The Effect of Open Reading Frame 3 of Hepatitis E virus on Cellular NF-κB Activity

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Abstract

NF-B (Nuclear Factor Kappa B) is a crucial transcription factor that is essential for host survival during pathogen infection in animal and human. Therefore, it has been a main goal for numerous pathogens to modify cellular NF-κB activity to create an environment conducive to their survival within the host. In the present study, the cell line type (SW480) was used as a model of colon cancer cells. Subsequently and through using western blotting, the effect of ORF3 protein on TNF- induced NF-B activation was observed by detecting the NF-B p65 subunit in the nuclear and cytoplasmic extracts. The results appeared that p65 translocated into the nucleus when stimulated by TNF-α in the control groups (GFP and mock cells). However, weak nuclear translocation was seen in ORF3-expressing cells. Moreover, the experiment revealed the ORF3 protein in SW480 inhibited the nuclear translocation of the p65 protein. After TNF-α stimulation, the difference in the band intensity of NF-κB protein in the nuclear fraction of control groups cells from each GFP group (A3) and mock cells (B3) was significantly higher compared with nuclear extract from Orf3expressed cells (C3 , P = 0.0001). In conclusion, the current study was confirmed that HEV ORF3 protein inhibits the activity of cellular NF-κB in human colon cancer cells.

Introduction

Virus-caused hepatitis has arisen as a global public health crisis affecting hundreds of millions of individuals (1). There are different types of hepatitis virus, such as A, B, C, D, E, and G. All regional studies focused on studying types A, B, and C (2–6). Hepatitis E is a major etiologic agent of enterically transferred form of hepatitis virus in humans and animals. Since the zoonosis of HEV was confirmed, HEV-induced hepatitis has been a worry for the public health not just in industrialized countries but also in developing ones. (7). Moreover, many studies were focused on their pathological pathway and their relatedness with nuclear factor kappa-B (NF-κB) activity (8) .

Nuclear factor kappa-B (NF-κB) is a key transcription factor that regulates several cellular pathways that, depending on the stimuli, lead to cell survival or death. In cells that are unstimulated, the (NF-κB) dimers (p50/p65 hetero-dimer or p50/p50 homo-dimer) are kept in the cytoplasm in an inactive state due to their interaction with I kappa B (IκBα) family members (9). Activators like tumor necrosis factor-alpha (TNF--α) and interleukin-1 (IL-1) trigger signaling cascades that involve the activation of different protein kinases. This leads to the activation and recruitment of the "IκBα kinases" (IKKs), which phosphorylate IκBα, causing it to be broken down. The breakdown of IκBα reveals the sequence of the p50/p65 nuclear localization (10). NF-κB dimers may then migrate into the nucleus and control transcription by binding to B motifs in the promoters of many genes. It is well known that extracellular pathogens often change the activity of NF-κB during an infection so that either the pathogen or the host can live.

The activation of "nuclear factor-kappa B" (NF-κB) is required for resistance to many different types of viral, bacterial, and parasite diseases (8). A large number of viruses, including HIV, take advantage of this trait by using κB response elements in their promoters to control gene expression (10). However, numerous pathogens, including African swine fever virus, HIV-1, and cowpox virus (11) have devised methods to counteract host NF-κB responses. It has been established that the pathogens' ability to inhibit NF-κB activity is crucial to the development of disease.

Three "open reading frames" (ORFs) are attributed to the hepatitis E virus (HEV) (12). whereas ORF2 encodes the major HEV capsid protein, ORF3 encodes a phosphoprotein that may be critical for viral survival and propagation by impacting several cellular processes in the host after infection, and ORF1 encodes nonstructural proteins required for viral replication.(13,14). Although infection of HEV typically limits itself, it causes fulminant hepatic failure and has a significant death risk in pregnant women (15,16). Recently it has been shown that NF-κB activity is downregulating in PBMC and liver biopsy samples from pregnant patients with fulminant hepatic failure. However, the mechanism behind these phenomena is still a mystery. In the present study, the capacity of the ORF3 protein has been confirmed to

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decrease cellular NF-κB activity using colon cancer cells (SW480) from humans. In turn, this resulted in decreased DNA binding of the p65 protein and nuclear localization, the main subunit of the (NF-κB) transactivation complex lead to improve the survival of HEV infection.

