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Proteomic profile of human monocytic cells infected with dengue virus

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ABSTRACT

Objective: To identify the changes in the proteome of U937 cells infected with dengue virus (DENV).

Methods: In this study, differentiated U937 cultures were infected with two DENV-2 strains, one of which was associated with dengue (DENV-2/NG) and the other one with severe dengue (DENV-2/16681), with the aim of determining the cellular proteomic profiles under different infection conditions. Cellular proteins were extracted and separated by two-dimensional electrophoresis, and those proteins with differential expression profiles were identified by mass spectrometry. The obtained results were correlated with cellular viability, the number of infectious viral particles, and the viral DNA/protein quantity.

Results: In comparison with non-infected cultures, in the cells infected with the DENV-2/NG strain, nine proteins were expressed differentially (five were upregulated and four were downregulated); in those cultures infected with the DENV-2/16681 strain, six proteins were differentially expressed (two were downregulated and four were upregulated). The downregulated proteins included fatty acid-binding protein, heterogeneous nuclear ribonucleoprotein 1, protein disulfide isomerase, enolase 1, heat shock 70 kDa protein 9, phosphotyrosyl phosphatase, and annexin IV. The upregulated proteins included heat shock 90 kDa protein AA1, tubulin beta, enolase 1, pyruvate kinase, transaldolase and phospholipase C-alpha.

Conclusions: Because the monocyte/macrophage lineage is critical for disease pathogenicity, additional studies on these proteins could provide a better understanding of the cellular response to DENV infection and could help identify new therapeutic targets against infection.

1. Introduction

Dengue is the most common disease in humans that is transmitted by arthropods. It is caused by the dengue virus (DENV) [1], which is transmitted by the bite of infected mosquitoes, principally those of the species *Aedes aegypti* and *Aedes albopictus* [2]. This virus, whose genome is made up of positive-strand RNA, belongs to the Flaviviridae family and the *Flavivirus* genus [3]. Although, in general, the existence of four serotypes that share antigenic similarities but are genetically different has been reported in the urban viral cycle setting (DENV-1 to DENV-4), the existence of a fifth serotype has recently been reported (DENV-5) in a wild cycle setting [4].

Dengue affects the populations of tropical countries, where more than 2 500 billion people are at risk of being infected, and it is endemic in over 100 of these countries [5]. Its prevalence has increased dramatically in the last few decades, and recent studies estimate that 96 million cases occur on a yearly basis, although this number may be underestimated in some regions due to poor

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clinical surveillance [1]. From a clinical perspective, dengue is a self-limiting febrile illness characterized by headaches, muscle aches, and skin eruptions. The most severe form of the disease is known as severe dengue in which there is increased vascular permeability that results in plasma leakage; other symptoms include thrombocytopenia, hemorrhagic manifestations, and compromised organs, such as the liver, heart, and central nervous system [6].

Although the pathogenesis of severe dengue is not completely clear, some of the risk factors involved in its development have been described (some related to the host and some to the virus). Of the factors related to the virus, it has been reported that the viral genetic variations partly determine virulence and, therefore, epidemic potential [7], which allows certain strains with higher replicative potential to spread more easily in a primary infection. In addition, some serotypes and strains have been associated with the development of severe forms of the disease [8]. In this regard, some DENV strains within serotype 2 have been reported to be associated with the development of mild or severe forms of the disease, as is the case for the New Guinea strain (DENV-2/NG) (originally isolated from a dengue patient) [9], and the DENV-2/16681 strain (isolated from a patient with severe dengue) [10]. Based on their origin and etiology, the DENV-2/NG strain has been associated with the development of dengue [11], and the DENV-2/16681 strain has been associated with severe dengue [12]. Regarding host-associated factors, much has been discussed regarding the pathogenic role of the immune response to a secondary infection. In this regard, the principles of antibody-dependent enhancement and original antigenic sin are the most studied [13,14]. Independent of the factors favoring the development of severe dengue, whether in a primary or secondary infection, there are some cells that play a very important role in the initial capture of the virus, its transport to specific sites within the host, and its replication and propagation.

Monocytes, macrophages, and dendritic cells are considered the primary targets of DENV infection *in vivo* [15,16], although the presence of the virus in different cell types such as hepatocytes, lymphocytes, endothelial, epithelial, neuronal, and muscle cells has also been reported [17]. The monocyte/macrophage lineage, which is normally involved in the processes of the innate and adaptive immune responses, is successful in the elimination of most pathogens, although pathogens sometimes use this strategy to their advantage. In the case of dengue, macrophages are not only the main targets of DENV for its replication but also responsible for spreading the virus to different parts of the hosting organism after its transmission and for enhancing proinflammatory cytokine production, responsible in great measure for the development of severe dengue [15,18]. Within this lineage, the U937 cell line has been widely used to study different aspects of DENV infection.

The U937 cell line, isolated from human histiocytic lymphoma, shows characteristics of immature monocytes, but its differentiation promoted with phorbol esters converts it to a cell line with the morphologic and functional characteristics of macrophages [19]. This cell line can be infected with DENV by two different mechanisms: through cell surface receptors (heat shock 70 kDa protein and heat shock 90 kDa protein), thus allowing the study of conventional forms of viral entry into the host cell during primary or secondary infection, and through Fc receptors that bind viral complexes with antibodies during secondary infections [20,21]. This cell line has been used for studies on viral replication [22], on the evaluation of antiviral agents [23–25], on the immune response [26], *etc*.

