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Original article

NEWCASTLE DISEASE VIRUS SUPPRESSES ANGIOGENESIS IN MAMMARY ADENOCARCINOMA MODELS

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Summary

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Cancer cells heavily utilise angiogenesis process to increase vascularisation for tumour mass growth and spread, so targeting this process is important to create an effective therapy. The AMHA1 strain of Newcastle disease virus (NDV) is an RNA virus with natural oncotropism. NDV induces direct tumour cytolysis, apoptosis, and immune stimulation. This work aimed to test NDV anti-angiogenic activity in a breast cancer model. To evaluate NDV's antitumour effect *in vivo*, NDV was tested against mammary adenocarcinoma AN3 transplanted in syngeneic immunocompetent mice. *In vivo* antiangiogenic activity was evaluated by quantifying the blood vessels in treated and control tumour sections. *In vitro* experiments that exposed AMN3 mammary adenocarcinoma cells and Hep-2 laryngeal carcinoma cells to NDV at different time intervals were performed to identify the exact mechanism of anti-angiogenesis by using angiogenesis microarray slides. *In vivo* results showed significant tumour regression and significant decrease in blood vessel formation in treated tumour sections. The *in vitro* microarray analysis of 14 different angiogenesis factors revealed that NDV downregulated angiopoietin-1, angiopoietin-2, and epidermal growth factor in mammary adenocarcinoma cells. However, NDV elicited a different effect on Hep-2 as represented by the downregulation of inducible protein 10, intracellular adhesion molecule-1, and basic fibroblast growth factor beta in NDV-infected tumour cells. It was found out that microarray analysis results helped interpret the *in vivo* data. The results suggested that the NDV oncolytic strain reduced angiogenesis by interfering with angiogenesis factors that might reduce tumour cell proliferation, infiltration, and invasion.

Key words: angiopoietin, bFGF, ICAM, IP-10, protein microarray, virotherapy

INTRODUCTION

Angiogenesis is defined as the formation of new blood vessels and is essential for supplying nutrients and oxygen to tumour tissues (Folkman, 1971). It is a multi-step process that involves endothelial cell (EC) proliferation, migration, and tubule forma-

tion (Bouïs *et al*., 2006). Tumour cells and tumour stroma cells support tumour growth by inducing neo-angiogenesis factors, whereas solid tumours require neovascularisation for proliferation, invasion, and metastasis (Ucuzian *et al*., 2010). Understanding cancer angiogenesis allows the discovery of better therapeutic targets and more effective treatment strategy (Al-Shammari *et al*., 2015).

It requires an agent that can inhibit tumour cells without interfering with normal processes. However, this requirement is highly difficult to achieve through conventional therapies (Castañeda-Gill & Vishwanatha, 2016; Tysome *et al*., 2013b). Oncolytic viruses selectively target cancer cells and avoid normal cells by either natural processes or genetic engineering (Al-Shammari *et al*., 2014c). The Newcastle disease virus (NDV) is an oncolytic virus that has been tested as an experimental therapeutic agent with many promising antineoplastic properties (Schirrmacher, 2016). The AMHA1 strain of NDV (Al-Shammari *et al*., 2014b) is oncolytic (Al-Shammari *et al*., 2010) and able to induce DNA fragmentation and Fas ligand upregulation in cancer cells. Apoptosis is induced by the AMHA1 NDV strain through intrinsic and extrinsic pathways (Al-Shammary *et al*., 2014a). NDV can increase the antitumour activity of standard therapeutic agents *in vitro* and *in vivo* (Al-Shammari *et al*., 2016a,b). This finding may suggest the use of NDV in combination with specific angiogenesis inhibitors.

Several tumour angiogenesis inhibitors, such as small molecules, aptamers, antibodies, and peptides, have been examined (Niu & Chen, 2010). Understanding tumour neovascularisation and vascular targets can lead to the advancement of cancer virotherapy that inhibits new tumour vessel formation, along with transcriptional and transductional endothelial inhibition. These viruses can suppress angiogenic factors or express anti-angiogenic molecules (Toro Bejarano & Merchan, 2015). In our study, the influence of NDV on angiogenesis and proliferation factors in NDV-infected tumour cells was investigated to explore the anti-angiogenesis mechanism involved in the oncolytic effect of NDV against epithelial cancer cells.

