行政院國家科學委員會專題研究計畫　成果報告

人類乳突瘤病毒的 E7 蛋白對肺癌細胞的 CDK 活化激素以及細胞週期的影響

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計畫主持人： 邱慧玲

計畫參與人員： 謝明儒、謝易修

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行政院國家科學委員會補助專題研究計畫 □ 成 果 報 告 □期中進度報告

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共同主持人：
計畫參與人員：　謝明儒、謝易修

成果報告類型(依經費核定清單規定繳交)：☑精簡報告　□完整報告

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執行單位：中山醫學大學 醫學檢驗暨生物技術學系

中　華　民　國　95 年 10 月 30 日

中文摘要：

慢性肝炎及肝硬化一直是影響國人健康及造成死亡的主要原因之一。除了酒精及藥物之外，病毒感染也是重要肇因，其中B型肝炎已有疫苗可以有效預防，而C型肝炎病毒尚未有疫苗或有效治療方式來控制，因此C型肝炎病毒感染所導致的慢性肝炎或肝癌在台灣將是越來越重要的醫療議題。當宿主細胞受到C型肝炎病毒感染的時候，可藉由C型肝炎病毒本身外套膜蛋白的高度變異來躲避宿主免疫系統的攻擊；另外一種方式則是透過病毒蛋白的作用來抑制宿主免疫系統的產生或是促使細胞凋亡來幫助病毒繁殖擴散，導致組織產生持續性的發炎反應，進而引起慢性肝炎、肝硬化以及肝癌。之前的研究指出C型肝炎病毒套膜蛋白E2會導致細胞凋亡，但是確實的相關機制卻還不是很清楚。因此我們先探討C型肝炎病毒的套膜蛋白E2是否有導致Huh-7人類肝癌細胞株發生細胞凋亡的能力，再進一步研究探討其相關的分子機制。經由MTT assay、DNA fragmentation以及western blot等分析方式，我們發現套膜蛋白E2確實會導致Huh-7細胞產生細胞凋亡，並且促進procaspase 8、procaspase 9及procaspase 3的活化以及 使得PARP蛋白受到切割，細胞色素C、Bax及tBid蛋白表現量也因而增加，同時Bid及Bcl-2蛋白表現量減少。根據上述結果可以發現，套膜蛋白E2會使細胞凋亡抑制蛋白(Bcl-2)的蛋白表現量減少，並且藉由透過切割活化procaspase 8，將Bid的C端切除而形成tBid蛋白，tBid蛋白移動到粒線體細胞膜上使得Bax蛋白的活化，導致粒線體膜電位改變，而將細胞色素C釋放至細胞質當中，促使procaspase 9蛋白活化，並繼續活化下游的procaspase 3蛋白，並導致procaspase 3的受質PARP蛋白受到切割而進行細胞凋亡。

關鍵詞：C型肝炎病毒、套膜蛋白E2、細胞凋亡、caspase蛋白、細胞色素C

英文摘要：

Introduction: one unusual characteristics of HCV is to establish chronic infection and the precise mechanisms remain unclear. Materials and methods: Huh-7 cells were transiently transfected with E2 and subjected to MTT assay, DNA fragmentation assay and western blotting to see the impact of E2 protein on apoptosis. Results and Discussion: E2 may inhibit cell proliferation by inducing apoptosis and pro-caspases 3, 8 & 9 were cleaved and activated to result in the presence of active forms in a time-dependent fashion, which suggesting that E2-induced apoptosis is caspase-dependent. Furthermore, the cytosolic level of cytochrome c was increased together with a gradually down-regulated Bcl-2 and up-regulated Bax protein expression. The continuing reduction of Bid protein and the gradual increase of tBid protein also indicated that a time-dependent increased turn-over of Bid protein into tBid. Taken together, our data suggested that HCV E2 may induce apoptosis through a mitochondrial damage-mediated caspase pathway.

Keywords: Hepatitis c virus, Envelope protein 2, Apoptosis, Cytochrome c, Caspase

Introduction

Hepatitis C virus (HCV) infection has been one of major causes of chronic hepatitis and liver failure worldwide. One unusual characteristics of hepatitis C is its capability to establish chronic infection in most infected individuals. The precise related mechanisms of carcinogenesis and mechanisms involved in evading host immune surveillance remain to be elucidated, however, analysis of peripheral blood mononuclear cells and liver biopsies from chronic patients suggested that HCV infection could induce apoptosis, which may help the virus escape the immune surveillance and cause liver injuries. The involvement of other HCV proteins in the induction of apoptosis has not been clearly identified.