Despite the importance and broad use of the U937 cell line, no studies have been performed in which protein expression changes have been determined in this cell line when infected by DENV. For this reason, the present study identified the modifications in the U937 proteome after infection with two strains of DENV-2 clinically associated with dengue: the New Guinea strain DENV-2/NG (associated with dengue) and the DENV-2/16681 strain (associated with severe dengue) under primary infection conditions.

2. Materials and methods

2.1. Cell maintenance and viral stock production

Differentiated-U937 cells (human monocyte derived from lymphoma), and C6/36HT cells (derived from *Aedes albopictus*) were a kind gift from Dr. Jaime E. Castellanos at the El Bosque University (Bogotá, Colombia) and Dr. Guadalupe Guzman at the Pedro Kouri Institute (Havana, Cuba). Both cell lines were grown in Dulbecco's modified Eagle medium (Gibco/Invitrogen, Grand Island, NY, USA) supplemented with 0.25 μg/mL of amphotericin B, 100 μg/mL streptomycin, 100 units/mL penicillin, and either 2% (U937 cells) or 10% (C6/36HT cells) fetal bovine serum (FBS, Gibco). Cell cultures were incubated with 5% CO_2, at either 37 °C (U937 cells) or 34 °C (C6/36HT cells). For all assays, two serotype 2 reference strains were used: strain DENV-2/NG (dengue reference strain) and strain DENV-2/16881 (severe dengue reference strain). Both strains were a kind gift from Dr. Jorge Osorio at the University of Wisconsin (Madison, WI, USA). The viral stocks were expanded in the C6/36HT cell line (five passages for each viral strain) and stored at −70 °C until used.

2.2. MTT viability assay

A colorimetric MTT assay was used to measure the effect of the infection on U937 cell viability. For this assay, 2.5×10^4 cells were seeded in 96-well plates and were infected [multiplicity of infection (MOI): 1] at 24 h. The infected cells were incubated for an additional 48 h. Then, 50 μL of MTT (0.5 mg/mL) were added, and the cultures were incubated for 3 h at 37 °C. Then, 100 μL of dimethyl sulfoxide (Fisher Scientific Inc., Rockford, IL, USA) were added, and the absorbance was read at 450 nm using a Varioskan Flash reader (Thermo Scientific Inc., Rockford, IL, USA). The data were processed by comparing the absorbance of the infected cultures with the absorbance of the uninfected control cultures. The results were expressed as the mean of at least three independent experiments, each with two replicates ($n = 6$).

2.3. Cell infection

A total of 3×10^6 U937 cells were seeded in 25 cm^2 cell culture flasks. After 24 h, the cultures were incubated with each of the reference stocks (DENV-2/NG or DENV-2/16681) for 2 h at 37 °C at an MOI of 1. Afterwards, the virus was removed, and the cultures were cultured for an additional 48 h, after which the supernatant of each culture was collected and stored at −70 °C for subsequent infectious viral particle quantitation

(by the plaque method). The cell monolayers were used for viral genome quantitation (by real-time RT-PCR) and for protein extraction. There were five replicates of each experimental condition (DENV-2/NG- or DENV-2/16681-infected cultures), as well as five replicates for the uninfected control cultures.

2.4. Fluorescence microscopy

U937 cells were grown on glass coverslips at a density of 2.5×10^4 cells per coverslip and infected with DENV-2/NG or DENV-2/16881 at a MOI of 1. At 48 h after inoculation, cell monolayers were washed with phosphate buffered saline (PBS) and fixed with 3.8% paraformaldehyde in PBS at 37 °C for 30 min. The monolayers were permeabilized with 0.5% Triton X-100, blocked with 5% FBS and incubated with primary anti-DENV antibody (1:500) followed by incubation with anti-mouse secondary antibody conjugated with fluorescein isothiocyanate and Hoechst 33258 dye. Finally, the cells were washed with PBS and the coverslips were mounted. Images were obtained using an inverted microscope (IX-81 Olympus).

2.5. Viral quantitation by the plaque method

A total of 5×10^4 U937 cells were seeded in 24-well plates. After 24 h, the cells were inoculated with 200 μL serial dilutions (1×10^{-1} to 1×10^{-5}) of collected previously viral supernatants. After 2 h of incubation, the virus was removed and Dulbecco's modified Eagle medium was supplemented with 2% FBS, and 1.5% carboxymethyl cellulose (Sigma–Aldrich, St. Louis, MO, USA) was added. The plates were incubated for 8 days at 37 °C and 5% CO_2. Then, the monolayers were fixed with 4% para-formaldehyde (Sigma–Aldrich) and stained with a crystal violet solution (Sigma–Aldrich). The plaques were counted to determine the plaque-forming units (PFU/mL) of the viral inoculum. The results were expressed as the mean of five independent experiments, each with two replicates ($n = 20$).

2.6. Viral quantitation by reverse transcription, followed by real-time PCR (RT-qPCR)

The viral genome was quantified in the collected previously cell monolayers using real-time RT-PCR according to the methodology previously described [27]. For this, total RNA was extracted using the modified RNAzol method and a total of 0.5 μg of RNA was reverse transcribed using 0.5 μg/mL of random primers (Promega Corp., Madison, WI, USA) and 200U of M-MLV reverse transcriptase (Promega). The cDNA was amplified by real-time PCR (qPCR) using SYBR Green and DENV-2-specific primers (mD1 and mTS2). These primers amplify a 119 bp segment of the C-prM region. Amplification was conducted in a SmartCycler (Chepeid, Sunnyvale, CA, USA), and the genomic copy amounts were calculated with an absolute quantitation, using a specific standard curve for DENV-2. The results were expressed as the mean of five independent experiments ($n = 5$).

