中 國 醫 藥 大 學
醫學研究所
碩士學位論文

乙醛經由活化 nuclear factor-κB 和 activator protein 1 訊息傳導路徑活化 MMP-9 表現而造成腫瘤轉移

Acetaldehyde induces matrix metalloproteinase-9 gene expression and promotes hepatocarcinoma cells invasion through nuclear factor-κB and activator protein 1 signaling pathways

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論文題目

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本論文係張 藝 馨 於中國醫藥大學醫學研究所完成之碩士論文，經考試委員審查及口試合格，特此證明。

考試委員

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所長：[illegible]

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藝馨 瑾誌

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中文摘要

乙醛是酒精主要的氧化代謝產物，有研究指出乙醛與肝癌的發展有關，致病機轉可能為乙醛可活化轉錄因子 activator protein 1 (AP-1)與 nuclear factor-κB (NF-κB)。由於 Matrix metalloproteinase-9 (MMP-9)啟動子具有 AP-1 與 NF-κB 的結合位，且會破壞細胞外的基質，而參與腫瘤轉移的過程。然而乙醛是否會經由轉錄因子 AP-1 與 NF-κB 誘發 MMP-9 的活性而促使腫瘤轉移，這部分不是研究的非常透徹。在這個研究當中，我們證實了乙醛可經由轉錄因子 AP-1 與 NF-κB 調控 MMP-9 基因的轉錄作用而誘發 MMP-9 的活性。進一步利用西方墨點法觀察 AP-1 與 NF-κB 上游訊息傳遞的相關蛋白發現乙醛是透過 c-Jun N-terminal kinase (JNK)/β-transducin repeat-containing protein (β-TrCP)及 inhibitory κB-α (IκB)訊息傳導路徑活化 NF-κB，促使 NF-κB 從細胞質進入到細胞核當中活化 MMP-9 基因的轉錄及進一步的表現。另外，乙醛也會經由 p38 訊息傳導路徑活化 AP-1，進而活化 MMP-9。綜合以上結果，我們的結果顯示乙醛與腫瘤的轉移有關。而機轉為乙醛可以經由 IκB、 JNK 及 p38 訊息傳導路徑同時活化轉錄因子 NF-κB 與 AP-1 進入細胞核中與 MMP-9 基因上游的啟動子結合而活化 MMP-9 基因表現。

英文摘要

Acetaldehyde, the very reactive intermediate of oxidative metabolism of ethanol, is potentially associated with alcohol-induced liver diseases. Matrix metalloproteinase-9 (MMP-9) is directly involved in human hepatic tumorigenesis and metastasis. However, the relationship between acetaldehyde and MMP-9 expression in liver diseases is currently poorly understood. Herein we demonstrated that acetaldehyde increased MMP-9 gelatinolytic activity and promoted cell invasion through the up-regulation of MMP-9 gene transcription in HepG2 cells. The transcription of MMP-9 gene was regulated by acetaldehyde via inductions of nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) activities. Western blot analysis indicated that acetaldehyde stimulated the translocation of NF-κB into nucleus through inhibitory κB-α (IκB-α) and c-Jun N-terminal kinase (JNK)/β-transducin repeat-containing protein (β-TrCP) signaling pathways. Acetaldehyde also induced AP-1 activity via the phosphorylation of p38 kinase. In conclusion, our findings demonstrated that acetaldehyde activated NF-κB and AP-1 activities via IκB, JNK/β-TrCP, and p38 signaling pathways, resulting in the induction of MMP-9 gene expression and the increase of cell invasion. On the basis of these data, we suggested that acetaldehyde plays an important role in tumor invasion and metastasis.

目錄

圖目次

第一章 文獻探討

乙醛簡介及其致病機轉

乙醛(acetaldehyde)是酒精在肝臟中經由酒精去氫酶 (alcohol dehydrogenase, ADH)代謝所產生的，其被證實具有致癌性，會導致細胞癌化、抑制 DNA 修補以及增強細胞傷害 (Obe and Ristow, 1979; Garro, et al., 1986) ，國際癌症組織協會鑑定乙醛為致癌物質。乙醛有可能引起肝臟疾病，例如肝炎及肝癌 (Morgan et al., 2004)。近年來有研究報告指出其可能與一些蛋白質結合而影響肝臟結構與功能(Lieber, 1994; Pares et al., 1994; Carter and Wands, 1988) 。除此之外，乙醛在肝癌細胞株 HepG2 中也可活化轉錄因子 activator protein 1 (AP-1)與 nuclear factor-κB (NF-κB) (Roman et al, 2000)。而 AP-1 與 NF-κB 可能與腫瘤侵襲及轉移相關 (Huber et al., 2004)。然而乙醛是否會經由 AP-1 與 NF-κB 促進腫瘤轉移，到目前為止還不是非常清楚。

腫瘤的侵襲與轉移與 MMP 的相關性

腫瘤的侵襲與轉移是造成癌症病患治療失敗及死亡的主要原因，腫瘤細胞的轉移需將細胞外基質重組，過度破壞細胞外的基質是癌症的特點，也是癌症惡化的主因 (Lukashev and Werb,1998)。許多蛋白質水解酶都能破壞細胞外基質的成分，其中以 Matrix metalloproteinase (MMP)為主。MMP 是 Zinc-binding endonuclease 的一員，其功用是破壞基底膜及調控細胞生長及分化，不同種類的

