行政院國家科學委員會專題研究計畫 成果報告

C 型肝炎病毒核心蛋白及 NS5A 蛋白對於胰島素作用相關分子的影響

研究成果報告(精簡版)

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中文摘要及關鍵詞

關鍵詞：C 型肝炎病毒、NS5A 蛋白、細胞凋亡、caspase

C 型肝炎病毒的慢性感染可導致肝臟纖維化，但其相關機制並不清楚。在此年度研究中，我們將含有 NS5A 基因片段的質體送入 Huh-7 細胞中，進行 MTT assay、DNA fragmentation assay 及 western blotting 等分析來探討 NS5A 蛋白是否具有導致細胞凋亡的能力。結果顯示 NS5A 可藉由引發細胞凋亡來抑制細胞增殖，此蛋白表現可導致 pro-caspases 3、8 及 9 被切割後成為活化態，且此現象具有時間相關性，顯示 NS5A 引發細胞凋亡的作用與 caspase 有關。此外 cytochrome c 在細胞質內濃度增加，同時伴隨著 Bcl-2 蛋白濃度減少及 Bax 蛋白增加。隨著時間增加，Bid 蛋白逐漸減少，同時 tBid 蛋白逐漸增加。統整以上之結果顯示，C 型肝炎病毒的 NS5A 蛋白可經由與 caspase 相關的路徑來引發細胞凋亡。

Abstract

Keywords: hepatitis C virus, NS5A protein, apoptosis, caspase

Chronic HCV infection may lead to hepatic fibrosis and the precise mechanisms remain unclear. In this study, Huh-7 cells were transiently transfected with NS5A and subjected to MTT assay, DNA fragmentation assay and western blotting to see the impact of NS5A protein on apoptosis. Results showed that NS5A 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 NS5A-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 NS5A may induce apoptosis through a mitochondrial damage-mediated caspase pathway.

Introduction

Hepatitis C virus (HCV) infection may lead to liver diseases of various severities, from acute hepatitis to fatal hepatocellular carcinoma (HCC). While most studies on HCV-related pathogenesis focused on that the anti-apoptosis potential of viral proteins leading to inhibition of cell death and uncontrolled cell growth may be the underlying mechanism for HCC, fewer studies have been conducted to address the molecular mechanism of acute hepatitis. Viral hepatitis is evidenced by death of hepatocytes causing by virus replication or immunity during the early stage of disease and the mechanism of HCV replication, persistence and pathogenesis is still unclear.

Apoptosis is one of the typical processes of cell death and generally considered to be a mechanism of host defense against viral infection since induction of apoptosis will interrupt viral replication as viral infected cells are eliminated. Therefore, viruses have evolved various mechanisms to evade or inhibit the process of apoptosis to lead to a chronic infection. On the other hand, it is now known that some viruses are able to induce apoptosis, resulting in the dissemination of viral particles with minimum host immune response as well as protecting progeny viral particles from host neutralizing antibodies, since apoptotic cells are usually vacuolized and endocytosed by phagocytic cells. Furthermore, recent reports have pointed out the contribution of the mitochondria-dependent apoptotic pathway involving the Bcl-2 family of proteins in the development of liver diseases (Taya, et al., 2000; Toubi et al., 2001) and our recent study also revealed that HCV E2 may induce apoptosis of Huh-7 cells via a mitochondrial-related caspase pathway (Chiou et al., 2006) The impact of other HCV viral proteins on cell growth should be clarified.

Among HCV structural (core, E1, E2, and P7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins, NS5A has been demonstrated to be a versatile protein involved in many cellular events and interfering various pathways related to viral persistence or pathogenesis. This protein is well known to block the innate immunity against viruses by directly interacting with and inhibiting the activity of protein kinas K (PKR), which is interferon-induced and double-stranded RNA-dependent. Furthermore, studies with NS5A expressing cell lines revealed that NS5A may interfere cell cycles to lead to a reduced S phase and an increased G_2/M phase (Honda et al., 2000). NS5A has also been demonstrated to interfere the process of apoptosis, for instance, protecting cells against TNF-α- and p53-mediated apoptosis (Avirutnan et al., 1998; Duarte dos Santos et al., 2000; Hijikata et al., 1991; Prikhod'ko et al., 2002; Wang et al., 2002; Zhu et al., 1998). The anti-apoptotic property of NS5A has been widely investigated to imply that NS5A may contribute to the persistent infection and carcinogenesis, while the involvement of NS5A in apoptosis induction to lead to hepatic injury has been less investigated. In this study, we aim to see the impact of NS5A on apoptosis-related events to address the involvement of NS5A in the process of acute hepatitis.