2.7. Protein extraction

The cells obtained from infected and uninfected cultures were dislodged using 0.25% trypsin, spun at 1 500 r/min for 5 min, and washed twice with PBS. The cells were lysed using hypotonic PBS (13.6 mmol/L NaCl, 0.27 mmol/L KCl, 0.4 mmol/L Na_2HPO_4, and 0.15 mmol/L KH_2PO_4) and subjected to five freezing (in liquid nitrogen) and thawing (using ultrasound) cycles. The insoluble material was removed by centrifugation, and the supernatant was used to precipitate proteins using acetone. The precipitate was resuspended in hydration buffer (9 mol/L urea, 4% CHAPS, 1.6% 3–10 ampholytes, 1% 4–7 ampholytes, and 40 mmol/L dithiothreitol), and the protein concentration was determined using a 2D Quant kit (GE Healthcare, Piscataway, NJ, USA).

2.8. Two-dimensional electrophoresis

A total of 200 μg of protein obtained from each of the experimental conditions was loaded onto 7-cm DryStrip strips with a non-linear pH of 3–10 (GE Healthcare) and allowed to hydrate passively for 12 h. The first dimension (isoelectric focusing) was conducted at 20 °C and with a 50 μA current per strip, using the Ettan IPGphor 3 system (GE Health-care) until 14 800 volt-hours was reached. The strips were equilibrated by incubating them in buffer I (6 mol/L urea, 2% sodium dodecyl sulfonate, 375 mmol/L Tris–HCl pH 8.8, 20% glycerol, and 10 mg/mL of dithiothreitol) for 20 min, followed by incubation in buffer II (6 mol/L urea, 2% sodium dodecyl sulfonate, 375 mmol/L Tris–HCl pH 8.8, 20% glycerol, 25 mg/mL of iodoacetamide) for 20 min. After equilibration, the strips were placed on 12.5% acrylamide/bis-acrylamide gels, and the second dimension was conducted using the Mini-PROTEAN system (Biorad, Hercules, CA, USA) at 100 V for 90 min. The gels were stained using the Oriole fluorescent kit (Biorad, Hercules, CA, USA) for 90 min. A total of 5 gel replicates for each experimental condition was obtained ($n = 5$).

2.9. Image analysis and protein expression comparison

Gel images were captured using a ChemiDoc XRS scanner (Biorad), and the images were analyzed using ImageMaster 2D Platinum 7.0 software (GE Healthcare). The parameters used to detect spots were as follows: smooth, 4; saliency, 8; and minimal area, 3. To determine the spots displaying differential expression, the relative volume of spots expressed in U937 cells infected with each of the reference stocks and in uninfected cultures were compared using the image analysis software.

2.10. Protein digestion and MALDI-TOF/TOF mass spectrometry

The differentially expressed protein spots were excised from the gel, washed 3 times with 50% acetonitrile (ACN) and 50 mmol/L ammonium bicarbonate (NH_4HCO_3) for 10 min, and dehydrated with 100% ACN for 10 min. Then, 20 μg/mL trypsin in 25 mmol/L NH_4HCO_3 was added, and the proteins were digested overnight at 36 °C. The next day, ACN was added for 10 min, and the extracted peptides were dried in a vacuum centrifuge. After centrifugation, the peptides were resuspended in a 50% methanol/0.1% trifluoroacetic solution, and each sample was dropped on a MALDI plate containing a matrix solution (50% alpha-cyano-4-hydroxycinnamic acid in ACN, 0.1% trifluoroacetic, 10 mmol/L ammonium citrate). The samples were analyzed with an ABI 4800 MALDI-TOF/TOF mass spectrometer (MS), with a mass range of 700–4 000 Da. Peptides

with a signal-to-noise ratio above 20 in MS mode were selected for tandem mass spectrometry (MS/MS). MS/MS was conducted using air as a collision gas and 2 kV collision energy. A maximum of 45 MS/MS spectra were allowed for each spot.

2.11. Protein identification

Protein identification based on the obtained MALDI-TOF/TOF spectra was conducted using GPS Explorer™ protein analysis 3.6 software (Applied Biosystems, Foster City, CA, USA). This software conducts a combined search of the MS and MS/MS data against the non-redundant database of the National Center of Biotechnology (NCBI nr), using the MASCOT® search engine (Matrix Science, Boston, MA, USA). Trypsin was defined as the digesting enzyme, whereas methionine oxidation and lysine acetylation were defined as peptide modifications. Mass tolerances of 80 mg/kg for precursor ions and 0.5 Da for the corresponding fragments were allowed. The bioinformatics analysis was conducted by sequence homology of proteins belonging to *Homo sapiens*. The biological functions of the identified proteins were assigned according to gene ontology, and the identified proteins were classified according to their cell function.

2.12. Statistical analysis

Student's *t*-test was used to compare the cell viability between infected and uninfected cultures. Similarly, this test was used to compare the number of infectious viral particles and the number of genomic copies between DENV-2/NG-versus DENV-2/16681-infected U937 cells. In both cases, $P < 0.05$ indicated a statistically significant difference. For the proteomic analysis, an ANOVA test was used to compare the level of intensity (volume intensity) for each spot in each condition (infection with DENV-2/NG, infection with DENV-2/16681 and non-infection). Finally, comparisons with $P < 0.05$ were further assessed with Tukey's *post-hoc* test.