MMP 具有不同受質特異性，例如：interstitial collagenase (MMP-1)能夠分解第一、二、三型的 collagens (Collier et al., 1988; Glodberg et al., 1986) ，而 stromelysin (MMP-3) 具有分解第三、四型的 collagens、proteoglycan、fibronectin 以及 laminin 的活性 (Chin et al., 1985; Okada et al., 1986; Wilhelm et al., 1987; Saus et al., 1988) 。另外 72kDa 及 92kDa 的 Gelatinase/type IV collagenase(MMP-2 及 MMP-9) 可分解第四、五型的 collagens、fibronectin、laminin 以及 gelatins(Collier et al., 1988; Murphy et al., 1989; Wilhelm et al., 1989; Okada et al., 1990) 。因為基底膜含有許多 MMP 的受質，所以 MMP 異常分泌時會破壞基底膜的組成。

MMP 參與許多生理過程中結締組織的重組，例如胚胎的發育、排卵及傷口修復。此外這些蛋白質水解酶的異常分泌可能產生疾病，例如，類風濕性關節炎、心血管疾病 (Nagase and Woessner,1999; Hulboy et al., 1997; Stetler-Stevenson et al., 1993) 。另外因為 MMP 會破壞基底膜，所以腫瘤的侵襲與轉移必須依賴其表現上升(Westermarck and Kahari, 1999; Stamenkovic, 2000)。近年來 MMP 可當成肝癌的血清指標 (Paradis et al., 2005)，尤其是 MMP-9 在肝癌表現量最高，MMP-9 會破壞基底膜中的第四型膠原蛋白，造成肝癌的轉移 (Arii et al., 1996)。MMP-9 啟動子上含 AP-1 與 NF-κB 結合位 (Sato and Seiki, 1993; Sato et al., 1993) ，在本實驗室之前的報告當中已經證實了乙醛可以活化 NF-κB 而增加細胞激素的表現。接下來我們想了解的是乙醛是否可經由誘發 NF-κB 或 AP-1 的活

性增加 MMP-9 的表現，而在腫瘤轉移的進程中扮演重要的角色。

NF-κB 及 AP-1 在基因調控的重要性

真核生物的基因受到結合至啟動子上游序列的轉錄因子調控，其中 NF-κB 及 AP-1 在基因調控扮演重要角色 (Brach et al., 1993; Liou and Baltimore et al., 1993) 。NF-κB 及 AP-1 受到許多刺激物例如生長因子、細胞激素、UV 及氧化壓力所活化 (Johnson and McNight, 1989; Abate et al., 1990; Sen and Packer, 1996) ，而活化的 NF-κB 及 AP-1 可調控許多基因的表現包含與免疫反應及腫瘤轉移的相關基因 (Johnson and McNight, 1989; Liou and Baltimore et al, 1993; Sen and Packer, 1996) 。

NF-κB 最早是由 Baltimore 於 1986 年發現。NF-κB 是由 p50、p52、p65 (Rel A)、Rel B 及 c-Rel 組成的雙倍體轉錄因子 (Siebenlist et al., 1994)。NF-κB 為誘發型的轉錄因子在發炎反應、免疫反應、腫瘤細胞惡化中扮演重要角色 (Karin and Ben-Neriah, 2000)。在正常情況下, NF-κB 與其抑制蛋白質 IκB 結合而存在於細胞質當中，但是如果細胞受到傷害，IKK 會將 IκB 磷酸化，接下來 β-TrCP 與磷酸化的 IκB 結合，將其送到 26S proteosome 分解， IκB 與 NF-κB 解離之後，使 NF-κB 次單位 p50 及 p65 蛋白質的 nuclear localization signals 暴露出來，而使得 p50/p65 heterodimer 送到細胞核內。活化態的 heterodimer 會進一步與基因啟

動子上的特定序列結合，而導致下游基因的表現 (Baldwin et al., 1996; Barnes et al., 1997; Manna and Aggarwal, 2000) 。

而 AP-1 是由 Fos (c-Fos, FosB, Fra-1, and Fra-2) 和 Jun (c-Jun, JunB, and JunD) 家族組成，其在細胞增生、分化以及凋亡作用中扮演重要角色(Brach et al., 1993; Liou and Baltimore, 1993)。AP-1 活性受到 mitogen-activated protein (MAP) kinases 像是 c-Jun N-terminal kinases (JNKs)、p38 和 extracellular signal-regulated kinases (ERKs)調控 (Karin et al., 1997)。

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研究動機

由於乙醛可以活化 NF-κB、AP-1(Roman et al, 2000)，而 MMP-9 啟動子上含 AP-1 與 NF-κB 結合位 (Sato and Seiki, 1993; Sato et al., 1993) 。在這個研究中，我們想要探討的是乙醛是否可經由誘發 NF-κB 或 AP-1 的活性增加 MMP-9 的表現，而在腫瘤轉移的進程中扮演重要的角色(圖 1)。我們的結果顯示乙醛與腫瘤的轉移有關。而機轉為乙醛可以經由 IκB、JNK 及 p38 訊息傳導路徑同時活化轉錄因子 NF-κB 與 AP-1 進入細胞核中與 MMP-9 基因上游的啟動子結合而活化 MMP-9 基因表現。這是第一次發現乙醛會活化 MMP-9 的訊息傳導路徑。