Materials and Methods

Cell culture

Huh-7, a human hepatocellular carcinoma cell line, was grown in Dulbeco's modified eagle medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum, 2 mM

glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, 0.1 mM nonessential amino acid and 1 mM sodium pyruvate and maintained at 37℃ in a humidified atmosphere of 5% CO_2.

Construction of a plasmid containing NS5A gene

A DNA fragment containing full-length NS5A coding region (aa 498-777) was obtained from a PCR amplification with a pair of primers (5'-GCAAGCTTCGCTCCGGCTCGTGGCTAAAG-3'; HindIII site underlined and 5'-GGGCCCTTAGGACATTGAGCAGCAGACGA-3'; ApaI site underlined) and then cloned into a expression vector pEGFP-N1 (Beckton-Dickinson) to yield pEGFP-NS5A, a NS5A expression vector.

Cell survival assay

At 12 or 24 hours after transfection as described above, cells were subjected to trypsin treatment and collected and then stained with an equal volume of trypan blue. Surviving cells were counted under microscope and survival rate was expressed as percentage with the number of untransfected cells being the control of 100%.

DNA fragmentation

Transfected or untransfected cells were harvested and incubated in lysis buffer (10 mM/L Tris, 1 mM/L EDTA, 100 mM/L NaCl, 5 g/L SDS, 1 μg/μL RNase A, pH 8.0) at 37℃ for 30 minutes. After the incubation, proteinase K (final concentration of 0.1 mg/mL) was added and the incubation was continued at 55℃ for 4 additional hours. At the end of incubation, DNA was extracted and precipitated with ethanol, and then dissolved in TE buffer followed by agarose gel electrophoresis.

Western blot analysis

For the preparation of whole cell lysate, cells were lysed in a lysis buffer (25 mmol/L hepes, 15 g/L Triton X-100, 10 g/L sodium deoxycholate, 1 g/L SDS, 0.5 mol/L NaCl, 5 mmol/L edetic acid, 50 mmol/L NaF, 0.1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 0.1 g/L leupeptin, pH7.8) at 4℃ and then centrifuged at 15,000 g for 15 minutes. For the detection of cytochrome *c* in cytosolic fraction, cells were washed twice with ice-cold PBS (pH 7.4) and then resuspended in 100 μl of ice-cold mitochondrial buffer (20 mM HEPES (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitors) containing 250 mM sucrose. The nuclei and unbroken cells were separated from the cytosol fraction by centrifugation at 10,000 g for 10 minutes. Samples were separated in a 12.5% polyacrylamide gel and transferred onto a nitrocellulose membrane as previously described (Liu et al., 2001). The blot was subsequently incubated with 5% non-fat milk in PBS for 1 hour to block non-specific binding, and probed with a corresponding antibody against a specific protein (C8A256M for NS5A was obtained from BIODESIGN International; B-10 for Fas, C-178 for Fas-L, H-181 for FADD, C-2 for Bcl-2, N-20 for Bax, N-19 for Bid, h71 for cleaved Bid, F-2 for PARP-1, H-104 for cytochrome c, H-38 for caspase 9, H-134 for caspase 8, and L-18 for caspase 3 were purchased from Santa Cruz, California, USA) for 2 hours, and then with an appropriate peroxidase-conjugated secondary antibody for 1 hour. All incubations were carried out at 37℃ and intensive PBS washing was performed between incubations. After the final PBS washing, signal was developed by ECL detection system and relative photographic density was quantitated by scanning the photographic

negatives on a gel documentation and analysis system (Alpha Imager 2000, Alpha Innotech Corporation).