3. Results

3.1. Effect of DENV-2 infection on U937 cell viability

An MTT assay was conducted to determine the effect of virus infection on cell viability. No significant differences were observed at 48 h post-infection when DENV-2/NG- or DENV-2/16681-infected U937 cells were compared to the uninfected control culture ($P < 0.05$) (Figure 1). DENV-2 infection did not affect U937 cell viability. Moreover, neither of two viral strains produced morphological changes in the cells U937 (Figure 2). Based on these results, subsequent assays were conducted with a 48-h incubation period to guarantee that any proteome changes were due to viral infection and not due to infection-induced cell death.
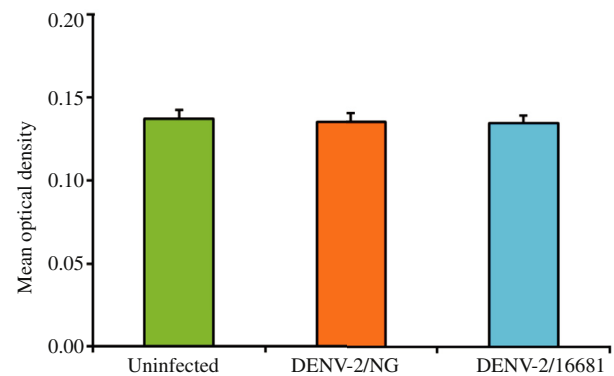

Figure 1. Effect of DENV serotype 2 on cell viability. The values represent the means of 3 independent experiments, each with 2 replicates for each condition ± SEM ($n = 6$).
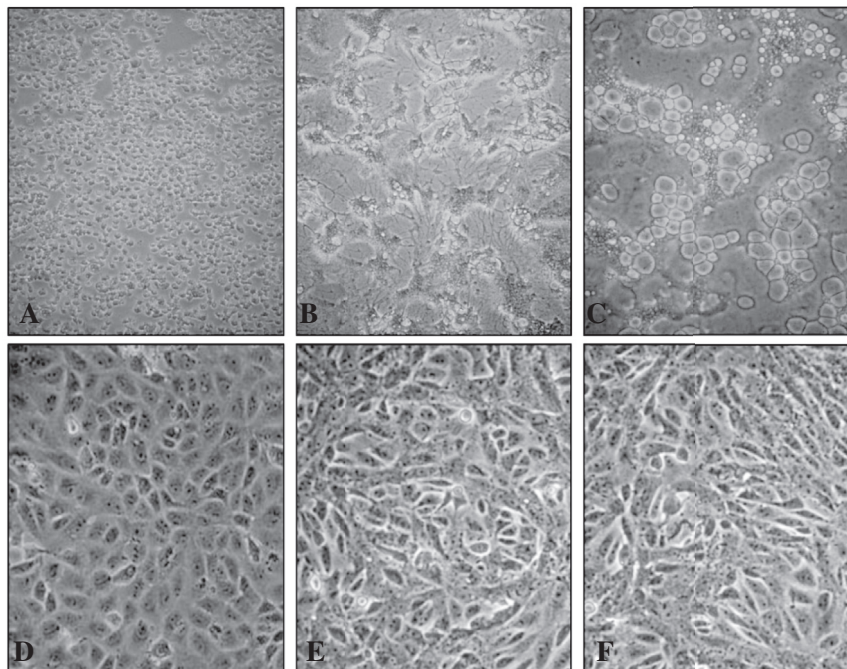

Figure 2. Effect of DENV serotype 2 on the morphology of U937 cells.
A–C: C6/36HT cells; D–F: U937 cells; A and D: Uninfected cell cultures; B and E: Cell cultures infected with DENV-2/NG; C and F: Cell cultures infected with DENV-2/16681. C6/36HT cells were used as a positive control of infection, and morphological changes were observed in these cells.

3.2. Comparison of the viral genome replication rates of the DENV-2/16681 and DENV-2/NG strains

To compare the infection in the cultures infected with either DENV-2/NG or DENV-2/16681, RT-qPCR was conducted to quantify the viral genome (in monolayers). Viral titering by the plaque method was used to quantify the infectious viral particles released into the medium (in the supernatant). Significant differences were observed in the number of genomic copies obtained from DENV-2/NG-infected cells (1.43×10^7 genomic

copies/mL) compared with the genomic copies from DENV-2/16681-infected cells (9.01×10^7 genomic copies/mL) ($P < 0.05$). Infection was greater in the latter strain (Figure 3A). On the other hand, no significant differences were observed in the number of infectious viral particles in the supernatant of DENV-2/NG-infected cells (2.19×10^6 PFU/mL) compared to DENV-2/16681-infected cells (2.54×10^6 PFU/mL) (Figure 3B). These results were consistent with the number of infected cells that was higher in cultures infected with DENV-2/16681 than those infected with DENV-2/NG (Figure 4).