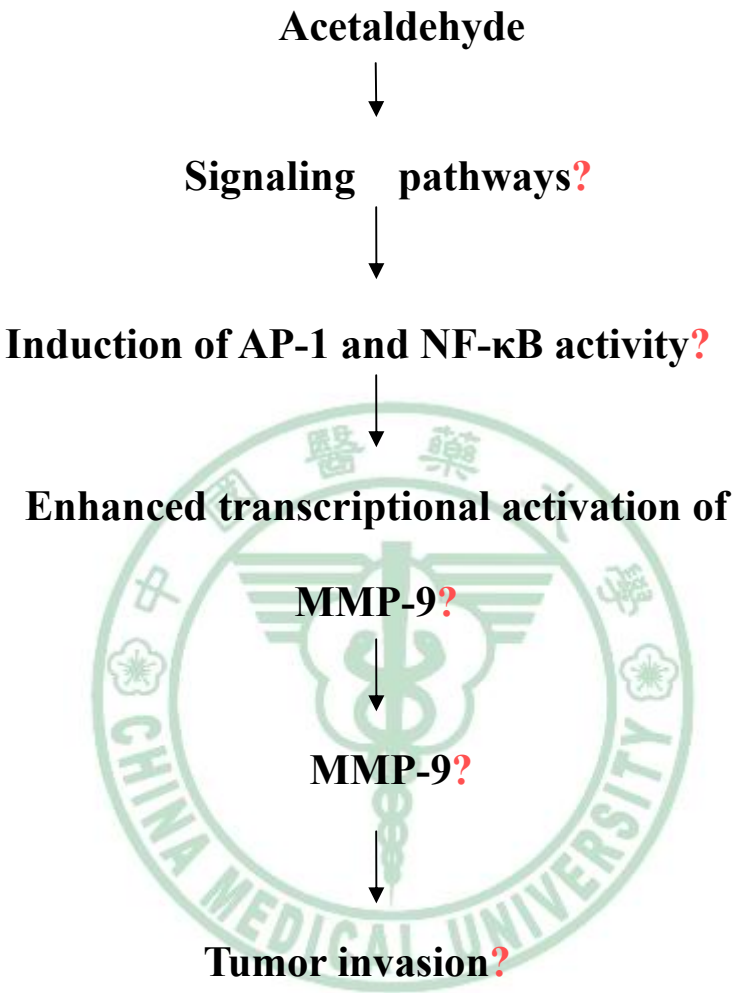


圖 1. 論文研究架構

探討乙醛對於 MMP-9 表現的影響及其機轉以及與腫瘤轉移的相關性

第二章 材料與方法

細胞培養及穩定性轉染 (Stable transfection)

HepG2 cells和Chang livr cells 以含有10%胎牛血清 (HyClone, Logan, Utah)的 Dulbecco modified Eagle medium (DMEM) (Life Technologies, Gaithersburg, MD) 37℃培養。HepG2 cells 以 SuperFect® transfection reagent (Qiagen, Valencia, CA) co-transfected 2.5 μg *Alw*NI-linearized pNF-κB-Luc 或 pAP-1-Luc DNA (Stratagene, La Jolla, CA) 和 2.5 μg *Eco*RI-linearized pSV3-neo DNA。48 小時後，繼代細胞並且利用 400 μg/ml G-418 (Promega, Madison, WI)篩選 (賴,2001)。重組細胞株命名為 HepG2/NF-κB 或 HepG2/AP-1，以含有 10%胎牛血清 (HyClone, Logan, Utah) 及 400 μg/ml G-418 的 Dulbecco modified Eagle medium (DMEM)培養。

乙醛的處理

乙醛 (Sigma, St. Louis, MO)利用 phosphate-buffered saline (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2)新鮮配置。HepG2 cells 培養於 25-cm^2 flasks 中 24 小時後，加入以 DMEM 稀釋不同濃度的乙醛。在 25-cm^2 flasks 的瓶口上封 parafilm 避免乙醛的揮發。

Gelatin zymography

在本實驗中以 gelatin zymography 測定細胞培養液中 MMP 的活性。HepG2 cells 以不同濃度的乙醛處理 24 小時後，收集細胞培養液加入等量的兩倍 non-reducing sample buffer (0.5 M Tris-HCl, 20% glycerol, 10% sodium dodecyl

sulfate (SDS), 0.2% bromophenol blue, pH 6.8)，在 4℃下利用含有 1 mg/ml gelatin 的 7.5% polyacrylamide gels 以 90 伏特電泳 120 分鐘分離蛋白質。Gels 之後再利用 2.5% Triton X-100 清洗 10 分鐘以去除 SDS 之後於 incubation buffer (50 mM Tris-HCl, 10 mM $CaCl_2 \cdot 2H_2O$, 50 mM NaCl, 0.05% Brij35, pH 7.6)中 37℃作用 48 小時，使 MMP 將 gels 中的 gelatin 水解。最後以 Coomassie brilliant blue R-250 染 30 分鐘，再以 40% methanol 和 10% acetic acid 退染。若具水解 gelatin 的活性會呈現透明與背景的藍色成對比 (圖 2)。Gels 上的 band 的密度是利用 Gel-Pro® Analyzer (Media Cybernetics, Inc., Silver Spring, MD)分析。

Reverse transcription-polymerase chain reaction (RT-PCR)

利用 acid guanidium-phenol-chloroform 法萃取 HepG2 cells 的 RNA 後 (Chomczynski and Sacchi, 1987)，1 μg 的 RNA 以 $oligo(dT)_{15}$ 的引子與 SuperScript™III (Invitrogen , Carlsbad, CA)進行反轉錄。之後 2 μl 的 cDNA 產物利用 Taq polymerase (Promega, Madison, WI)進行 PCR 以放大 MMP-9 和 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA 的量。PCR 中使用的 MMP-9 的引子序列正股為：5’-CGATGACGAGTTGTGGTCCCTGGGC-3’，負股為：5’-AATGATCTAAGCCCAGCGCGTGGC-3’；而 GAPDH 的引子序列正股為：5’-CACCCATGGCAAATTCCATGGCACC-3’，負股為：5’-CCTCCGACGCCTGCTTCACCACC-3’。

架構 MMP-9 啟動子冷光報導質體 (Construction of MMP-9 promoter/reporter plasmids)