MATERIALS AND METHODS

Virus

The oncolytic NDV (Iraq/Najaf/ICC MGR/2013) (Al-Shammari *et al*., 2014b) named AMHA1 strain was supplied by the Experimental Therapy Department, ICCMGR, Mustansiriyah University. Virus preparations were described previously (Al-Shammari *et al*., 2016b). NDV was injected in embryonated chicken eggs (Al Hanaa hatchery, Baghdad, Iraq), collected from allantoic fluid, and centrifuged at 3000 rpm for 30 min at 4 °C to be purified from debris. NDV was quantified on the basis of the tissue culture infective dose 50 (TCID50) (Wilden *et al*., 2009). The virus was aliquoted and frozen at −86 °C in a deep-freeze environment until it was used.

Animals

Albino Swiss mice were maintained in an animal house facility in accordance with the regulations of the Mustansiriyah University, Iraqi Center for Cancer and Medical Genetic Research (ICCMGR). All *in vivo* experiments were authorised by the ICCMGR Scientific Committee.

Mouse tumour model

AN3 mouse mammary adenocarcinoma tumour was obtained from a spontaneously occurring mammary adenocarcinoma of an albino Swiss mouse (Al-Shamery *et al*., 2008). AN3 was preserved by constant allografting in syngeneic inbred mice. The origin of the AN3 cell line was the same as that of the tumour used in our *in vitro* study (AMN3).

In vivo study

AN3 cells (10^6/100 µL per site) were inoculated into the right flanks of 2-month-old female Swiss albino mice. The mice were randomly distributed into two groups of five mice in each group when the tumour diameter was about 0.5 cm in any direction: one group was injected intratumourally (IT) with NDV at TCID50 of 7×10^7 in 100 µL of PBS, and the other group was not treated (control). The experiment was ended after 30 days. This experiment was repeated twice.

Antitumour efficiency assessment

Tumour diameters were measured using calipers twice weekly. Tumour size was calculated using the following formula: 0.5 × length × width × width (Al-Shamery *et al*., 2011). When the tumour burden reached a volume of approximately 10% of their body weight, the mice were sacrificed.

For tumour growth measurement, the tumour volume was normalised to the volume of each tumour on day 0 when the NDV therapy was first administered. Tumour growth inhibition (TGI) (Phuangsab *et al*., 2001) was calculated twice weekly during the assessment period as followed: GI (%) = 100×(tumour volume of untreated group − tumour volume of treated group) / (tumour volume of untreated group).

Tissue samples

After 30 days of NDV virotherapy, the experiment was finished, and the tumour was carefully dissected and fixed with 10% neutral buffered formalin. The tumour samples were embedded in paraffin and sectioned with a thickness of 5 µm for histological evaluation.

Quantitation of tumour angiogenesis

Tumour sections from the control and treated animals were observed in the uniformity of haematoxylin and eosin (H&E) staining under a light microscope. The three hot-spot areas comprising the maximum number of microvessels were recognised by examining the entire tumour section at ×40 and ×100. The areas of microvessel hot spots generally occurred at the tumour periphery. The vessels outside the tumour margin and the adjacent benign tissue were not counted. Individual microvessel counts were performed in a ×400 field (Fox, 2001). At least five independent microscopic fields per tissue section for four different mice per group were examined.

Cells and cell cultures

Mouse mammary adenocarcinoma (AMN3), human larynx squamous cell carcinoma Hep-2, and human glioblastoma ANGM5 cell lines were obtained from the Mustansiriyah University, Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), Cell Bank Unit. The cell lines were maintained in RPMI 1640 (US Biological, Salem, MA, USA) supplied with 5% foetal bovine serum, 100 µg/mL streptomycin, and 100 units/mL penicillin (Capricorn Scientific, Germany).

Oncolytic cytotoxicity assay of NDV

Cells (10,000 cells/well) were cultured into 96-well microplates and incubated at 37 °C overnight, infected with NDV at 512 HAU with two-fold serial dilution in triplicates, incubated at room temperature for 2 h, and washed with PBS. Serum-free media were added to the infected and non-infected cells. After 72 h of incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) viability assay was performed by removing the medium, adding 28 µL of 2 mg/mL MTT solution (Bio-World, USA), and incubating at 37 °C for 90 min. After the MTT solution was discarded, the crystals that remained in the wells were solubilised in 130 µL of dimethyl sulfoxide (DMSO; Santa Cruz Biotechnology, USA) and subsequently incubated at 37 °C for 15 min while shaking. Absorbance was determined using a microplate reader (Biochrom, UK) at a 492 nm wavelength. Cell viability assays were performed to assess growth inhibition, which was calculated as: $A - (B/A)\times 100$, where A is the mean optical density of untreated wells, and B is the optical density of treated wells (Takimoto, 2003).