Materials and Methods

Transfection of Cell lines: The ORF3 pcDNA3.1 (+)-P2A-eGFP plasmid was constructed by GenScript (USA) and used as the expression vector. Hepatitis E virus (genotype 1) open reading frame 3 (ORF3) encoding protein sequence acquired from GenBank (Accession no.LC061267). The negative control was the empty vector pcDNA3.1 (+)-P2A-eGFP (GFP vector). This study used of the Human colon cancer cell line (SW480), which was procured from Rawafid al-Eloom company for scientific and research training in Hilla/Iraq. The medium that named Roswell Park Memorial Institute 1640 medium (RPMI 1640, Capricon Scientific GmbH, German) was used and supplemented with 10% fetal bovine serum (FBS), 80 mg/2 ml gentamycin and 1% (v/v) trypsin was used to cultivate the SW480 cell line. Transfection protocol achieved according to the supporting protocol attached to the kit of DNAfectin™ Plus transfection reagent (Applied Biological Materials Inc., Cat. No. G2500). The first plate was transfected with ORF3-GFP plasmid and the second plate transfected with GFP control vector. While, third one was left as mock cells (without transfected) then all plates were incubated for 24 h in Co2 incubator. After transfection, the cells were stimulated by a treatment with 20 ng/ml TNF (diluted in the RPMI 1640) to induce NF-κ B activity and incubated for a further 2h (17). For fluorescence assay, cells were seeded on 8-well sterilized chamber slide (SPL life science, Korea) at density 1×10^5 and incubated in the same condition. Then, cells were viewed under fluorescence microscope when cells irradiated with UV at a wave length of 450 -490 (18).

Subcellular fractionation: Cytoplasm and nucleus isolation

Untransfected cells in addition to GFP vector was used as a control. Cells of each plate were scraped and lysed with 200μl of cytoplasmic lysis buffer (20 mM Tris/Cl, pH7.4; 5mM MgCl2; 100 mM NaCl; 0.5% NP 40; 1x Protease inhibitor cocktail). The lysate was incubated on ice for 30 min. Pipetting was then used extensively before being centrifuged at 9600g for 20 min at 4°C to separate the cytoplasmic fraction from the cell nuclei-containing pellet. To get the nuclear fraction, pellets were rinsed three times with cytoplasmic lysis liquid prior to being resuspended in 200μl radio immunoprecipitation assay (RIPA) buffer (50mM Tris/Cl, pH7.5; 150 mM NaCl 0.5% w/v sodium deoxycholate; 1% NP 40; 0.1% SDS; 1 x Protease inhibitor cocktail). The suspensions were cleared by centrifugation at 96,000 x g for 10 min at 4°C and the supernatant represented the fraction of nuclear material (19). Both the two fractions were examined using western blotting and immunoblotting with the appropriate antibody. Anti HistonH3 (No. E-AB-20031, Elabscience) was used as a marker for nuclear fraction and Anti beta actin (No. E-AB-22003, Elabscience) for the cytoplasmic (soluble) fraction.

Protein concentration estimation: The concentrations of total proteins in homogenized cell line lysates were determined using the bicinchoninic acid assay kit (No. E-BC-K318, Elabscience) instructions. Then, the total protein concentration was estimated.

Immunoprecipitation and Western Blot Analysis: For immunoprecipitation, the lysates were incubated with the indicated primary antibodies at 4 °C overnight and after being boiled in 2x SDS loading buffer. The immunoprecipitated proteins were separated using SDS-polyacrylamide gel electrophoresis (PAGE) and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were immunoreacted with the appropriate primary antibodies and HRP-conjugated secondary antibodies following blocking with 5% BSA in Tris-buffered saline containing 0.1% Tween-20 (13,20). Twenty micrograms of protein were separated by SDS-PAGE and transferred to PVDF membranes. Two hours of blocking with 5% skim milk (diluted in TBST) was followed by an overnight incubation with specific primary antibodies. Following washing with TBST containing 0.1% Tween 20, membranes were incubated with secondary antibodies coupled to horseradish peroxidase and ECL. Following the exposure of the PVDF membranes to film, an X-ray cassette was utilized to observe the protein bands and band scan software version 5.2. (LI-COR Biosciences, Java). Using Histon (nuclear protein) and –actin (cytoplasmic protein) as a loading control protein and normalization. the densitometric

ratios of experimental bands were then calculated (21).

Results

Detection of protein concentration of cell lysate

The initial standard curve was drawn in Microsoft Excel with an r-squared value of (0.9926) (Figure 1). Standard curve for BSA to quantify the total protein concentrations of cells lysates for three groups (ORF3 - GFP, GFP and mock cells) (Table 1). Then, equal number of proteins (25μg) for the three previous groups were analyzed by SDS-PAGE. The blue line is the linear fit determined by least square regression, with the adjacent equation used to calculate the sample protein concentration.