Results

NS5A expression induces cell death and DNA fragmentation of Huh-7 cells

To see the if NS5A could affect the growth of Huh-7 cells, a NS5A-expressing plasmid, pEGFP-NS5A, was constructed by inserting a DNA fragment containing the entire NS5A gene into pEGFP-N1, a plasmid containing EGFP gene, and then transiently transfected into cells. Cell survival assay revealed that cell number was significantly reduced by NS5A expression and such reduction was time-dependent with cell viability being reduced to less than 10% at 48 hours post transfection, as compared with that of mock transfection (Figure 1A).

To determine if such reduction of cell number by NS5A was related to apoptosis, cells transfected with pEGFP-NS5A or control vector pEGFP-N1 were subjected to DNA fragmentation assay. As shown in Figure 1B, the formation of DNA ladder in NS5A expressing cells was evident while cells untransfected or transfected with pEGFP-N1 showed no sign of DNA fragmentation. Therefore, the reduction of cell number caused by NS5A expression could be through the induction of apoptosis.

NS5A affects the expressing levels of apoptosis-related caspases

To explore the molecular mechanism responsible for NS5A-induced apoptosis, expression levels of several apoptosis-related genes, including Fas, Fas ligand, FADD, Pro-caspases and caspases 3, 8 & 9 were analyzed by western blots. The unaltered expressions of Fas, Fas ligand or FADD before and after NS5A-transfection indicated that these molecules were not involved (data not shown). Since it has been well established that caspases are involved in and indispensable for the final stage of apoptosis and pro-caspases would be cleaved divided into active forms to carry out their functions upon responding to various stimuli, the involvement of caspases in the apoptotic response induced by NS5A was studied. As shown in Figure 2, the presence of active forms of caspases 8 & 9 indicated that pro-caspases 8 & 9 were cleaved and activated in NS5A expressing cells. Furthermore, caspase 3, a downstream protease of caspases 8 & 9, was also cleaved and activated. These results suggested that NS5A-induced apoptosis is caspase-dependent.

NS5A-induced apoptosis involves cytochrome c release and caspase-dependent Bcl-2 cleavage

The release of cytochrome *c* from mitochondria to cytosol is a hallmark of apoptosis and such release leads to a caspase-9-dependent activation of caspase-3, as well as the presence of a cleaved form of PARP (with a molecular weight of 89 kDa). As shown in Figure 3, the cytosolic level of cytochrome *c* increased in a time-dependent manner in NS5A-transfected cells while the cleaved form of PARP clearly appeared with a concomitant decrease of pro-PARP. Since mitochondrial cytochrome *c* release could be induced through a serious of actions involving members of Bcl-2 family, such as Bax, Bcl-2 and Bid, we thus examined the impact of NS5A expression on these proteins. As shown in Figure 4, after NS5A transfection, the protein level of Bcl-2 was reduced, while a gradual increase of Bax protein expression was detected. Furthermore, the continuing reduction of Bid protein and the gradual increase of tBid protein indicated that Bid protein was

truncated to be tBid. These results indicated that NS5A may induce apoptosis via Bid- and Bcl-2-involved mitochondrial pathway.

Discussion

One of the most irritated and unresolved problem caused by HCV is the establishment of chronic infection. In addition to that the high mutation rate of viral envelope proteins (Ray et al., 1996; Ray et al., 1998) and the suppression of host immune system (Honda et al., 2000; Marusawa et al., 1999; Ray et al., 1997) are generally believed to be the main causes, apoptosis has been proposed to be one contributor to viral chronic infection. Although such programmed death may appear suicidal, apoptosis of infected cells may lead to a minimum host immune response while apoptotic cells are usually fragmentized and then endocytosed by phagocytic cells, therefore progeny viral particles may escape from neutralizing antibodies. Therefore, apoptosis has been suggested to be an effective mean of facilitating virus dissemination and responsible for hepatic injury.