Cell line exposure to NDV for microarray study

AMN3, Hep-2, and ANGM5 cells were plated in 25 cm^2 Nunc™ Cell Culture Treated EasYFlasks (Thermofisher, USA). They were seeded at 1×10^6 cells in a growth medium and incubated overnight at 37 °C. When the cells were in the exponential growth, the medium was removed, and 512 HAU of NDV was added to the flask for 2 h. The cells were then washed, and a serum-free medium was added. Duplicates were made for each treatment. Control cells were untreated. Afterward, the flasks were re-incubated at 37 °C for 6, 12, and 18 h.

Extraction of total protein from NDV-infected cells for microarray study

Infected cells were collected after certain time using a cell scraper and centrifuged to form pellets. The supernatant was discarded, and the pellets were suspended in a lysis buffer for 10 min at 4 °C. The cell lysates were centrifuged at 12,000 × *g* for 10 min. The pellets were then incubated in a lysis buffer at 4 °C for 40 min and centrifuged at 3000 rpm for 10 min. The supernatant was collected and stored in a deep freeze at −86 °C until it was used (Al-Hilli *et al*., 2009).

Angiogenesis factor measurements

Human angiogenesis microarray slides (Allied Biotech, Inc., MD, USA) containing 16 identical arrays of 14 capture antibodies (i.e., EGF-HB, TIMP-1, TIMP-2, HGF, ANP-1, ANP-2, VEGF-A, IP-10, PDGF-BB, KGF, ANG, VEGF-D, ICAM-1, and FGF-B) in quadruplicate were used. In accordance with the manufacturer's protocol, treated and nontreated samples were aliquoted to each array on the slide. The resulting microarray reacted with a biotinylated detection antibody. Angiogenic spotted slide glasses reacted with the sample proteins and subsequently with a streptavidin–alkaline phosphatase solution. The addition of an alkaline phosphatase solution induced colour development, measured using an Arrayit® scanner (Arrayit, CA, USA). The image was captured with SpotWare ver. 1.1 (Arrayit, CA USA) that utilised 10 µm scan gain for spot intensity. The resulting captured image was analysed using AGScan software Version 0.2. Sigenae.

Statistical analysis

Statistical analyses for the *in vivo* study were performed using ANOVA, and multiple comparison tests were conducted to compare between groups. The differences were considered significant at P=0.05. The MTT assay was analysed using one-way ANOVA in GraphPad Prism (GraphPad Software, Inc. San Diego, California). Unpaired t-tests were carried out to compare microarray data, and P<0.05 was considered significant.

RESULTS

Antitumour activity against mouse mammary adenocarcinoma tumour

The tumour-bearing mice were divided randomly into two groups of five in each group, the tumour diameter reached 0.5–1 cm. The relative tumour volumes were plotted over a 24-day period (Fig. 1A). The NDV treatment significantly reduced the tumour volume compared with that of the untreated control group (P<0.0001).

NDV-induced angiogenesis inhibition in transplanted tumours

Angiogenesis was histologically assessed in mouse mammary adenocarcinoma tumour. The microvessels in the stained sections were quantitated to examine angiogenesis in these tumours by counting the microvessels in the tumour vascular hot spots in the tumour periphery. NDV therapy induced a significant decrease in the number of microvessels compared with that of untreated mammary adenocarcinoma (Fig. 1B). The control group showed a massive tumour mass compared with that of the treated tumour tissue after intratumoural injection and revealed a small growth sheet in the tumour periphery and few microvessels.