Effect of HEV ORF3 on NFκB activation

After transfection, the colon cancer cell line SW480 was stably established to express the ORF3-GFP fusion protein, which successfully indicated green fluorescent cells by fluorescence microscopy (Figure 2). Using Western blotting, the effect of ORF3 protein on TNF- induced NF-B activation was observed by detecting the NF-B p65 subunit in nuclear and cytoplasmic extracts. The result appeared that p65 translocated into the nucleus when stimulated by TNF-α in the control groups (GFP and mock cells). However, weak nuclear translocation was seen in ORF3-expressing cells (Figure3). The experiment revealed the ORF3 protein in SW480 inhibited the nuclear translocation of the p65 protein. After TNF-α stimulation, the difference in the band intensity of NF-κB protein in nuclear fraction of control groups cells from each GFP group (A3) and mock cells(B3) was significantly higher compared with nuclear extract from Orf3expressed cells (C3, P = 0.0001).

Table 1: Total protein content in cells lysates

Standard concentrations of BSA[1] (mg/mL)	[2] Reading data (replication) Absorbance unites (nm)		Samples Codes	[2] Reading data (replication) Absorbance unites (nm)		Final Concentration of total protein (µg/µL)
2	1.11	1.092	A2	0.465	0.422	3.4
1.5	0.771	0.779	A3	0.528	0.464	4.0
1	0.555	0.55	B2	0.561	0.497	4.4
0.75	0.465	0.457	B3	0.447	0.508	3.8
0.5	0.357	0.348	C2	0.447	0.427	3.4
0.25	0.269	0.238	C3	0.452	0.481	3.7
0.125	0.195	0.183				
0.025	0.147	0.148				
0	0.135	0.132				

[1] Standard concentrations of bovine serum albumin. [2] Reading of absorbance using microplate reader at (562 nm) wavelength.
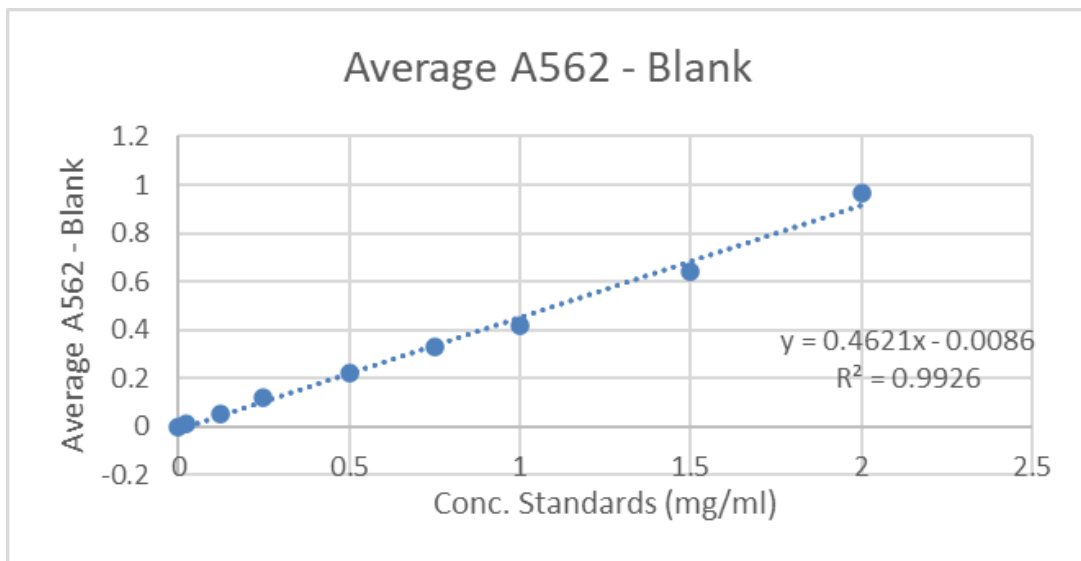

