 30 sec, 55 ºC for 30 sec, 72 ºC for 45 sec, and a final extension at 72 ºC for 5 min. Amplification of Ace2 was an exception as it required 42 cycles. All RT-PCR were repeated more than three times for consistent results.

Myod, or anti-sense Ace2 (ACE2R), Plf (PLFR) and ZAKI-4 (ZAKI-4R) were amplified using Expand High Fidelity PCR System (Roche), and same PCR conditions were used except for Myod which required extension of 90 sec at 72 ºC. Representative RT-PCR products were sequenced to confirm their identity. PCR template for Myod was from mouse skeletal muscle cDNA [10], for PLFR and ZAKI-4R from C2C12-SG cDNA and for ACE2R from C2C12 cDNA. Primers used for construction and comparison of Myod, ACE2R, PLFR and ZAKI-4R are listed in SD1.

Plasmid Constructs and Establishment of Stable Cell Lines

C2C12-SG and C2C12-E2G cell lines were established as previously reported [5]. Plasmid pAM (SD1) containing Myod, PLFR, ZAKI-4R and ACE2R was transfected into C2C12-SG or C2C12 cells using lipofectamine (Invitrogen). Stable transfectants C2C12-ACE2R were selected on Hygromycin B medium while C2C12-SG-MyoD, C2C12-SG-PLFR and C2C12-SG-ZAKI-4R were selected on medium containing both G418 and Hygromycin B. C2C12-SG derivative cell lines were established by pooling maximum of 10 colonies and surface expression of S protein was observed using cell-cell fusion assay [5].

RESULTS AND DISCUSSION

Defective Myogenic Phenotype of C2C12-SG Cells

Myogenesis of C2C12 cells is characterized by growth factor depletion (cultured in DM) and cell cycle exit followed by myoblast cell-cell fusion to form myotubes. C2C12-SG cell line, cultured in DM did not form myotubes whereas cell line C2C12-E2G [5] expressing ectodomain of Hepatitis C virus E2 protein showed normal myogenesis (SD1 Fig. 1). C2C12-SG and C2C12-E2G cells express ectodomains of S [1,173 amino acid (a.a.) residues] and E2 (310 a.a. residues) respectively, apart from that both the fusion proteins were identical. Hence, we conclude that the ectodomain of S protein was solely responsible for myogenic inhibition in C2C12-SG cells.

Differential Myogenic Gene Expression in C2C12 and C2C12-SG Cell Lines

Myogenesis involves dynamic and complex set of processes. Microarray technology was exploited to analyze the

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Fig. (1). Comparative analysis of microarray and RT-PCR results for nine genes. RNA samples for both analyses were prepared from C2C12 (A & B) and C2C12-SG (C & D) cells cultured in GM and DM respectively. RNA samples from C2C12-SG-MyoD cells are shown in lane E & F represent cells grown in GM and DM respectively. Bolded number represent highest microarray signal of the gene.
differential expression of genes in C2C12 and C2C12-SG cells grown in GM or DM (SD2). Microarray analysis between C2C12-SG and C2C12 cells cultured in DM medium showed 1.188 genes (SD2-1) have log2-ratios (LR) below -1 (two fold downregulated) and 944 genes (SD2-2) above 1 (two fold upregulated). Skeletal muscle genes such as *Tncs* (troponin C, fast skeletal; LR-10.4), *Myhl* (myosin heavy chain 2X, LR-10.2), *Tnnl* (troponin I, skeletal, slow 1; LR-10.2) and *Myh2* (myosin, heavy polypeptide 2; LR-10) were the most downregulated genes in DM cultured C2C12-SG cells. *Myod* (LR-7), one of the key players in myogenesis, was strongly downregulated as well. Our data indicated that the expression of myogenic marker genes in C2C12-SG cells were remarkably inhibited, however, the cell line still retained myoblast characters as seen by the expression of *Des* (desmin), the gene for muscle-specific intermediate filament protein. The reliability of the microarray data (SD2) was further confirmed by studying the expression of enzymes involved in TCA cycle to confirm that relevant signals among all 4 samples were uniform.

Rhabdomyolysis refers to disintegration of striated muscle. Pathogenesis of myolysis is often associated with bacterial, fungal and viral infections. This is thought to be the result of direct cell invasion and cellular degeneration by the pathogen. Influenza A and B are most common viruses associated with myolysis, studies estimate that up to 25% of AIDS (Acquired immuno deficiency syndrome) patients suffer from a myopathic disease that may be complicated by rhabdomyolysis [11].