Apoptosis is a tightly regulated progress under the control of several signaling pathways, such as caspase and mitochondrial pathways (Bassett et al., 1999; Calabrese et al., 2000; Hiramatsu et al., 1994) and it has been reported that apoptosis is involved in the pathogenesis of hepatitis C. Iken et al recently reported that HCV structural proteins may enhance apoptosis of activated T cells by upregulating FasL, which could amplify the ability of the liver to down-modulate T cell responses, leading to attenuation of anti-viral responses and facilitating viral persistence (Iken et al., 2006). Analysis of peripheral blood mononuclear cells and liver biopsies from chronic patients also suggested that HCV infection could induce apoptosis, which may cause liver injuries (Calabrese et al., 2000; Emi et al., 1999; Hiramatsu et al., 1994; Pianko et al., 2001; Taya, et al., 2000; Toubi et al., 2001). *In vitro* studies with either HCV full length RNA or cDNA (Kalkeri et al., 2001) have demonstrated that apoptosis could be induced by viral proteins. Several independent studies suggested that HCV core protein could modulate cellular apoptosis (Honda et al., 2000; Marusawa et al., 1999; Ray et al., 1996; Ray et al., 1998) and is involved in cell growth promotion and immortalization (Honda et al., 2000; Ray et al., 1997; Ray et al., 2000; Zhu et al., 1998). A study has indicated that expression of HCV E2 protein could induce apoptosis in cultured mammalian cells to contribute the liver injury (Zhu et al., 2004) and our previous revealed that HCV E2 may induce apoptosis of Huh-7 cells via a mitochondrial-related caspase pathway (Chiou et al., 2006). Therefore in this study, we examined the expression levels of apoptosis-related molecules in a HCV NS5A-expressing Huh-7 cell line to elucidate the role of HCV NS5A protein in the formation of acute hepatitis and liver injury.

With a molecular weight of 56- to 58-kDa, HCV NS5A gene product is a phosphorylated protein that participates in various events related to enhancement of viral pathogenesis. Containing a putative interferon (IFN) sensitivity-determining region, NS5A has been shown to directly interact with the IFN-induced double-stranded RNA-dependent protein kinase (PKR) to inhibit IFN-related immunity against viruses. Interactions of NS5A with molecules involved in cellular signaling, transcription activation and cell cycle regulation have also been described, such as growth factor

receptor-bound protein 2, p53, p21/waf and cyclins. Furthermore, NS5A expression could alter intracellular calcium and reactive oxygen species levels to lead to the activation of STAT-3 and NF-κB (Ray et al., 1996; Ray et al., 1998) and inhibit the activity of the mitogenic and stress activated transcription factor activating protein-1 (AP1) by interfering with Ras-ERK pathway (Marusawa et al., 1999). Furthermore, NS5A could protect cells against TNF-α and p53-mediated apoptosis (Avirutnan et al., 1998; Duarte dos Santos et al., 2000; Hijikata et al., 1991; Prikhod'ko et al., 2002; Wang et al., 2002; Zhu et al., 1998) and many of these interactions may contribute to an inhibition of apoptotic cellular response to persistent HCV infection.

In this study, to clarify the impact and underlying mechanism of HCV NS5A protein on apoptosis, as well as to elucidate the role of HCV NS5A protein in liver injury, HCV NS5A was transiently expressed in Huh-7 cells. Microscopic observation revealed signs of death of NS5A-transfected Huh-7 cells, viable cell number of NS5A-transfected cells was significantly reduced, compared to that of vector-transfected cells. Further analysis for DNA fragmentation demonstrated that reduced viability of NS5A-transfected cells was apoptosis-related. Results of subsequent western blotting indicated that NS5A protein caused activations of caspase-3, caspase-8 and caspase-9, to result in increased expressions of activated caspases. 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 NS5A-induced apoptosis is caspase-dependent. Moreover, a time-dependent release of cytochrome *c* was also observed after the NS5A transfection indicating a mitochondria-initiated apoptosis. Such apoptosis process is believed to be related to the expression of proteins of Bcl-2 family, which consists of anti-apoptotic proteins and pro-apoptotic proteins, and have been reported to regulate the induction of apoptosis at least through the control of mitochondrial function (Ma et al., 2002; Zhu et al., 2004). In the present study, the reduction of Bcl-2 protein level was observed in NS5A-transfected Huh-7, accompanied wit a reduced Bid protein level and an increased tBid level, which are concordant and reflect the increased apoptosis. Since it has been shown that Bid could activate multidomain members, Bax or Bak, by stimulating its oligomerization in the outer membrane, our result showing an increased Bax level caused by NS5A transfection was consistent with such regulation. Furthermore, protein-protein interactions among members of the Bcl-2 family may affect apoptosis, the Bcl-2/Bax expression ratio could be a useful marker of the outcome of an apoptoic stimulus. A decreased Bcl-2/Bax ratio was associated with increased apoptosis. Our quantitatively determination of protein level at 48 hours post-transfection showed that Bcl-2/Bax ratio of NS5A expressing cells was reduced to 2.2, compared to 6.76 of vector-transfected cells. Such ratio reduction further indicated the occurrence of apoptosis from NS5A expression.