Figure 1: Standard curve for bovine serum albumin as standard concentrations
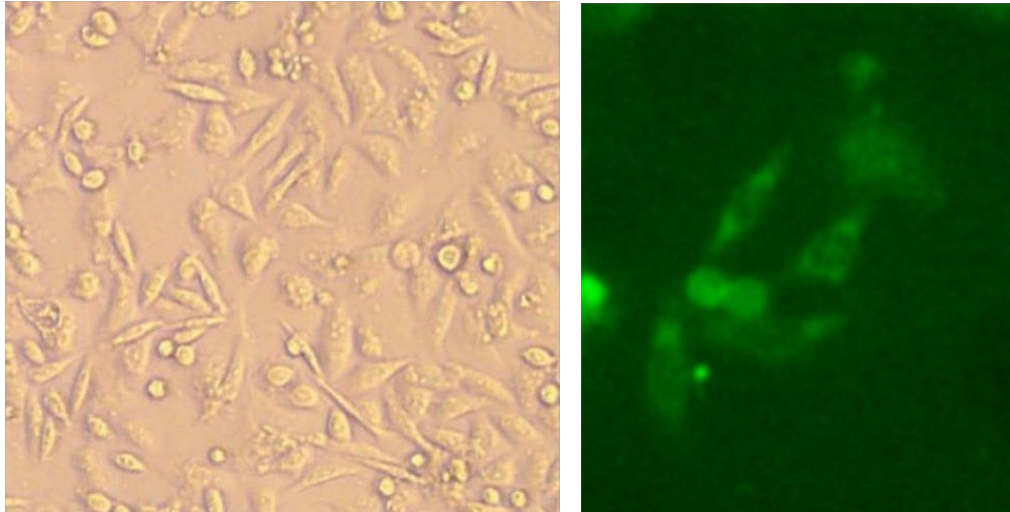
Figure 2:A. Monolayer culture of SW480 cells after transfected at (20X) magnification power under Inverted microscope. B. Detection of green fluorescein protein in ORF3 expressing cells using a fluorescence microscope with a set of filters that can detect fluorescein (Filter Set 9; excitation, BP 450-490 nm; emission, LP 520 nm).
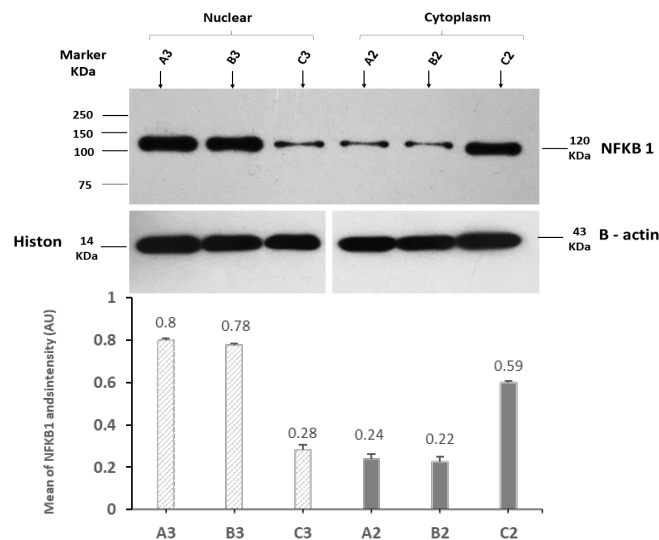

Figure 3: NF-kB activation. SW480 cells were transfected with either HEV ORF3 – GFP plasmid (C) or GFP control vector (A) with another untreated group (B) for 24 h, stimulated with TNF-α (20ng/ml) for 2 h. Western blotting was then performed. Nuclear and cytoplasmic extracts were used to measure the activity of NF-kB by examining the distribution of p65 using a p65-specific monoclonal antibody (120 KDa). Histon bands (14 KDa) and B-actin (43 KDa) were used as a house keeping gene (loading control) for nuclear and cytoplasm extract, respectively. Bar chart, showing the bands intensity of NFKB protein (mean±SE) in nuclear and cytoplasmic three different groups (as mentioned above). The differences between levels of NFKB protein in nuclear extract of Orf3 expressing cells (C3) and nuclear extract of control groups (A3,B3) were significantly higher (mean±SE: n=3, *P*<0.0001).