3.3. DENV-2 infection modifies protein expression in a strain-dependent manner

To obtain the proteomes, the proteins extracted from infected and uninfected U937 cell monolayers were separated by two-dimensional electrophoresis. On average, 189 spots were identified in uninfected U937 cells, 156 spots in DENV-2/NG-infected cells, and 200 in DENV-2/16681-infected cells. The different proteins detected had isoelectric points (pI) between four and eight and molecular weights between 11 and 84 kDa. The differential expression analysis was conducted using the relative volumes of the expressed spots of the infected versus uninfected cultures. Only proteins with a differential expression
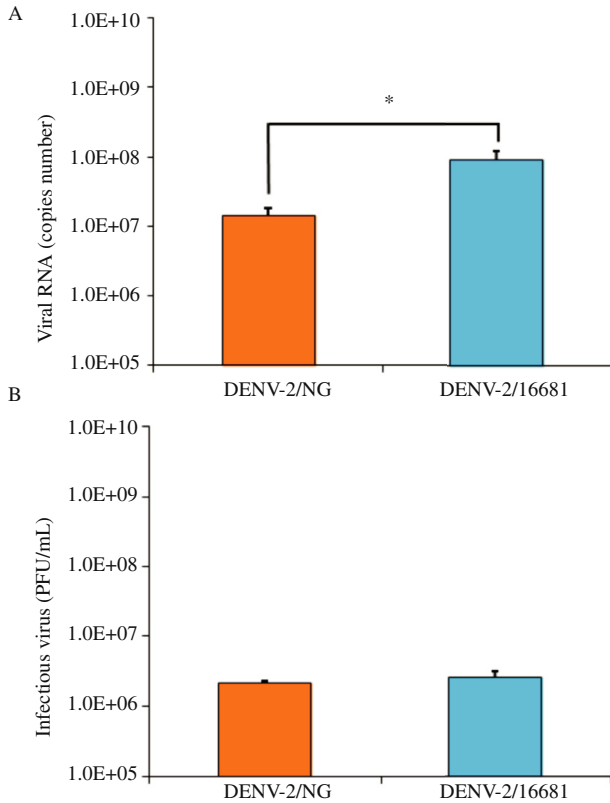

Figure 3. Comparison of the viral genome replication rates of the DENV-2/16681 and DENV-2/NG strains.
Viral production was measured 48 h after cultures were infected with an MOI of 1. A: Viral genome quantitation measured by RT-qPCR in monolayers; B: Infectious viral particle quantitation was measured using a supernatant plaque assay. Significant differences were only detected when the genome copy numbers were compared. The asterisk indicates $P < 0.05$ (student's *t*-test). The values represent the means of 3 independent experiments, each with 2 replicates for each condition ± SEM ($n = 20$).
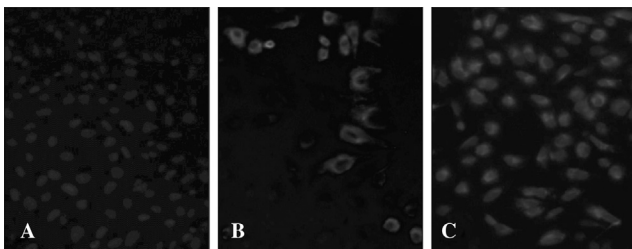

Figure 4. Comparison of the number of infected cells between cultures infected with DENV-2/16681 and cultures infected with DENV-2/NG.
A: Uninfected cell cultures; B: Cell cultures infected with DENV-2/NG; C: Cell cultures infected with DENV-2/16681.
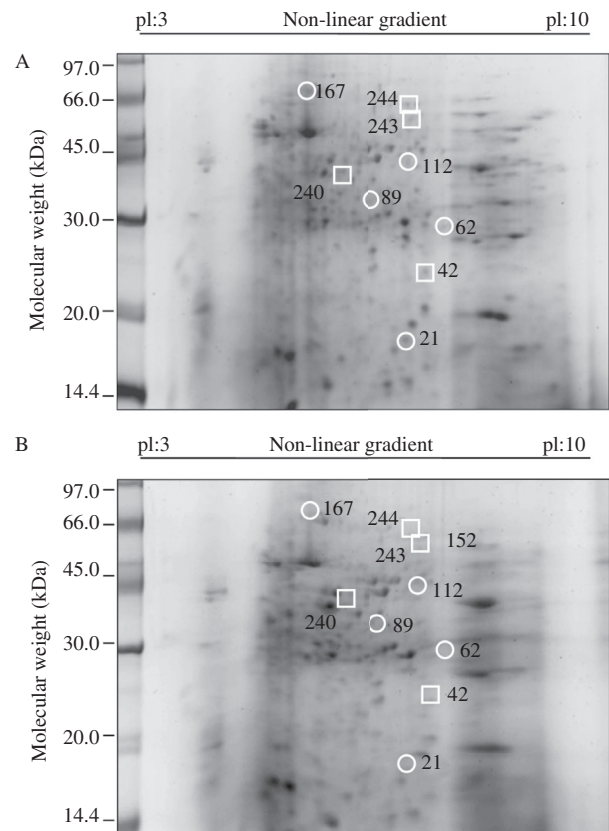

Figure 5. Differentially expressed proteins in uninfected cultures (A) compared with DENV-2/NG-infected cultures (B). A total of 200 μg of protein was separated by two-dimensional electrophoresis for each sample. The differentially expressed spots are indicated in the figures. The squares indicate proteins with increased expression in infected cultures, and the circles indicate proteins with decreased expression in infected cultures. A representative gel of the 5 gels that were analyzed is shown for each biological condition.