MMP-9 wild-type 及 mutants 啟動子是由 Douglas D. Boyd (MD Anderson Cancer Center, University of Texas, Houston)提供。而最先架構 MMP-9 啟動子的是 Hiroshi Sato (Cancer Research Medicine, Kanazawa University, Kanazawa, Japan) (Sato and Seiki, 1993)。 MMP-9 冷光報導質體是將 MMP-9 啟動子上的 -670 到 +54 片段以 PCR 放大後，將其架接至 pGL3-basic vector 。Wild-type 和 mutant 的 NF-κB 和 AP-1 結合位序列分別為: NF-κB (wild-type) GGAATTCCCC, NF-κB (mutant) TTAATTCCCC; AP-1 (wild-type) TGAGTCA, AP-1 (mutant) TATGTCA (圖 3)。 最後以 DNA 定序確認並利用 Qiagen plasmid midi kit (Qiagen, Valencia, CA)製備質體。

NF-κB 及 AP-1 活性測試 (Luciferase assay)

HepG2/NF-κB、AP-1 經不同濃度的乙醛處理 8 小時之後，以 350 μl Triton lysis buffer (50 mM Tris-HCl, 1% Triton X-100, 1 mM dithiothreitol, pH 7.8)使細胞溶解，在 4 ℃下 12,000 xg 離心 2 分鐘。之後加入 20 μl 的細胞溶解液與 20 μl 冷光試劑(470 μM luciferin, 33.3 mM dithiothreitol, 270 μM coenzyme A, 530 μM ATP, 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, pH 7.8) 以 luminometer (FB15, Zylux Corp., Maryville, TN)測定冷光值，單位以 relative luciferase unit (RLU)表示。活化倍率的計算方式是以乙醛處理細胞的 RLU

除以未受乙醛處理細胞的 RLU。

Biotinylated electrophoretic mobility shift assay (EMSA)

HepG2 cells 處理不同時間的乙醛後，收集細胞核的萃取液(Hsiang et al, 2002)。定量 10 μg 的 DNA 後加入會與 NF-κB 結合的 biotin 標定的雙股核苷酸進行反應。以 6% polyacryamide gel 電泳後，利用硝酸纖維膜轉印再以 blocking solution 阻斷非特異性的結合位後加入 alkaline phosphatase-conjugated streptavidin (Chemicon, Australia)，以 chemiluminescence (ECL system, Amersham, Buckinghamshire, UK)呈色，再利用 X 光影片顯影。

蛋白質轉漬法 (Western blot analysis)

HepG2 cells 經不同時間的乙醛處理後利用 250 μl sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue, pH 6.8)將細胞溶解，以超音波震盪器(Sonics and materials,Inc)160 W 震盪 10 秒擊破細胞，之後在 4℃下以 12,000 xg 離心 5 分鐘，最後以 100℃煮沸 5 分鐘。定量 10 μg 的蛋白質以 10% SDS-polyacrylamide 蛋白質電泳，並以硝酸纖維膜轉印後以 blocking buffer (20 mM Tris-HCl, 140 mM NaCl, 0.1% Tween-20, 5% skim milk powder, pH 7.6) 阻斷非特異性的結合位。之後加入 anti-IKK, anti-IκB-α, anti-p65, anti-JNK, or anti-β-TrCP 的抗體 (Cell Signaling Technology, Beverly, MA)進行雜

合作用後，加入 peroxidase-conjugated anti-rabbit antibody 以 chemiluminescence (ECL system, Amersham, Buckinghamshire, UK)呈色，再利用 X 光影片顯影。

腫瘤細胞轉移能力的測試 (Invasion assay)

細胞轉移測試是分析腫瘤細胞是否具有破壞細胞外基質的能力，將 Matrigel-coated film insert (8-μm pore size)上層覆蓋一層 Matrigel matrix，再將 Matrigel-coated film insert 置於 24-well invasion chambers (Becton-Dickinson Bioscience, Franklin Lakes, NJ)測定 (圖 4)，若腫瘤細胞具轉移能力則可破壞 matrix 轉移至下層。HepG2 cells (5×10^4) 與含或不含乙醛的 200 μl DMEM 混合後加到 Matrigel invasion chambers 的上層，Invasion chamber 下層加入 500 μl DMEM，培養於含 5% CO_2 的 37^0C 培養箱 24 小時後, 利用棉棒刮去 Insert chamber 上層的細胞，下層的細胞染色後利用 200x 顯微鏡計數細胞數目，以上實驗為三次實驗的平均值±標準差。

統計分析

本研究的數據使用 Excel XP (Microsoft)計算多次重複實驗中數據的平均值與標準差，並利用 Student's t test 分析有無顯著差異存在。