Known as the viral attachment protein responsible for the viral entry, HCV envelope protein 2 (E2), a structural protein, has been interested for its potential to induce apoptosis since this protein shares a similar genetic characteristic as the envelope proteins of certain Flaviviruses, which have been reported to be able to induce apoptosis in cultured mammalian cells to maintain persistent infection [18-22]. In this study, we examined the expression levels of apoptosis-related molecules in a HCV E2-expressing Huh-7 cell line to elucidate the role of HCV E2 protein in establishing chronic infection and liver injury.

Materials and Methods

The cell line used in this study is Huh-7, a human hepatocellular carcinoma cell line. A DNA fragment containing full-length E2 coding region (aa 498-777 of the HCV polyprotein) was obtained from a PCR amplification with a set of E2-specific primers and then cloned into a expression vector pEGFP-N1 to yield a E2 expression vector, pEGFP-E2, which was then transfected into cells with a lipofectamine reagent. At 24 and 48 hours post-transfection, cells were harvested and analyzed for cell viability (MTT assay), apoptosis (DNA fragmentation), and Western blot for apoptosis-related proteins.

Results

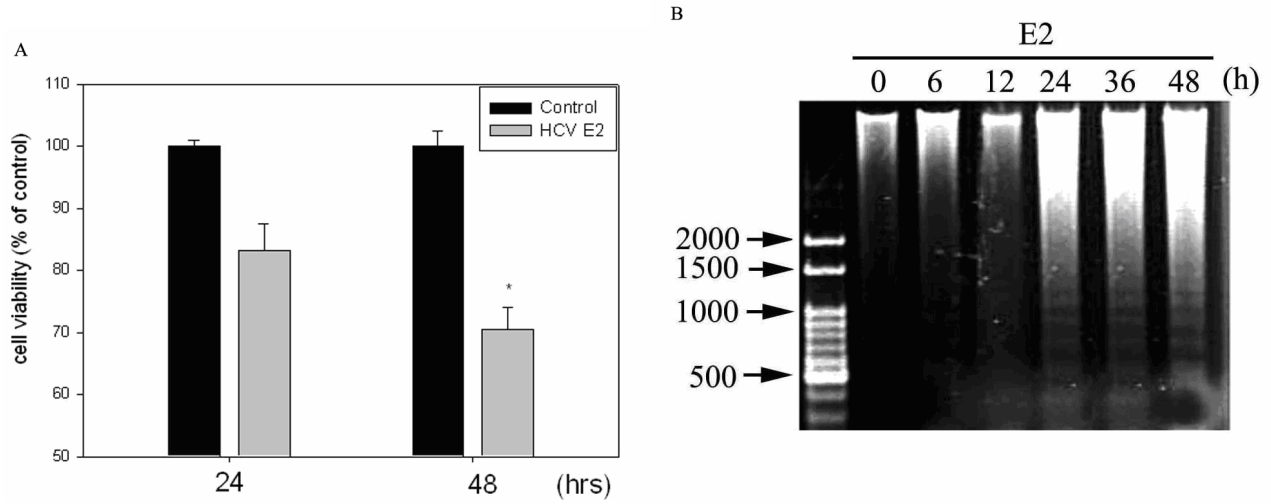


Fig. 1. HCV E2 inhibits the proliferation of Huh-7 cells by inducing apoptosis. (A) At 24 or 48 hours after E2 transfection, 0.5 mg/mL MTT in fresh medium was added and incubated for additional 4 hours. The survival of cells was detected by measuring the absorbance at a wavelength of 563 nm and expressed as the ratio of control. Data represent means ± SD of at three independent experiments. (B) At the indicated time after transfection, cells were harvested and subjected to DNA extraction. The DNA fragments were separated on 1% agarose gel electrophoresis, stained with ethidium bromide, and then visualized under ultraviolet light.