Figure 6. Differentially expressed proteins in uninfected cultures (A) compared with DENV-2/16681 infected cultures (B). A total of 200 µg of protein was separated by two-dimensional electrophoresis for each sample. The differentially expressed spots are indicated in the figures. The squares indicate proteins with increased expression in infected cultures, and the circles indicate proteins with decreased expression in infected cultures. A representative gel of the 5 gels that were analyzed is shown for each biological condition.
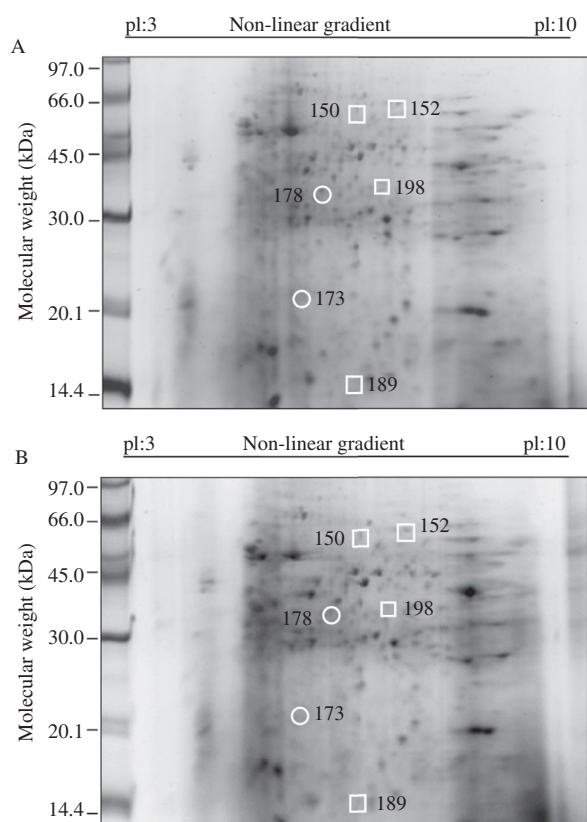
that was reproducible in at least four of the five analyzed gels were included. The relative expression change was considered significant if the ANOVA and Tukey's *post-hoc* tests had *P*-values < 0.05.

The protein analysis indicated nine differentially expressed proteins in DENV-2/NG-infected U937 cells. The expression of five of these proteins (Spots 21, 62, 89, 112 and 167) decreased in the infected cultures and four proteins (Spots 42, 240, 243 and 244) displayed increased expression compared with uninfected cultures (Figure 5A and B). The main percentage of differential expression was represented by proteins presenting decreased expression in the infected cultures (55.5%).

On the other hand, the protein analysis indicated that six proteins were differentially expressed in DENV-2/16681-infected U937 cells. The expression of two of these proteins (Spots 173 and 178) decreased in the infected cultures, whereas the expression of the remaining four proteins (Spots 150, 152, 189, and 198) increased in the infected cultures compared to the uninfected ones (Figure 6A and B). The main percentage of differential expression was represented by proteins displayed increased expression in the infected cultures (66.6%).

3.4. Protein identification by MS

Proteins with differential expression were identified by MALDI-TOF/TOF MS. The biological functions of these proteins were assigned according to gene ontology, and the identified proteins were classified into several different categories.

The proteins with decreased expression in DENV-2/NG-infected cultures compared with the uninfected cultures were as follows: fatty acid-binding protein (21), heterogeneous nuclear ribonucleoprotein 1 (hnRNP 1) (62), protein disulfide isomerase (89), enolase 1 (112) and heat shock 70 kDa protein 9 (167). The proteins that displayed increased expression in the infected cultures were heat shock 90 kDa protein AA1 (42), tubulin beta (240), enolase 1 (243) and pyruvate kinase M2 (244) (Table 1).

In DENV-2/16681-infected cultures, the proteins that displayed decreased expression were as follows: phosphotyrosyl phosphatase (173) and annexin IV (178). The proteins that displayed increased expression were as follows: pyruvate kinase (150), pyruvate kinase M2 (152), transaldolase (189) and phospholipase C-alpha (198) (Table 2).

Table 1

Comparison of proteins expressed in uninfected *vs.* DENV-2/NG-infected cultures.

Spot no.	Protein ID	NCBI accession	Molecular weight (kDa)	Number of matched peptides	Score MS & MS/MS	% Cover sequence	Volume intensity		Cell function
							Uninfected	DENV-2/NG infected	
21	Fatty acid-binding protein	gi|4557581	15.15	9	247	45.19	0.34	0.00	Lipid-binding protein
62	hnRNP 1	gi|48145673	49.10	16	322	30.07	0.28	0.00	RNA splicing
89	Protein disulfide isomerase	gi|119597640	54.05	17	512	37.50	0.16	0.00	Cell redox homeostasis
112	Enolase 1	gi|62897945	47.17	22	522	54.61	0.13	0.00	Carbohydrate metabolism
167	Heat shock 70 kDa protein 9	gi|12653415	73.68	21	601	32.99	0.47	0.00	Protein folding
42	Heat shock 90 kDa protein AA1	gi|38196932	49.16	14	534	24.64	0.29	0.67	Protein folding
240	Tubulin beta	gi|119608775	48.79	14	345	34.55	0.00	0.30	Cytoskeleton
243	Enolase 1	gi|203282367	47.01	24	526	52.66	0.00	0.32	Carbohydrate metabolism
244	Pyruvate kinase M2	gi|169404695	56.52	18	232	48.65	0.00	0.27	Carbohydrate metabolism

Table 2

Comparison of proteins expressed in uninfected *vs.* DENV-2/16681-infected cultures.