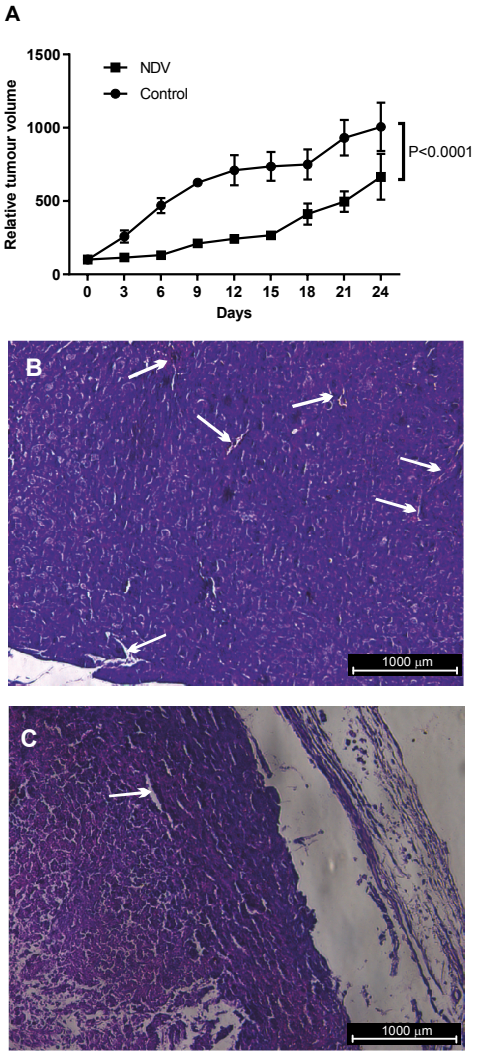

Fig. 1. *In vivo* experiments on an AN3 mammary adenocarcinoma transplantable tumour model. **A.** Relative tumour volumes (mean± SEM) plotted over a 30-day period; **B.** Quantitative analysis of microvessels of the histological sections showing microvessels in the control group; **C.** NDV intratumorally injected tumour tissue showing few microvessels.

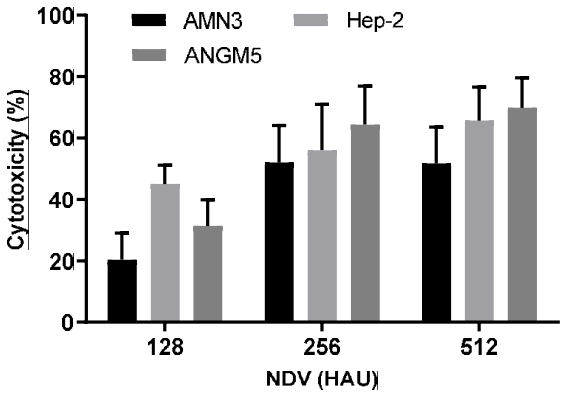

Fig. 2. Oncolytic effects on the NDV-infected cancer cell lines. MTT cell viability assay for studying the growth inhibition caused by NDV treatment after 72 h of virus exposure.

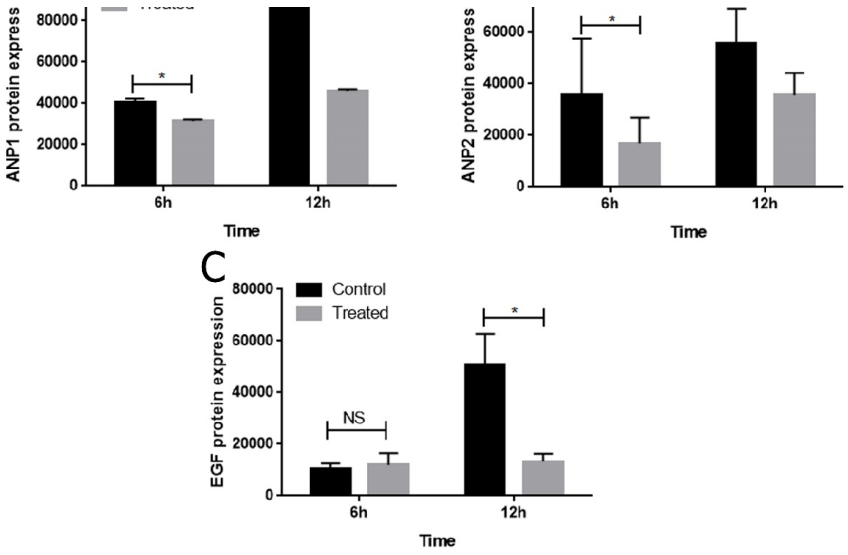

Fig. 3. Angiopoietin-1 (A), angiopoietin-2 (B) and epidermal growth factor (C) expression, AMN3 mouse mammary adenocarcinoma cell line. Average spot intensity of NDV-treated and control cells for 6 and 12 h (mean ± SEM).