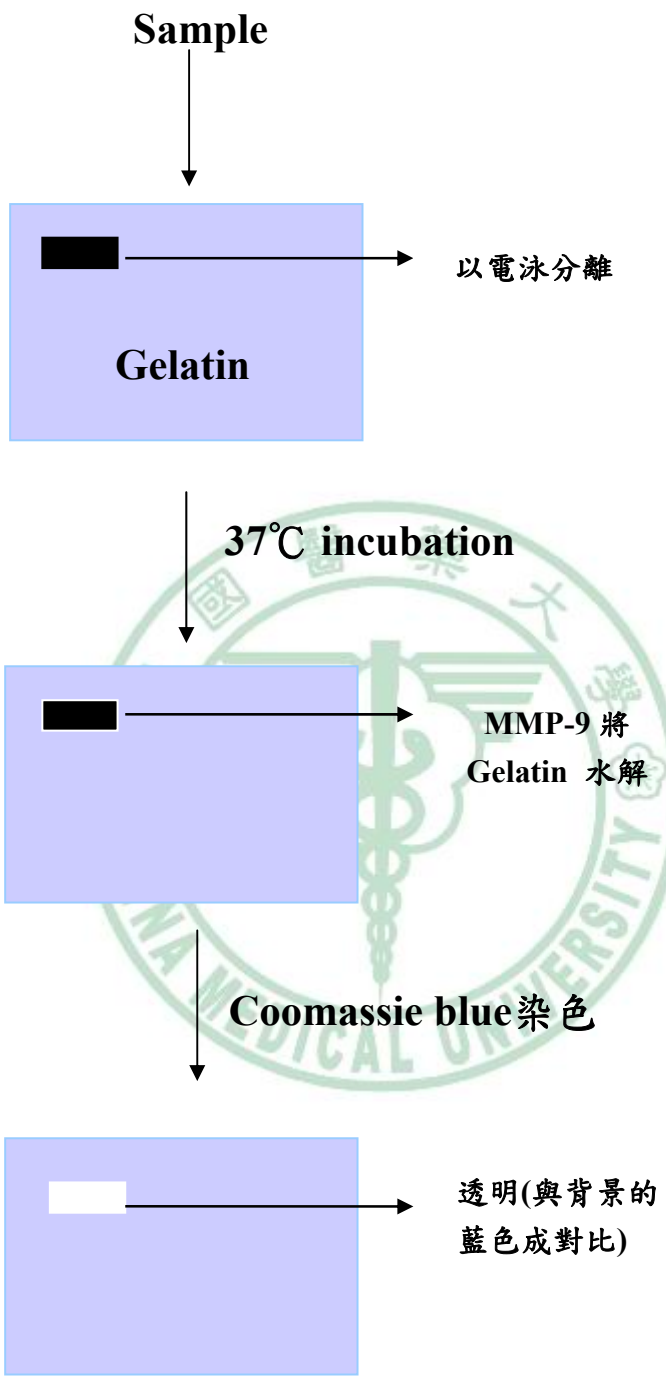


圖 2. Gelatin zymography 流程圖

MMP9p-wt

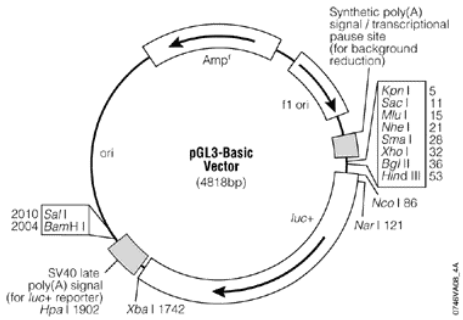
MMP9p-NF - κ B-CAT

MMP9p-AP-1-CAT

PCR

MMP-9 promoter mutant

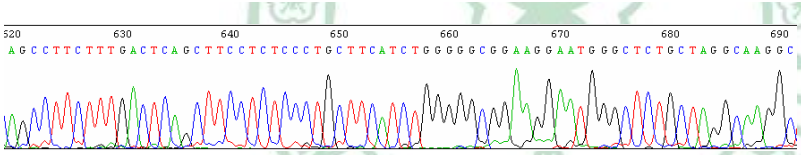
(730 bp)