Recently, a BH3 motif was identified in the X protein of hepatitis B virus (HBx) and proven to be responsible for the pro-apoptotic activity (Lu et al., 2005). Furthermore, the conserved BH3-like regions of many pro-apoptotic Bcl-2 family of proteins are critical for their induction of cell death and for their interactions with anti-apoptotic proteins, including the Bcl-2. It has been reported that NS5A contains three BH domains (Bcl-2 homology domains), locating at aa 121-129, 152-168, and 261-275. Therefore, it is possible that NS5A may induce apoptosis of hepatocytes via these BH

domains, which warrants more detailed molecular study.

Taken our data together, HCV NS5A protein may first activate caspase-8 to lead to Bid truncation and Bcl-2 reduction, and subsequently promote the release of cytochrome *c* and activation of caspase-9 and caspase-3 to induce cellular apoptosis via a mitochondria-mediated pathway. However, other pathways related to apoptosis, such as JNK and p38 pathways, were not examined in this study yet and therefore the impact of NS5A on these pathways should be clarified. These results indicated a direct involvement of HCV NS5A in cellular apoptosis, which may contribute to liver pathogenesis.

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Figure 1 (A) & (B)

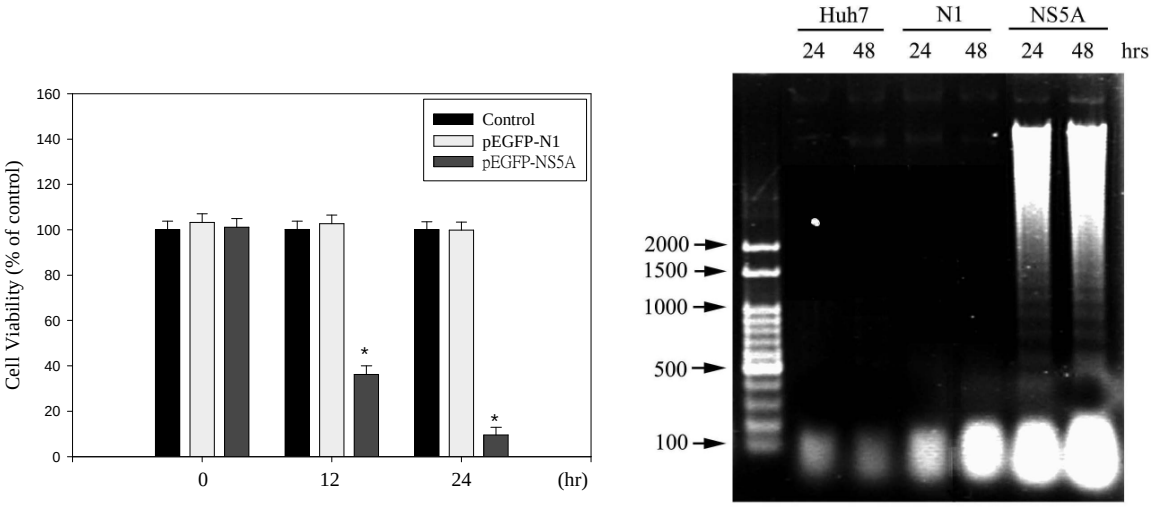


Figure 2

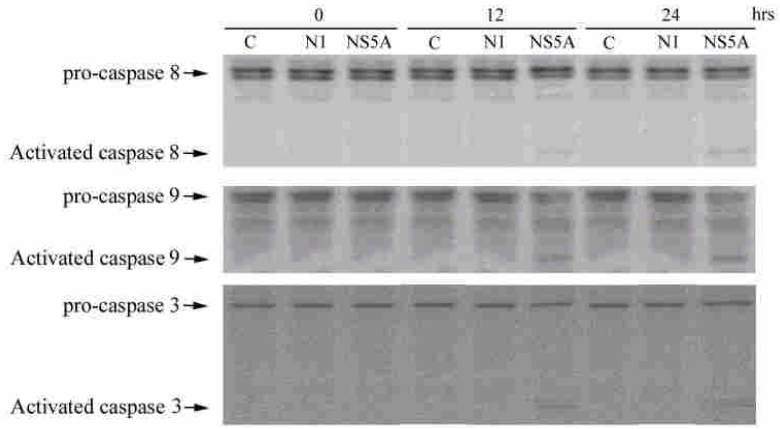
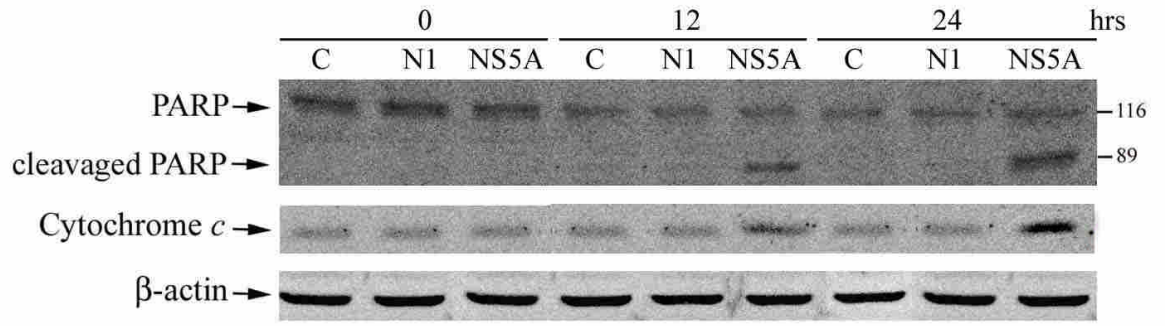


Figure 3



行政院國家科學委員會補助國內專家學者出席國際學術會議報告

95 年 10 月 2 日

附件三

報告人姓名	邱慧玲	服務機構及職稱	中山醫學大學 醫學檢驗暨生物技術學系 副教授
時間 會議 地點	95 年 9 月 15-19 日 愛爾蘭 都柏林	本會核定 補助文號	95-2320-B-040-016- 計畫內編列之預算
會議名稱	(中文) 第 27 屆世界醫學檢驗師大會 (英文) The 27th World Congress of Biomedical Laboratory Science		
發表論文題目	(中文) 乳癌患者之 Cystatin C 血清濃度及臨床特性 (英文) Relationships between the level of Cystatin C and clinical features of breast cancer		

一、參加會議經過

本次與醫檢師公會全國聯合會（全聯會）之理監事組團參加此會議。於 9 月 14 日抵達韓國首都首爾後即前往會議地點（COEX 會議中心）報到及領取大會議程及摘要手冊，將準備好的論文海報張貼於指定位置。因全聯會為本大會之會員國，15 日之開幕典禮中有中華民國國旗進場，足見大會之誠意。於 16-19 日前往會場聆聽多場特別演講，並在下午於展出海報處說明研究成果，與多位學者進行深入討論。

二、與會心得

本次大會的主題涵蓋檢驗醫學各領域，藉由此次會議讓我有機會接觸到更實際的臨床領域，獲得不少新觀念及之前未曾有過的一些想法。另外也與各國從事醫學檢驗的專家學者多有接觸，瞭解他國醫學檢驗的現況。

三、考察參觀活動(無是項活動者省略)

此次大會安排參觀三星醫學中心的檢驗醫學部，該中心有全自動的檢體運送及貼標籤系統，並且有多項最新的全自動分析儀，讓醫檢師能有心力及時間從事更高階的品管或研究工作，這也將是台灣醫檢界朝向的目標。

四、建議

國內應多加舉辦如此大型會議、增加補助出國額度、或盡量補助博士班學生出國開會或短期研究之經費，讓年輕研究學者有機會與國際接軌。

五、攜回資料名稱及內容

會議議程手冊

會議摘要手冊

六、其他