Newcastle disease virus oncolytic activity in vitro

The infected cell lines showed significant oncolytic cytopathic effects. The number of the infected cells decreased compared with that of the untreated cells, which grew to form overgrowth layers. The MTT assay revealed that the NDV significantly induced oncolysis in mouse mammary adenocarcinoma, laryngeal carcinoma, and glioblastoma cell lines (Fig. 2).

Significant oncolytic effects were detected at 256 and 512 HAU titres.

Angiogenesis proteins response to NDV infection

A human angiogenesis microarray was used to study the transcription and translation response of AMN3, Hep-2, and ANGM5 cells infected with the Iraqi NDV strain. Hep-2 and ANGM5 cancer cell lines were chosen because they exhibit high susceptibility to NDV infection and support virus replication. Protein microarrays were used to compare the protein expression of specific factors in the infected cells with the same factors in the mock-infected cells and study the angiogenesis factors of transcription and translation responses during NDV infection. The origin of the AMN3 mouse mammary adenocarcinoma cell line is the same as that of the AN3 transplantable tumour *in vivo*, so the response of angiogenesis factors to NDV infection 6 and 12 h post-infection was investigated. The results showed that NDV infection downregulated angiopoietin 1 and 2 significantly 6 and 12 h after infection and the epidermal growth factor after 12 h compared with those of the control cells (Fig. 3).

The response of Hep-2 laryngeal carcinoma cell to NDV virus infection was further tested, and the factors that changed are presented in Fig. 4. The expression of three of these factors was significantly upregulated or downregulated at different time points after NDV infection. Nevertheless, general analysis revealed that some factors were downregulated at different NDV infection time points. These

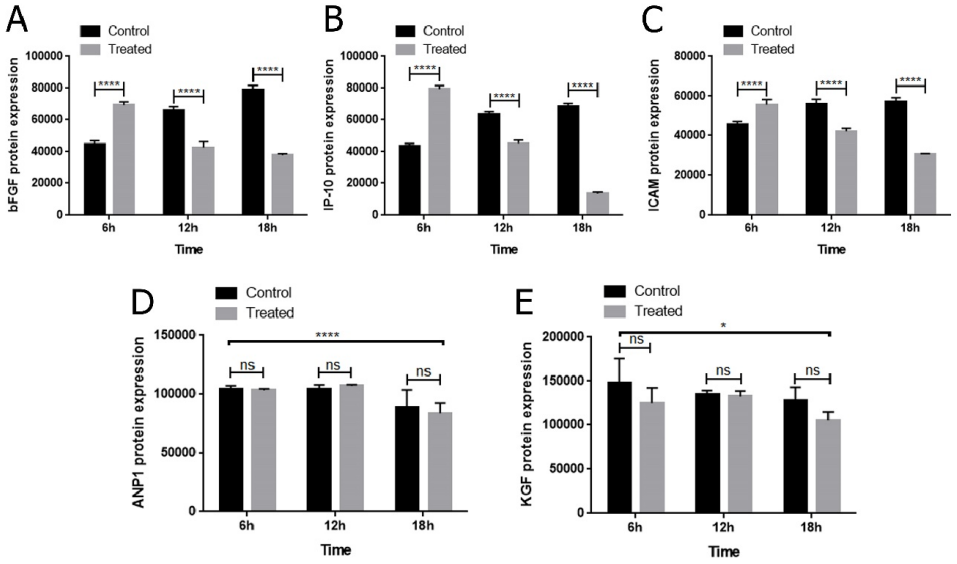

Fig. 4. Microarray spot analysis of ANGM5 glioblastoma cell line and Hep-2 laryngeal carcinoma cell line and effects of NDV infection on cancer cells. **A.** ANGM5, IP-10-fold change with an upregulation pattern. **B.** ANGM5, bFGF upregulated only in the first 6 h of exposure and then downregulated at 12 and 18 h. **C.** ANGM5, ICAM upregulated at 6 and 12 h and downregulated at 18 h. **D.** Hep-2 cells after 18 h of virus exposure showed an insignificant reduction in angiopoietin-1 expression. E. Insignificant reduction of keratinocyte growth factor at 6 and 18 h post-infection for Hep-2.

results might represent factor-specific regulation patterns depending on cell type.