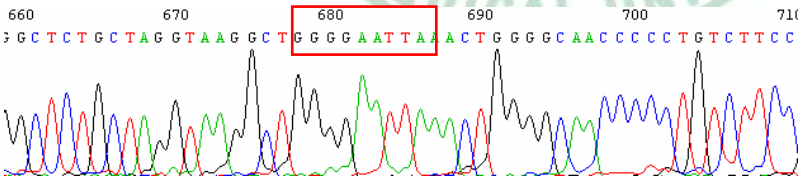
pGL3-basic

MMP9p-NF - κ B mutant

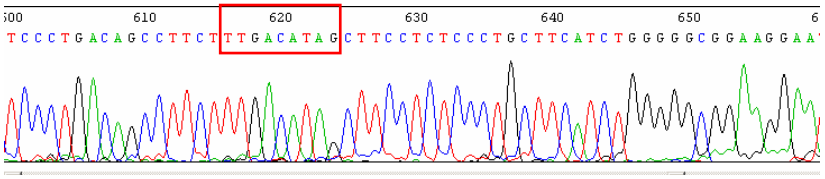
MMP9p-AP-1 mutant



MMP9p-Wild type



MMP9p-NF-κB mutant



MMP9p-AP-1 mutant

圖 3. 架構MMP-9啟動子的冷光報導質體流程圖

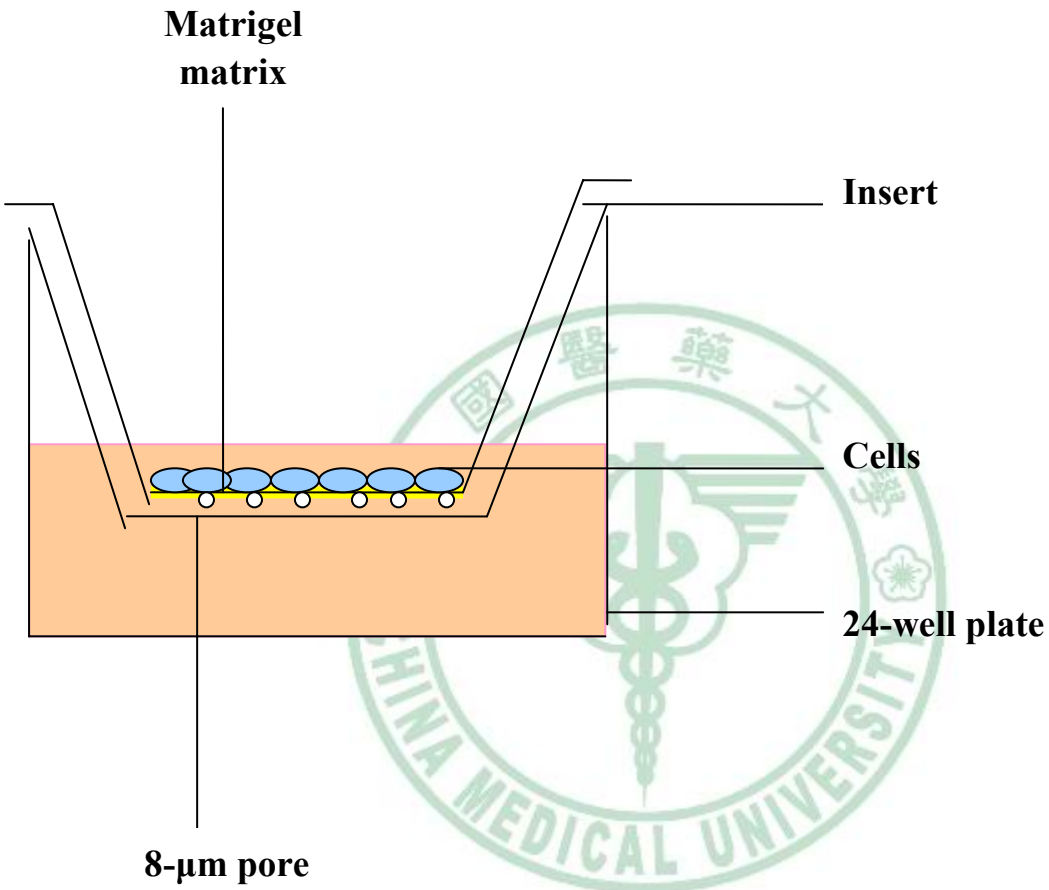


圖 4. Matrigel 示意圖

第三章 實驗結果

乙醛在 HepG2 及 Chang liver cells 中可增加 MMP-9 的表現

腫瘤的侵襲與轉移須 MMP 的表現上升。為了探討乙醛可否藉由增加 MMP-9 的表現而造成腫瘤的轉移，我們利用 Zymographic analysis 觀察乙醛對於 MMP 表現的影響。Zymographic analysis 是利用 MMP 具水解 gelatin 的能力，於是在 PAGE 加入 gelatin，若有 MMP 存在則可在 37 度培養 48 小時後將 gelatin 水解，被水解的部分則無法被 coomassie blue 染色，再依照分子量得知是哪一種 MMP。圖 5 的結果顯示，乙醛在 HepG2 cells 能增強 92 kDa MMP-9 的表現，10 μM 的乙醛活化倍率最高，為 mock 組的 12.9 倍；而 100 μM 的乙醛活化倍率比 10 μM 低。另外乙醛也可在 Chang liver cells 中增加 MMP-9 的表現，但活化倍率較低。這些結果證實了乙醛在 HepG2 及 Chang liver cells 中可增強 MMP-9 的表現。

乙醛可以增加 MMP-9 mRNA 的表現量

為了了解乙醛增加 MMP-9 的表現是否經由增加 MMP-9 mRNA 的量，我們利用 RT-PCR 觀察乙醛對於 MMP-9 mRNA 的影響。圖 6 是 RT-PCR 的結果，上圖是 MMP-9 mRNA 變化量，中圖以 GADPH 證實 RT-PCR 條件相同，下圖以 18S 及 28s rRNA 當 internal control 證明 RNA 等量。活化倍率計算方式為未加乙醛的 MMP-9/GAPDH 比上加入乙醛的 MMP-9/GAPDH 的比值。從圖 6 的結果可得知

HepG2 cells 在處理乙醛 16 小時之後，MMP-9 mRNA 明顯增加，10 μM 的乙醛活化倍率最高，為 mock 組的 9.4 倍。這些結果證實了乙醛經由增加 MMP-9 基因轉錄而增加 MMP-9 的表現。

乙醛可促進 NF-κB、AP-1 的活性

由於 MMP-9 啟動子上含 AP-1 與 NF-κB 結合位(Sato and Seiki, 1993; Sato et al., 1993) ，於是我們利用冷光報導系統分析乙醛對於這兩個轉錄因子的影響。結果顯示乙醛可活化 NF-κB 的活性，最高活化倍率為 1.6 倍;而 AP-1 為 1.2 倍(圖 7)。NF-κB、AP-1 活化後會進入到細胞核中與特殊 DNA 序列結合，而增加下游基因的表現。因為之前的實驗中，10 μM 乙醛活化 MMP-9 及上游轉錄因子 NF-κB、AP-1 的倍率最高，所以將之後實驗乙醛濃度定為 10 μM。為了確定乙醛能夠活化 NF-κB、AP-1 的活性，於是我們利用 EMSA 測定乙醛對於是否可增加 NF-κB、AP-1 進入細胞核中與 DNA 結合。圖 8、9 的結果顯示 10 μM 的乙醛可增加細胞核中 NF-κB、AP-1 與 DNA 結合。隨著時間的增加，細胞核中 NF-κB 與 DNA 結合越多，直到加入乙醛後的第 8 小時，可達到最高的活化倍率 (圖 8)。而 AP-1 與 DNA 的結合的量，加入乙醛後的 15～45 分鐘也會增加 (圖 9)。這些結果證實了乙醛可增加 NF-κB、AP-1 進入細胞核中與 DNA 結合。

乙醛可經由MMP-9啟動子上的NF-κB及 AP-1結合位調控MMP-9的表現

為了探討乙醛可否藉由促進 MMP-9 基因上游啟動子的活性而使轉錄作用增加，我們利用 MMP-9 啟動子的冷光報導試驗分析。首先利用 PCR 放大 MMP-9 基因啟動子的位置 -670 到+54，之後架接到 pGL3-Basic vector (圖 10A)。將架接好的質體轉染到 HepG2 cells，處理乙醛 24 小時之後以冷光測定 MMP-9 啟動子的活性受影響之情形。圖 10B 的結果顯示乙醛可誘發 MMP-9 啟動子的活性，約為 pGL3-Basic vector 的 5.2 倍。由於 MMP-9 啟動子上含 AP-1 與 NF-κB 結合位，於是我們利用 MMP-9 啟動子 NF-κB 或 AP-1 結合位的單點突變的冷光報導質體分析乙醛對於這兩個轉錄因子的影響。圖 10B 的結果顯示 NF-κB 或 AP-1 結合位的突變可明顯的使乙醛所活化的 MMP-9 啟動子的活性下降。由以上結果證明乙醛可經由 MMP-9 啟動子上的 NF-κB 及 AP-1 結合位調控 MMP-9 的表現。