It has been shown that *Myod* plays a key role in conversion of normal dermal fibroblasts and chondroblasts into elongated post-mitotic mononucleated striated myoblasts [12]. It has also been reported that overexpressed MyoD was able to rescue the myogenic defect [13]. As a first attempt to rescue the myogenesis in C2C12-SG cells, we developed another cell line C2C12-SG-MyoD that expressed *Myod* ectopically, however, the myogenic phenotype could not be rescued. Amongst all the up and down regulated transcripts, we further selected upregulated *Plf* (LR 5.6), *Thbs2* (thrombospondin 2, LR 5.4), *ZAKI-4* (calcineurin inhibitory protein, LR 2.3) and *Nfl* (neurofilament-L, LR 5.6) genes and myogenic markers *Myod*, *Myf5* (LR 7.7). *Ckm* (muscle type creatine kinase, LR -4.8), *Myh1* and *Des* genes for confirmation by RT-PCR. However, RT-PCR amplification results of *Myf5* and *Nfl* did not match with the microarray signals (Fig. 1) as RT-PCR amplifications showed the relatively constant level of both the RNA samples. Both microarray and RT-PCR analyses correlated well with respect to upregulated genes *Plf*, *Thbs2* and *ZAKI-4*, as well as downregulated genes *Myod*, *Ckm* and *Myh1* (Fig. 1, lanes A-D). The ectopic *Myod* expression reduced the upregulation of *Plf*, *Thbs2* and *ZAKI-4* when grown in DM. The downregulation of *Ckm* was also similarly reversed, however *Myh1* remained unaffected (Fig. 1, lanes E, F).

We also checked expression of other genes that are involved in myogenesis or important in muscle development by RT-PCR, most of the genes tested were very weak or absent in the microarray data such as *Mstn*, *Pax7* and *Agr2* (primers are listed in SD1 Table 1). We did not observe any signals relative to these genes either from C2C12 or C2C12-SG cDNA samples, but we detected high expression levels of myostatin (*Mstn*) from mouse skeletal muscle cDNA sample (data not shown). C2C12 cells cultured in DM showed upregulation of P21, cyclin-dependent kinase inhibitor, which facilitates cell cycle withdrawal in differentiating myoblasts. Microarray data (SD2) showed that irrespective of the medium used for culturing C2C12-SG cells expressed same levels of P21 as undifferentiated C2C12 cells, however the results of RT-PCR (SD1 Fig. 2) showed reverse P21 regulation of C2C12 and C2C12-SG cells.

Partial Myogenic Rescue of C2C12-SG Cells by Antisense *Plf*

*Plf* is a secreted glycoprotein in the prolactin-growth hormone family. It has been shown to partially inhibit the differentiation of C2C12 cells by reducing the level of *Myod* mRNA [14] and downregulating reporter driven expression by *Ckm* promoter [15]. ZAKI-4, inhibits calcineurin which regulates skeletal muscle differentiation. To determine the myogenesis recovering ability of C2C12-SG cells we used antisense RNA of these two upregulated genes. However, we could not establish C2C12-SG clones expressing antisense *Thbs2*, which was one of the target genes.