NDV interfered with the kinetics of the gene activation and repression of three different proteins. Inducible protein 10, ICAM-1, and FGF-B were the genes most affected by NDV infection, which presented changes in their protein expression compared with that of the untreated cells at all indicated time points.

Basic fibroblast growth factor-β was one of the proteins upregulated during the first 6 h post-infection compared with that of the control cells (Fig. 4A). The induction pattern of this protein significantly declined from 12 h to 18 h post-infection and reached a lower value than the baseline level of the control cells at 6 h. The protein induction of the control cells increased with time and reached a significantly spiked point at 18th h. The FGF-B protein expression significantly changed.

Inducible protein 10 spot analysis showed significant spiked upregulation during the first 6 h of infection (Fig. 4B). Afterward, the expression pattern decreased and maintained from 6 h to 18 h post-infection. On the contrary, the control cells showed an upregulation pattern but never reached the same 6 h spiked point as the infected cells even after 18 h of infection. Two-way ANOVA and multiple comparison analysis revealed that the expression in the infected cells significantly changed during the time course compared with that of the control cells ($P<0.001$). The ICAM-1 spot analysis showed no significant upregulation pattern at 6 h post-infection compared with that of the control cells. The expression also decreased from 12 h to 18 h post-infection (Fig. 4C). Intracellular adhesion molecule-1 protein in the control cells had an upregulated pattern from 6 h to 18 h post-infection, whereas the infected cells exhibited a downregulated pattern. Intracellular adhesion molecule-1 protein expression was significantly repressed in the infected cells at 6 and 18 h post-infection and reached a value lower than the baseline level at hour 6.

The microarray spot analysis of the ANGM5 glioblastoma cell line revealed that the angiopoietin-1 expression in the cancer cells infected with NDV decreased after 18th h of infection, but this decrease was not significant. ANOVA showed significant differences depending on the time of expression ($P<0.001$). The analysis of keratinocyte growth factor spot showed a nonsignificant upregulation during the first 6 h of infection (Fig. 4E). Subsequently, its expression also decreased at 18th h post-infection compared with that of the control cells, but this result was not significant. Two-way ANOVA revealed that the expression significantly changed during the time course ($P<0.001$).

DISCUSSION

Oncolytic viruses have been used for targeted delivery for local expression to angiogenesis inhibitors to increase antitumour activity (Tysome *et al*., 2013a). In the current work, the direct anti-angiogenic effects of oncolytic DNV AMHA1 strain were investigated *in vivo* by using an AN3 mammary adenocarcinoma mouse model and an *in vitro* model against selected human and mouse cancer cell lines. The *in vivo* and *in* vitro results provided evidence that oncolytic virotherapy (NDV) could suppress cancer angiogenesis. The *in vivo* experiments revealed that NDV significantly inhibited tumour growth and induced the shrinkage of mammary adenocarcinoma tumour mass in the syngeneic mouse model. Histological findings showed that the microvessel

formation in the treated tumours significantly decreased compared with that of the control group. These results were further analysed to determine the exact mechanism related to this reduction through a microarray assay and study the expression of 14 angiogenesis factors during the NDV infection of AMN3 cells that share the same origin as that of the AN3 tumour model. These cells were analysed, and the proteins expressed in and belonging to tumour angiogenesis, proliferation, and metastasis pathways were identified. The microarray experiment was conducted to describe the changes in the cellular protein expression levels in response to NDV infection. The analysis showed that infection with the AMHA1 NDV strain significantly affected the expression pattern of the three cellular proteins identified. Expression levels were measured at 6 and 12 h post-infection, and substantial changes in the levels of specific proteins (angiopoietin-1, angiopoietin-2, and EGF) in the NDV-infected cells were observed, i.e., these levels decreased compared with those in the uninfected control cells. Angiopoietins are essential angiogenesis factors that play coordinated roles with vascular endothelial growth factor (VEGF) in support of a new vasculature to sustain tumour growth. Their overexpression in breast cancer is correlated with poor prognosis (Ahmad *et al*., 2001). The majority of breast cancer cases express a measurable level of angiopoietins 1 and 2; moreover, tumour aggressiveness is associated with angiopoietin 2 expression (Sfiligoi *et al*., 2003). Therefore, targeting ANP1 and ANP2 is a promising antitumour activity, and agents are currently in phase II clinical trials showing evidence of efficacy and safety profile (Herbst *et al*., 2009). Therapeutic inhibitors that target ANP2 can attenuate the progression of hepatocellular carcinoma (Lefere *et al*., 2019). The angiopoietin pathway has been identified as a key mediator in the pathologic angiogenic switch in breast cancer (Ramanathan *et al*., 2017). NDV suppressed EGF levels in the infected cells after 12 h of infection. EGF is an important cytokine that plays a leading role in cancer cell progression and proliferation; therefore, it is an attractive target for anticancer treatment (Ganti & Potti, 2005). Another study has investigated the regulating molecules in vasculogenic mimicry (VM), which are vascular-like channels that lack the involvement of endothelial cells and are detected in the aggressive activity of breast cancer stem/progenitor cells (BCSCs). Furthermore, they discovered that EGF can stimulate the VM activity of BCSCs. The stimulation of heat shock protein 27 (Hsp27) is essential for EGF-induced angiogenesis in endothelial cells (Tate *et al*., 2013 Lee *et al*., 2014).