乙醛磷酸化 IκB 後使得 NF-κB 進入到細胞核中

接下來藉由蛋白質轉漬法往上推測乙醛活化上游的 NF-κB 訊息傳導路徑的位置。NF-κB 原本會與 IκB 結合呈不活化而存在於細胞質中，若 IκB 被 IKK 磷酸化後會與 NF-κB 分離，此時 NF-κB 會活化進入到細胞核中影響基因的轉錄。我們利用 NF-κB 的次單位 p65 觀察 NF-κB 在細胞核質的比例。從圖 11 中可以看出乙醛處理的細胞，p65 進入到細胞核中增加。由於 IκB 的磷酸化和分解為 NF-κB 活化的主要訊息傳導路徑，所以接下來想往上探討的是乙醛是否會經由增加 IκB

的磷酸化和分解而活化 NF-κB。從圖 11 的結果可知 IκB 磷酸化的量在乙醛加入的 45 分鐘到 24 小時皆有明顯的上升。這些結果證明了乙醛磷酸化 IκB 後使得 NF-κB 進入到細胞核中。

乙醛可能可以經由 JNK 及 β-TrCP 訊息傳導路徑加速磷酸化的 IκB 分解

在之前的研究中 我們利用蛋白質轉漬法證實了乙醛可以經由 IκB 的磷酸化和分解進而活化 NF-κB。然而加入乙醛後 IKK 的活性並無明顯改變， 許多訊息傳導路徑都能調控 NF-κB 的活性，包含 ras/raf-1、 MAP kinases 或 Akt (Beaupre et al., 1999; Madrid et al., 2001; Kurland et al., 2003; Nawata t al., 2003) 。近年來有報告指出，JNK 可以經由增加 β-TrCP 使得 IκB 磷酸化加速分解而讓 NF-κB 持續活化(Spiegelman et al., 2001)。從圖 12 中蛋白質轉漬法的結果可得知經乙醛處理的細胞 JNK 及 β-TrCP 的量明顯的升高，這些結果證明了乙醛可能可以經由 JNK 及 β-TrCP 而增加 MMP-9 基因的表現。

乙醛可以經由 JNK 和 p38 訊息傳導路徑活化 AP-1 的活性

因為 AP-1 的活性受到 MAP kinases 的調控 (Karin et al., 1997), 所以接下來我們利用蛋白質轉漬法觀察乙醛對於 MAP kinases 的影響，從圖 12 結果中可得知乙醛對於 JNK, p38, and ERK 蛋白質的量影響不大。而乙醛可刺激 JNK 及 p38

的磷酸化但並無法誘發 ERK 的磷酸化增加，這些結果證明了乙醛可能可以經由 JNK 及 p38 活化 AP-1。

乙醛可促進腫瘤細胞轉移

腫瘤細胞轉移需增加MMP-9的表現 (Nelson et al., 2000)。 我們在之前的研究中已經證實乙醛可增加MMP-9的表現，所以接下來我們想要測試乙醛是否經由活化MMP-9而可增加腫瘤細胞轉移。如圖 13所示,利用Matrigel-coated filter測試，乙醛可促進細胞的轉移能力約為16倍。這些結果證明了乙醛可能可以經由活化MMP-9而增加腫瘤細胞的轉移。

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第四章 討論

肝臟是酒精氧化的主要部位，人體內只有百分之十到十五的酒精在其他組織氧化。在肝臟中酒精被酒精去氫酶氧化成乙醛，此反應需要 NAD 當輔酶；由於細胞內 NAD 的含量有限，因此代謝以零級反應進行，亦即每小時代謝 7-10 公克酒精。人體肝細胞內的微粒體含有特殊的酵素稱細胞色素 p450 還原酵素(cytochrome p450 reductase)來完成藥物在體內的代謝過程。微粒體 Cytochrome p-450 還原酵素中有一個族群(特別是 cytochrome p450 2E1 酵素)可以將乙醛進一步氧化產生各種自由基。在慢性酒精中毒的過程中,此酵素的活性會大大的提昇,因而產生大量自由基傷害肝細胞並促進肝臟傷害(Morgan et al., 2004)。

酒精造成的疾病，其發生和演變有多種基因參與，包括基因與基因之間及基因與環境之間交互影響。酒精去氫酶和乙醛去氫酶(aldehyde dehydrogenase，*ALDH*)是人體主要的酒精代謝酶系統，酒精的藥理作用及細胞毒性和酒精及代謝產物乙醛濃度相關。*ADH*2 和 *ALDH*2 對偶基因的功能多形性影響乙醇和乙醛的藥物動力學及對腦、心臟血管、肝、上下消化道的藥物效力學，進而影響人類的飲酒行為、酒癮和酒精器官傷害的發生。酒精在人體內分解，90-95% 以上經由氧化途徑，包括酒精去氫酶、細胞色素、過氧化酶(catalase) 和乙醛去氫酶，其中以酒精去氫酶和乙醛去氫酶途徑為主((Yin et al., 1999)。*ADH* 和 *ALDH* 均呈複雜的酵素家族 (family)，其對偶同功酶具種族差異。人類乙醛去氫酶巨族 (superfamily) 至少有 12 個基因，其間的胺基酸序列相同性約在 15% 和 80% 之