To drive the ectopic expression of targeted genes, promoter of *Takifugu rubripes* beta actin1 (GenBank accession no. U37499) was used for the expression in C2C12 or C2C12-SG cells. EGFP expression of both undifferentiated C2C12 myoblasts and differentiated C2C12 myotubes is shown in SD1 Fig. (3). The expressions of ectopic *Myod*, antisense *Plf* (*PLFR*) and *ZAKI-4* (*ZAKI-4R*) were driven by the promoter. C2C12-SG-PLFR and C2C12-SG-ZAKI-4R are two C2C12-SG derivative cell lines expressing *PLFR* or *ZAKI-4R*. Our RT-PCR results showed that antisense genes were expressed only in modified cell lines but not in their respective controls (Fig. 2A). Expression of *Myod*, *Ckm*, *Myhl*, *Plf* and *Myf5* in cell lines C2C12-SG, C2C12-SG-PLFR and C2C12-SG-ZAKI-4R cultured in DM (Fig. 2B) were studied as a next step. Among the three downregulated myogenic genes *Myod*, *Ckm* and *Myh1* studied, only *Ckm* was fully recovered in C2C12-SG-PLFR cells while *Myod* was partially recovered where as *Myh1* changed trivially. The myogenesis inhibition in C2C12-SG cells were partially rescued with *PLFR* and indicating that downregulation of *Ckm* in C2C12-SG cells is certainly linked to the expression of *Plf* which is in concordance with *Ckm* promoter studies [15]. As for the *Plf* expression, C2C12-SG-PLFR had the lowest level of expression when compared to other two cell lines C2C12-SG and C2C12-SG-ZAKI-4R, therefore the expression of *PLFR* and *Plf* were compared (Fig. 2C) using cDNA samples from C2C12-SG-PLFR cells. C2C12-SG-PLFR cells cultured in DM showed partial recovery of myogenic phenotype (Fig. 2D). Shorter and lesser myotubes differentiated from C2C12-SG-PLFR culture which, may be due to partially recovered myogenic genes. Our data suggested that the myogenic defect in C2C12-SG culture was partly due to upregulated *Plf* or more efficient *Plf* downregulation is required for complete recovery of myogenesis.

**Uregulation of *Plf* in Ace2 Antisensed C2C12 Cells**

Ectodomain of S protein is much bulkier than ectodomain of E2 protein by 863 a.a residues. Because of this major difference between C1C12-SG and C2C12-E2G cell
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Fig. (2). Expression of (A) PLFR (antisense Plf) and ZAKI4R (antisense ZAKI-4) in C2C12-SG-PLFR and C2C12-SG-ZAKI-4R cells, (B) expressions of Myod, Ckm, Myh1, Plf, Myf5 genes in C2C12, C2C12-SG, C2C12-SG-PLFR and C2C12-SG-ZAKI-4R cells, (C) PLFR and Plf in C2C12-SG-PLFR cells after RT-PCR. PLFR and Plf (SD1) has 70 nt difference between them. Des was used as the internal control for (A) and (B). (D), Shorter myotube formation from C2C12-SG-PLFR cells cultured in DM. The photos were taken under 400 x magnifications. Left panel indicates brightfield and right panel photo indicates fluorescence microscopy fitted with FITC filter. The presence of green fluorescence is due to the S protein tagged with EGFP at C-terminus.

hACE2: GIDGRKKKNAKRSGENPASYIDISKGHENPGFQNTDDVQTSF
mAce2: GIKKRKKKNETRREEENPYSMDKGEKSNAGFQNSDDAQTSF
cAce2: GIKRRKKNQARSEENPYSVLKSGHENPGFQHADDVQTSF
bAce2: GIRRRRKKNQASSEENPYSGLPNKGENNSGQNOIDDVQTSF
gAce2: GQRDRKKKARGRNEGSCNCEVNPYEDGRSNKGFQSEETQTFTS
xAce2: GYKERTKQKSGDKEAVELSQANNPENPATLEITQENTGQMNKAF
fAce2: GVRERRKPIAVNPNYIDNDDGHNKAYDSDNEQTGF
tAce2: GVRERRKKPKAVHNPYIEDDGQINKAYDSDNEQTGF

Fig. (3). Cytoplasmic domains of ACE2s and ACEs. The source of ACE2s is prefixed by h (human, GenBank accession no. BAB40370), m (mouse, GenBank accession no. AAH26801), c (cat, GenBank accession no. Q56128), b (cattle, GenBank accession no. AAI05341), g (chicken, GenBank accession no. XP_416822), x (Xenopus tropicalis), f (Fugu) and t (Tetraodon). The sequences of Xenopus tropicalis, Fugu, and Tetraodon ACE2 are listed in the Supplementary Data 1 of [22]. ACEs are prefixed by h (human, GenBank accession no. EAW94312), m (mouse, GenBank accession no. AA10363), r (rat, GenBank accession no. AAP80809) and o (rabbit, GenBank accession no. AAA31153; the domain of ACE in sheep, pig, horse and dog etc. are highly similar to oACE). Amino acid residues represent the consensus motif of tyrosine kinase phosphorylation sites are bolded and italicized (K/RXXE/DXXY), bolded residues (S/TXXE/D) are for casein kinase II and bolded and underlined (S/TXXE/D) are for protein kinase C.
lines, we suspected that S protein may impinge on the surface membrane remodeling which is very much required for myoblasts cell-cell fusion. However, microarray and RT-PCR analysis clearly showed that myogenic inhibition of C2C12-SG cells were not just morphological but was due to altered global gene expression patterns, thereby leading to the possibility of altered downstream signaling events.