The NDV infection of Hep-2 cells were analysed and measured at 6, 12, and 18 h post-infection. Surprisingly, different sets of proteins were downregulated. The IP-10 protein showed expression patterns characterised by spiked levels in the first 6 h compared with that of the control cells. The expression levels of cancer cell proteins that govern cell proliferation (FGF-B) and metastasis (ICAM-1) decreased through the infection pathway. The expression of other proteins was not detected. These findings supported the suggestion that NDV often leads to significant changes in the protein expression in infected tumour cells (Schirrmacher, 2017). Our results indicated that the Iraqi NDV virulent strain infection upregulated the expression of an IP-10 protein with a known angiostatic function (Yates-Binder *et al*., 2012), and this protein was previ-

ously described to be upregulated by other NDV strains (Washburn & Schirrmacher, 2002). IP-10 induces the dissociation of newly formed blood vessels (Bodnar *et al*., 2009) and downregulates microvessel density in tumour tissues (Wang *et al*., 2009).

Interestingly, the NDV infection increased the level of some particular host proteins early, i.e., at 6 h post-infection. However, they were eventually downregulated significantly at 12^{th} and 18^{th} h. This observation might be a nonspecific response of the cells to stress or may be a more specific cell response to viral replication (Guerra *et al*., 2003). FGF-B and ICAM-1 proteins showed downregulated patterns in the infected cells and an upregulated pattern in the control cells. The repression pattern in the infected cells likely interferes with cancer angiogenesis because FGFs can stimulate EC proliferation, migration, and neovascularisation by initiating blood vessel induction *in vivo* as tested in chick chorioallantoic membranes and corneas (Spinetti *et al*., 2001; Presta *et al*., 2009). The expression of adhesion molecules is correlated with oral cancer progression activities, such as cytokine production, proliferation, and invasion (Usami *et al*., 2013). The ICAM-1 expression in tumour cells influences metastatic potential that determines cancer lethality (Roland *et al*., 2007). Thus, interference with ICAM-1 expression has a prognostic value in reducing metastatic potential. In human oral cancer cells, NDV negatively regulates MMP-7 and β-catenin to promote apoptosis and cell migration inhibition (Morla *et al*., 2019).

The NDV-infected ANGM5 cells showed a lesser anti-angiogenic response; the ANP1 expression decreased at 18 h post-infection, but this decrease was not significant. In human and animal glioma, ANP1 stimulates tumour angiogenesis (Stratmann *et al*., 1998; Machein *et al*., 2004). Interfering with ANP1 signaling induces anti-angiogenic activity (Bhattacharya *et al.*, 2015). Moreover, our results in ANGM5 cells showed that NDV infection slightly suppressed the KGF levels. KGF plays a role in tumour angiogenesis through the upregulation of VEGF gene expression (Ferrara, 2004). NDV decreases other vital proteins such as glyceraldehyde3-phosphate (GAPDH) (Al-Shammari *et al*., 2019) in cancer cells.

In conclusion, the NDV-host interactions showed a cell-specific suppression of angiogenic protein expression. Furthermore, NDV possess an *in vivo* and *in vitro* anti-angiogenic activity. Mammary adenocarcinoma microarray analysis revealed an angiopoietin cellular protein with expression levels markedly modified by NDV infection and suggested the potential roles of NDV as an anti-angiogenic regulator, and these findings should be of great value.

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