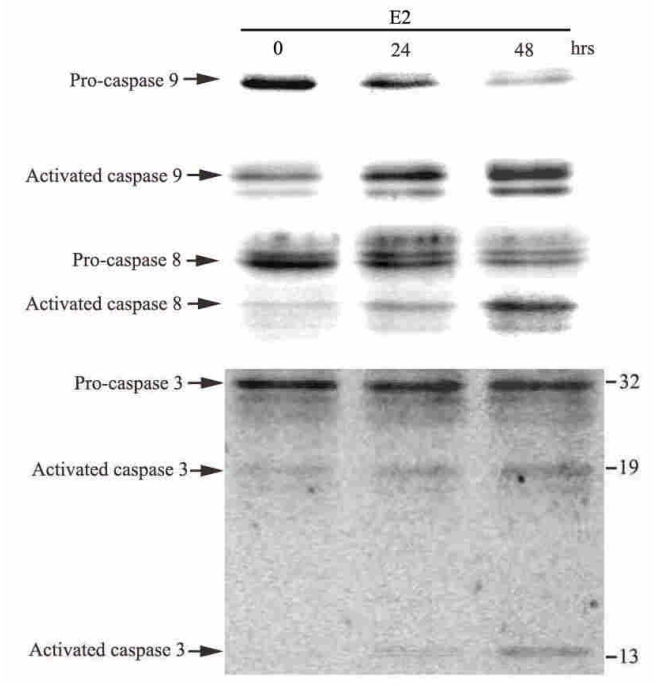
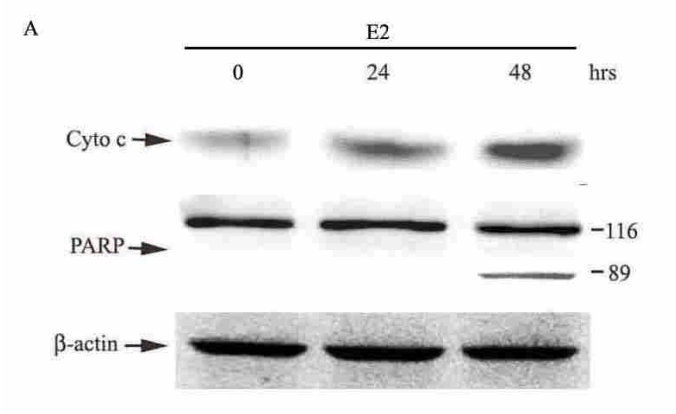


Fig. 2. Activation of caspases in E2-transfected Huh-7 cells. At 24 or 48 hours after E2 transfection, cells were harvested and the whole cell lysate were prepared for western blotting with an antibody against caspase 3, 8 or 9 as described in Materials and Methods.



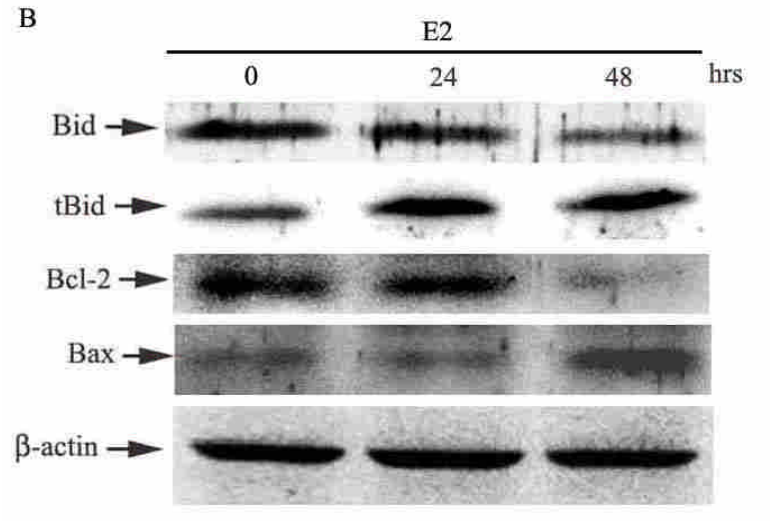


Fig. 3. Involvement of cytochrome c release and caspase-dependent Bcl-2 cleavage in E2-transfected Huh-7 cells. At 24 or 48 hours after E2 transfection, cells were harvested and the whole cell lysate were prepared for western blotting with an appropriate antibody against cytochrome c or PARP (A), or Bid, tBid, Bcl-2, or Bax (B) as described in Materials and Methods.

Discussion

One of the most irritated and unresolved problem caused by HCV is the establishment of chronic infection in most infected people despite of effective immune response and such chronic HCV infection may lead to cirrhosis and hepatocellular carcinoma. In addition to that the high mutation rate of viral envelope proteins and the suppression of host immune system are generally believed to be the main causes, apoptosis has been proposed to be one contributor to viral chronic infection. Although such programmed death of cells infected by viruses may appear suicidal, this has been suggested to be a response to virus infection as a mean of facilitating virus dissemination.