Spot no.	Protein ID	NCBI accession	Molecular weight (kDa)	Number of matched peptides	Score MS & MS/MS	% Cover sequence	Volume intensity		Cell function
							Uninfected	DENV-2/16681 infected	
173	Phosphotyrosyl phosphatase	gi|157837067	17.90	10	282	42.04	0.26	0.00	Signal transduction
178	Annexin IV	gi|189617	36.03	19	762	57.94	0.52	0.00	Calcium ion binding
150	Pyruvate kinase (human muscle)	gi|67464392	59.71	32	986	59.12	0.13	0.29	Carbohydrate metabolism
152	Pyruvate kinase M2	gi|169404695	56.52	31	841	62.16	0.13	0.27	Carbohydrate metabolism
189	Transaldolase	gi|5803187	37.52	6	92	17.56	0.00	0.10	Carbohydrate metabolism
198	Phospholipase C-alpha	gi|303618	56.67	11	190	28.71	0.00	0.20	Signal transduction

4. Discussion

Cells of the monocyte/macrophage lineage are of great importance during dengue's development because they are one of the primary targets for DENV, responsible for spreading it from the site of infection toward different sections of the body [15]. In addition, once infection has been established, these cells are the main responsible party for pro-inflammatory cytokine production, thus facilitating the development of severe forms of the disease [15,18]. Despite the important role that these cells have on the disease's pathogenesis (in both primary and secondary infections), to date, there is only one study that shows how some proteins from the undifferentiated monocyte cell line THP-1 are affected under primary infection conditions [28]. Therefore, this study evaluated the proteome changes in the differentiated monocyte cell line U937 after infection with two different viral strains, keeping in mind that the viral genetic variations have been associated with both the epidemic potential of the infection and disease severity.

Because it has been previously reported that different strains from the same serotype can promote significant changes in both morphology and viability in cell cultures [27], an initial comparison was performed on the viability of the non-infected cell cultures with those infected for 48 h, revealing that viability was not significantly impaired in the infected cultures (Figure 1), similar to the behaviors found for other cell lines of epithelial (VERO) or hepatic origin (HepG2) [29,30]. Significant changes in morphology were also not observed (Figure 2). This result offers assurance that the changes in the cellular proteome are caused by DENV infection and not cell death.

When performing the comparative analysis of the production of viral genome copies and infectious particles from each viral strain after infecting U937 cells, significant differences were detected only in viral RNA production. The cells infected with the DENV-2/16681 strain had a higher genomic copy number than those infected with the DENV-2/NG strain (Figure 3). This increased replication rate obtained with the strain associated with severe dengue had been previously reported in the VERO cell line [30] and could be related to the development of this clinical form of the disease, as some clinical studies have demonstrated higher levels of viral RNA in the plasma of patients with severe dengue in comparison with dengue patients [31]. However, a higher number of cells were immunolabeled against viral proteins in cultures infected with DENV-2/16681 (Figure 4), which could be correlated with the high viral protein levels detected in patients with severe dengue [32].

Previous studies have shown that the proteomes of epithelial cells (VERO) are significantly different when these cells are infected different viral strains [30]; a similar behavior was detected in this study when U937 cells were infected with either the DENV-2/NG (Figure 5 and Table 1) or DENV-2/16681 strains (Figure 6 and Table 2). The most relevant findings will be discussed next.

The fatty acid-binding protein was downregulated in U937 cells infected with the DENV-2/NG strain. Keeping in mind that lipids and fatty acids induce the formation of replication complexes in DENV infection, their downregulation would be unfavorable for viral replication in this cell type. This finding could be related with observations from other studies showing that the percentage of infected monocytes *in vitro* are much lower compared with the percentages obtained from epithelial, hepatic, and endothelial cell types [33]. However, keeping in mind that this same protein is upregulated in VERO cells infected with DENV-2/16681 [30], it could be surmised that the regulation of its expression could be affected by the infecting viral strain, which, in turn, could be correlated with differences in viral genome quantity, which is lower in cells infected with the DENV-2/NG strain compared with the DENV-2/16681 strain (Figure 3). Regardless, in the present study, there was a significant increment in the quantity of this protein in the cultures of monocytes infected with the DENV-2/1668 strain, which makes it impossible to prove this hypothesis.

Another protein that was downregulated in the cultures infected with the DENV-2/NG strain was disulfide isomerase, a protein that regulates platelet function and helps to maintain their structural integrity [34]. For this reason, its decreased expression could be related to the development of thrombocytopenia, which is characteristic of dengue [35]. However, decreased levels of this protein have also recently been detected in a pathologic condition known as macrothrombocytopenia [36], a finding that could be associated with what occurs in dengue infections, as it is known that platelet function is affected during disease development [37]. These results are also correlated with previous findings in VERO cells [30] in which downregulation of this protein was observed after infection with the DENV-2/16681 strain, which is related to platelet adhesion inhibition, thus allowing severe hemorrhage.