Discussion

It has been hypothesized that HEV's pathogenic mechanism is strongly immune-mediated and does not involve direct viral replication (22). HEV ORF3 can interact with diverse cellular proteins in multiple clotting-related pathways, this suggests that it may play a potential role in the establishment of an environment that is generally conducive to viral replication. These results in acute and chronic infection, as well as a wide variety of extra-hepatic symptoms, some of which have been linked to altered immunological states. To avoid viral infection, PRRs, particularly TLRs (Toll-like receptors), activate the host's innate immune responses. Within minutes of viral infection, the body's innate immune response normally converges and activates transcription factors like NF-κB. After being activated, factors are translocated to the nucleus, where they stimulate the production of inflammatory cytokines (17). Normally, NF-κB has broad transcriptional regulatory roles and is normally accumulated within the cytoplasm as a latent complex by IκBα (23). Work presented in this experiment to see if pORF3-mediated regulation of host genes were caused for a possible role of a major transcription factor (NF-κB). Many papers used cells that are not natural targets for HEV infection, such as lung epithelial cells, A549, and THP1 monocytes (17,24) as well as using HeLa, HEK293T, and S10-3 cells by (25). In addition to macrophages and dendritic cells, epithelial cells, endothelial cells, and fibroblasts, which are not specialized cells, are involved in the development of this response. Human colon cancer cells SW480 have been reported 59% efficiency at 24h post-transfection (26) and therefore considered a suitable cell line to use in the present study regarding the involvement of HEV ORF3 in the NF-kB pathway. Transfection Reagent or lipofectamine 2500 was used to deliver HEV ORF3 gene into the cells, because a cationic liposome reagent is capable of forming complexes with nucleic acids and delivering genes into cells via the cellular endocytosis pathway without severe damage without significant toxicity (26). When ORF3 protein expression was successful, green fluorescein protein (GFP) was observed in transfected cells with a fluorescent microscope, next step was the cellular levels of NF-κB checked in group of SW480 cells transfected with the ORF3-GFP plasmid (ORF3-pcDNA3.1 (+)-P2A-eGFP) or group transfected with GFP vector and in the group of mock cells. Analysis of intensity of the NF-κB protein bands after TNF α - stimulation and the cytoplasmic and nuclear lysates were prepared, western blotting was performed with anti- NF-κB (P65) antibodies. Although NF-κB protein was found to be quantitatively confined to the nucleus of control groups (GFP group and mock cells), it was primarily detected in the cytoplasm of ORF3 expressing cells indicating that the ORF3 protein interfered with the nuclear localization of NF-κB. Therefore, the present findings suggested that pORF3 prevented TNF- α-induced NF-κB activation, resulting in decreased levels of TNF-α, IFN-, IL-6 and other cytokines. These findings are in agreement with

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previous study (17). It has been found that there was an increase in the translocation of p65 at 1 and 2 h after treating mock cells with TNF-α to validate the participation of the NF-κB pathway in HEV infection. The active form of p65, which is the primary component of the NF-κB trans-activation complex, attaches to its DNA consensus site and forms a DNA-protein complex (27). Our findings indicate that pORF3 modulates the primary transcription factor (NF-κB), hence inhibiting its nuclear translocation and transcription factor activity. Another study demonstrated that the protein HEV ORF3 preferentially repressed the NF-κB pathway by lowering the quantities of IKKε which phosphorylates the inhibitory IκBα protein, resulting in the dissociation of IκBα protein from NF-κB. Now that NF-κB is released, it migrates into the nucleus and triggers the expression of at least 150 genes (28).

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تأثير إطار القراءة المفتوحة 3 لفيروس التهاب الكبد E على نشاط NF-κB الخلوي

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الخلاصة

العامل النووي كابا ب (NF-κB) هو من العوامل الضرورية والحرجة لبقاء المضيف أثناء العدوى الممرضة في الحيوانات والإنسان. وبسبب ذلك فان العديد من مسببات الامراض تعمل في الاساس على تعديل نشاط NF-κB الخلوي لإنشاء بيئة مواتية لبقائها داخل المضيف. في الدراسة الحالية تم استخدام الخلايا (SW480) كنموذج دراسي لخلايا سرطان القولون. ومن خلال استخدام لطخة ويسترن، لوحظ تأثير بروتين ORF3 على تنشيط NF-B الناجم عن TNF من خلال اكتشاف الوحدة الفرعية NF-B p65 في المستخلصات النووية والسيتوبلازمية. واظهرت النتائج بان p65 ينتقل إلى النواة عند تحفيزه بواسطة TNF-α في مجموعات السيطرة (GFP والخلايا الوهمية) بالرغم من وجود انتقال نووي ضعيف في الخلايا المعبرة عن ORF3. كما بينت نتائج التجربة بان بروتين ORF3 في SW480 قد منع النقل النووي لبروتين p65. وبعد تحفيز TNF-α ، كان الفرق في شدة النطاق لبروتين NF-κB في الجزء النووي من خلايا مجموعات السيطرة في المجموعة GFP (A3) والخلايا الوهمية (B3) أعلى بكثير مقارنة بالمستخلص النووي في الخلايا المعبرة عن (P = 0.0001, C3)ORF3). علية يمكن الاستنتاج أن إنتاج بروتين الخاص بشفرة للتنبؤ بإطار القراءة المفتوح3والمعروفة ب (ORF3) في خلايا سرطان القولون البشري يثبط نشاط NF-κB الخلوي في خلايا سرطان القولون البشري.

الكلمات المفتاحية: فايروس التهاب الكبد نوع ج HEV, ,نشاط.