Apoptosis is a tightly regulated progress under the control of several signaling pathways, such as caspase and mitochondrial pathways and it has been reported that apoptosis is involved in the pathogenesis of hepatitis C. There is evidence that immune response (cytotoxic T lymphocyte) might be involved in the apoptosis of hepatocytes in HCV infected patients. Iken et al recently reported that HCV structural proteins core, envelope 1 (E1) and envelope 2 (E2) may enhance apoptosis of activated T cells by upregulating FasL. Increased apoptosis of activated T cells induced by HCV structural proteins could amplify the ability of the liver to down-modulate T cell responses, leading to attenuation of anti-viral responses and facilitating viral persistence. However, another study indicated that HCV core-E1-E2 transgenic mice develop significantly larger tumors than transgenic mice expressing core alone or nontransgenic mice. The accelerated tumor phenotype is attributable to suppression of apoptosis rather than enhanced proliferation. These data implicate HCV E1 and/or E2 in conjunction with core as antiapoptotic, tumor accelerator proteins. Furthermore, a recent study suggested that HCV core protein induces the expression of FasL and the activity of FasL promoter in HepG2 cell system to induce apoptosis while other studies have obtained controversial results. Therefore, HCV core protein may not the only viral protein involved in HCV-induced apoptosis and the contribution of other HCV proteins should be clarified.

Among other HCV proteins, E2 protein has been suspected to possess a capability of inducing apoptosis due to its genetic similarity as the envelope proteins of dengue and Langat viruses, which could induce apoptosis during infection. As for the impact of HCV E2 protein on apoptosis, inconsistent results have been obtained, while a study has indicated that HCV E2 protein could induce apoptosis to contribute the liver injury, another study showed that E2 inhibits apoptosis by inhibiting TRAIL-induced cytochrome *c* release from the mitochondria, which may subsequently augment persistent HCV infection. Therefore, to clarify the impact and underlying mechanism of HCV E2 protein on apoptosis, as well as to elucidate the role of HCV E2 protein in establishing chronic infection and liver injury, HCV E2 was transiently expressed in Huh-7 cells. Microscopic observation showed that E2-transfected Huh-7 cells displayed a shrank morphology while cell proliferation of E2-transfected Huh-7 cells was significantly reduced, compared to those vector-transfected cells. Our further assay for DNA fragmentation demonstrated that reduced

proliferation of E2-transfected cells was apoptosis-related. Results of subsequent western blotting indicated that E2 protein caused activations of caspase-3, caspase-8 and caspase-9, to result in increased expressions of activated caspases, which is consistent with a study of Langat virus demonstrating that expression of envelope (E) protein could induce apoptosis via the caspase-3 pathway. Furthermore, the cleavage of PARP into 116 kDa and 89 kDa also proved the occurrence of apoptosis and the activation of caspase pathway. Taken together, we have proven that HCV E2-induced apoptosis is caspase-dependent. Moreover, a time-dependent release of cytochrome *c* was also observed after the E2 transfection indicating a mitochondria-initiated apoptosis. Such apoptosis process is believed to be related to the expression of proteins of Bcl-2 family, which have been reported to regulate the induction of apoptosis at least through the control of mitochondrial function. For examples, Bcl-2 could influence the permeability of intracellular membranes and cytochrome *c* release from mitochondria to control apoptosis. Furthermore, during apoptotic process, Bid is cleaved by caspase-8 and the resultant truncated Bid could cause mitochondrial cytochrome *c* releasing into the cytoplasm. In the present study, the reduction of Bcl-2 protein level was observed in E2-transfected Huh-7, accompanied wit a reduced Bid protein level and an increased tBid level, which are concordant and reflect the increased apoptosis. According to current understanding, BH3-only proteins, such as Bid, could activate multidomain members, Bax or Bak, which have the capability of altering the integrity of outer mitochondrial membrane, by stimulating its oligomerization in the outer membrane. Our result showing an increased Bax level caused by E2 transfection was consistent with such regulation.

An increased Fas-mediated apoptosis PBMC from chronically infected patients has been reported and core protein has been shown to be able to increase the sensitivity of a T cell line to Fas-mediated apoptosis. However in this study, expression levels of Fas and FasL were not significantly altered by the transfection of HCV E2 (data not shown). Furthermore, other pathways regulating apoptosis, such as JNK and p38 pathways, were not examined in this study yet and therefore the impact of E2 on these pathways could not be ruled out and should be clarified.

Taken our data together, HCV E2 protein may first activate caspase-8 to lead to Bid truncation and Bcl-2 reduction, and then activates mitochondria-mediated apoptotic pathway including cytochrome *c* release and subsequent activation of caspase-9 and caspase-3. These results indicated that HCV E2-induced apoptosis of Huh-7 cells may involve a mitochondrial-related caspase pathway. In summary, our data have revealed that E2 protein may involved in HCV-induced pathogenesis by inducing apoptosis of Huh-7 cells.