One of the isoforms of the enolase protein was upregulated in cultures infected with the DENV-2/NG strain, while another of the isoforms was downregulated in the same cultures. These findings agree with recent studies that showed increased levels of basic isoforms of enolase in hepatocytes, although its acid

isoforms had a decreasing tendency [38]. This multi-functional protein is not only involved in cytoplasmic processes such as glycolysis but is also expressed on the surfaces of diverse hematopoietic, epithelial and endothelial cells and serves as a receptor and plasminogen activator [39–41], thus fulfilling an important task in the intravascular coagulation and fibrinolytic system [41]. It has been proposed that enolase upregulation during DENV infection could be associated with the compromised vascular and coagulation systems present during severe dengue cases. The α-enolase protein could activate plasminogen, modify the coagulation-fibrinolysis balance and favor hyperfibrinolysis. In fact, several studies have reported increases in fibrin degradation products during DENV infection [42]. Enolase downregulation could be correlated with a decrease in the intracellular viral load due to an increase in the tumor necrosis factor-α level, a recently proposed mechanism in THP-1 cells [28].

hnRNP 1 is another of the downregulated proteins detected in cultures infected with DENV-2/NG. This finding contrasts with the results obtained from other groups that have shown that several isoforms of this protein are upregulated in endothelial cells infected with the DENV-2/16681 strain or in undifferentiated monocytic cells [28,43]. However, these differences could be explained considering that, as we previously demonstrated, the regulation of the expression of certain proteins is dependent on the cell type analyzed and the infecting viral strain [30]. However, decreased expression of this protein in monocytes could prevent the migration of infected cells, which would limit, eliminate or delay the development of severe forms of the disease, as it has been reported that this protein, when interacting with intermediate filaments, plays an important role in cellular migration [44].

Heat shock protein 70 kDa (Hsp-70) was upregulated in VERO cultures infected with DENV-2/16681 [30], and interestingly, downregulated in U937 cells. Keeping in mind that cells of epithelial origin replicate the virus more efficiently than monocytes as well as the role that this protein plays by interacting with the DENV E glycoprotein, facilitating viral replication and the production of viral particles [33,45], it could be postulated that its downregulation prevents the correct folding of viral proteins, making viral replication more difficult in these cells. In contrast, heat shock protein 90 kDa (Hsp-90) was upregulated; this protein is also required for viral replication, as has been previously reported [46]. Thus, it could be proposed that Hsp-70 downregulation would be compensated with Hsp-90 upregulation. While Hsp-90 plays a very important role in DENV internalization [20], suggesting that the capture and internalization of the virus in macrophages could be made more efficient by Hsp-90 upregulation, and viral replication would be less efficient due to Hsp-70 downregulation.

The only protein identified in this study whose expression was increased independent of the infecting viral strain (DENV-2/NG or DENV-2/16681) was pyruvate kinase. Keeping in mind that this enzyme is involved in the process of glycolysis for energy production in the cell, it has been proposed that modifications in its expression are associated with a higher energy requirement for DENV replication [30,38].

Infection with DENV-2/NG also increased the expression of β-tubulin, a cytoskeletal protein. Keeping in mind that it has been postulated that tubulin (the main component of microtubules) facilitates the trafficking of the DENV E protein [47], its

upregulation could facilitate viral assembly in these cells. However, its upregulation could also be correlated with a higher risk of developing severe forms of the disease, as increased tubulin expression has been detected in patient serum, an observation that has led to postulations on the suitability of tubulin as a biomarker for the development of severe forms of dengue disease [48].

The phosphotyrosyl phosphatase protein was downregulated, which could be related to an increase in nitric oxide (NO) production, as it has been demonstrated that this protein regulates NO production in macrophages [49]. In turn, the increase in NO could be considered a cellular defense mechanism, as a correlation between NO production and the development of non-severe forms of dengue disease has been reported [50].

Anexin 4 was also downregulated in cultures infected with the DENV-2/16681 strain. It was recently shown that anexin A4 regulates interleukin 8 (IL-8) production in macrophage cultures. When anexin is over-expressed in these cultures, IL-8 production is significantly decreased [51]. For this reason, keeping in mind that the results obtained in this study demonstrate a decrease in anexin A4 expression in cultures infected with the DENV-2/16681 strain, it would be postulated that this downregulation could lead to increased IL-8 expression. In turn, the increase in IL-8 has been associated with the development of severe forms of dengue disease, as higher levels of IL-8 have been detected in patients with severe dengue [52].

Transaldolase protein expression was increased in cultures infected with the DENV-2/16681 strain. This enzyme participates in the phosphate pentose pathway, which allows the production of ribose 5-phosphate, an important precursor in nucleotide synthesis required for RNA replication [53]. Thus, it is postulated that the increase in the expression of this enzyme may be associated with a higher replication rate of the DENV-2/16681 strain, which is correlated with the increase in the genome copy number of this strain (Figure 3A).

Finally, DENV-2/16681 infection also caused an increase in the expression of the phospholipase C protein, an enzyme that can cleave the viral non-structural protein 1 (NS1) on the surfaces of cells infected with DENV [54]. NS1 can be detected intracellularly, associated with the plasma membrane or in secreted form [55], the last of which is associated with the development of severe dengue, as the secreted NS1 protein can bind to prothrombin (a coagulation factor) and inhibit its activation, thus avoiding the development of a normal coagulation process and favoring hemorrhage [56]. Therefore, it is suggested that the upregulation of phospholipase C expression could induce an increase in the NS1 levels in patient serum and thereby contribute to the hemorrhagic symptoms characteristic of severe dengue.

This proteomic study identified changes in the expression levels of proteins in differentiated human monocytes in response to infection with two DENV strains. These proteins are involved in many biological processes, which may benefit viral replication or, alternatively, be related to disease development. Because monocytes are critical for the disease pathogenesis, additional studies on these proteins could lead to a better understanding of the cellular responses to DENV infection and to the identification of new therapeutic targets against this disease.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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