RAS enzymes ACE and ACE2 have been in main focus of study with respect to their ability to regulate blood pressure in cardiovascular, kidney and lung diseases. The role of RAS in myogenesis has been studied in stimulation of hypertrophy by Ang II in C2C12 myoblasts [16]. It was demonstrated that ACE inhibitor and AT2 antagonist significantly upregulated myogenesis in differentiating C2C12 cells resulting in hypertrophic myotubes [17]. The short cytoplasmic tail of ACE at a.a. residue Ser<sup>1270</sup>, phosphorylated by casein kinase II (CK2), was shown to physically interact with the kinase [18]. The phosphorylation site is involved in the regulation of enzyme shedding (cleavage/secrection). Mitogen-activated protein kinase 7 and c-Jun N-terminal kinase were also found to be associated with the cytoplasmic domain of ACE, hence in ACE signaling cascade [18]. Although ACE2 is relatively new player in RAS system, it is a well known cellular receptor of the S protein. To study the possibility of ACE2 in cell signaling, 8 ACE2 and 4 ACE sequences were compared (Fig. 3). Protein cytoplasmic domains of these proteins from different vertebrates showed conserved phosphorylation sites of tyrosine kinase, CK2 and protein kinase C. The motifs were predicted based on Motif Scan at http://myhits.isb-sib.ch/cgi-bin/motif_scan [19]. The consen-

![Fig. (4). Expression of (A) ACE2R (antisense Ace2) in C2C12-ACE2R cells, (B) ACE2R and sensed Ace2 in C2C12-ACE2R cells, (C) Plf expressions in C2C12-ACE2R, C2C12, C2C12-SG, C2C12-SG-PLFR cells that were cultured in GM, DM or 10 d in DM, (D) Ace2, Myod1, Ckm and Myh1 in C2C12-ACE2R, C2C12, C2C12-SG, C2C12-SG-PLFR cells cultured in DM for 10 days. Des was used as the internal control for (A) and (D).](http://myhits.isb-sib.ch/cgi-bin/motif_scan)
interesting issue to be explored. However, C2C12-ACE2R cells, with downregulated Ace2 and upregulated Plf, still differentiated, formed myotubes, and also expressed myogenic marker genes Myod1, Ckm and Myh1. In addition, the expression level of Plf increased in C2C12-ACE2R cells cultured in DM for 10 d. Ckm was not downregulated suggesting the regulation of ACE2R was not fully equivalent to the regulation of S protein.

CONCLUSION

In this paper, we show that ectodomain of SARS-CoV S protein inhibits myogenesis of C2C12 myoblasts, and the effect was partially due to upregulated Plf. We demonstrate that the downregulation of mouse Ace2 plays a role in upregulating Plf. Expression levels of Plf, also known as mitogen-regulated protein, is high during the transition from G1-S phase of cell cycle which prohibit myoblasts withdrawal from cell cycle, however reverse and time dependent upregulation in C2C12-ACE2R cells cultured in DM remains to be unveiled. Mxi1 had been shown to inhibit C2C12 myogenesis, it was identified to dedifferentiate C2C12 myotubes into mononucleated cells with the induced capability to redifferentiate into cells that express chondrogenic, adipogenic, myogenic, or osteogenic markers [20]. To test the hypothesis, we tried to work in same way with Tet-On system, the dedifferentiation effect did not appear suggesting the signaling roles of either Plf or Ace2 are relatively downstream. Finally, severe impairment in myocardial contractility with higher AngII levels has been observed in Ace2 null mice, and this cardiac defect was completely rescued by genetic ablation (double knockouts) of Ace2 [21]. The antagonist of antmyogenic (Ace2 downregulated by S protein) and hypermyogenic (ACE inhibitor) effects offers an alternative angle to study.

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ABBREVIATION

RT-PCR = Reverse transcription-polymerase chain reaction

SUPPLEMENTARY MATERIAL

This article also contain supplementary data and it can be viewed at www.bentham.com/open/toidj

REFERENCES