間，他們的基質專一性差異甚大。乙醛作為基質的 *ALDH*1 和 *ALDH*2 屬低 Km 類，*ALDH*3 則屬高 Km 類(Yin et al., 1995)。東方人族群約 50% 具 *ALDH*2 變異型對偶基因，它位於 12 號染色體 q24 的位置，在表現序列 12 有一點突變，造成 487 位置麩胺酸換成離胺酸，導致酶失去活性(Yin and Agarwal ,2001)。*ALDH*2 變異型在其他族群尚未發現，所以是東方人特有的對偶基因。變異型 *ALDH*2 在飲酒後 2 小時，血中乙醛仍持續高量堆積，造成心搏持續加速，心輸出量持續增加，舒張壓持續下降，全身血管阻力持續降低，面動脈、頸總動脈和內頸動脈血流速率持續降低，同時持續產生顯著的整體不舒適的感覺。因此即使變異型 *ALDH*2 飲少量酒，仍會造成血液中乙醛持續堆積，引發強烈心臟血管反應，產生整體不舒適感覺(Peng et al., 1999)。所以我們認為乙醛對於東方人造成的肝臟傷害可能比其他人種嚴重。

乙醛與肝癌的發展有關，有研究指出肝癌轉移病患的 MMP-9 基因的表現高於正常人(Arii et al., 1996)。在這個研究當中我們探討的是乙醛與 MMP-9 基因表現的相關性，我們的數據顯示出乙醛可以經由轉錄因子 AP-1 與 NF-κB 調控 MMP-9 的轉錄，進而造成 MMP-9 表現增加。由於 MMP-9 會分解與腫瘤轉移相關的基底膜主要成分-第四型膠原蛋白 (Nelson et al., 2000)，所以我們認為乙醛可能參與腫瘤的轉移與發展的步驟。

由於 MMP 會破壞基底膜，所以 MMP 的表現增加可能與腫瘤的侵襲與轉移有關。MMP-9 在肝癌的表現量最高，所以 MMP-9 可能與肝癌的轉移有關。許多肝病誘發物包含 B 型肝炎病毒、生長因子、TPA (12-*O*-tetradecanoylphorbol -13-acetate)等都會造成肝癌的惡化，近年來的研究證實了這些刺激物會經由不同的訊息傳導路徑增加 MMP-9 的表現而與腫瘤的轉移有關。B 型肝炎病毒的 X 蛋白會經由 PI-3K/AKT 及 ERK 路徑活化 AP-1 與 NF-κB 增加 MMP-9 的表現 (Chung et al., 2004)。另外 TGF-β 也可藉由 p38 傳導路徑活化 MMP-2 及 MMP-9 的表現 (Kim et al., 2004)。而 TPA 主要是經由 PKC 活化下游的 Ras/ERK 路徑誘發 MMP-9 基因表現增加 (Liu et al., 2002)。在這個研究當中我們首次証實乙醛可以經由 IκB 、JNK 及 p38 訊息傳導路徑活化轉錄因子 AP-1 與 NF-κB 而增加 MMP-9 的表現。

MMP-9 啟動子上含 AP-1、NF-κB、SP-1 及 AP-2 轉錄因子結合的位置(Sato and Seiki, 1993)，近年來有研究指出這些轉錄因子能夠增加 MMP-9 的表現，例如 v-src 可經由 AP-1 及 GT box 刺激 MMP-9 表現(Sato et al., 1993)。而在卵巢癌細胞則是經 MEK 訊息傳導路徑調控其基因表現(Gum et al., 1996)。另外也有研究利用基因轉殖老鼠證明 AP-1、NF-κB、SP-1 是調控 MMP-9 表現及受傷害的組織重組所需(Mohan et al., 1998)。所以乙醛也可能影響其他訊息傳導路徑以及增加其他基因表現而促進腫瘤轉移，在未來希望能夠結合基因晶片分析以及動物實驗

更加深入的了解乙醛促進肝癌轉移的分子機轉。

大多數的 ROS (reactive oxygen species)也可經由增加 MMP 的表現而造成腫瘤細胞的侵襲與轉移(Shi et al., 2004)，例如 H_2O_2 及 XXO(superoxide-generating system of xanthine plus xanthineoxidase)可以經促進 MMP 轉錄及後轉錄作用增加 MMP 及 pro-MMP 的表現，而與腫瘤的轉移有關 (Siwik et al., 2001)。在這個研究當中，我們證實酒精的氧化代謝產物乙醛經由 AP-1 及 NF-κB 增加 MMP-9 的轉錄，這些結果證明了乙醛也與腫瘤的轉移相關。

未受刺激的細胞，NF-κB 與其抑制蛋白質 IκB 結合形成不活化態，而存在於細胞質當中，但是如果 IKK 將 IκB 磷酸化，接下來 JNK 會活化 β-TrCP 使其與磷酸化的 IκB 結合，將其送到 26S proteasome 進行分解，而 IκB 與 NF-κB 解離之後，NF-κB 即可持續活化進入到細胞核中與基因上游的 NF-κB 的核苷酸序列結合，使下游基因的表現量增加 (Karin and Ben-Neriah, 2000)。在這個研究當中，我們利用蛋白質轉漬法證實了乙醛經由 JNK/β-TrCP 將 IκB 磷酸化及分解，而增加 MMP-9 的表現。在這個研究中我們證實乙醛除了活化 NF-κB 以外也可促進 AP-1 的活性，這是因為 JNK 及 p38 訊息傳導路徑皆會活化 AP-1 的活性 (Karin et al., 1997)，所以我們認為乙醛是透過 JNK 及 p38 訊息傳導路徑活化 NF-κB 及 AP-1。總而言之，我們的結果證明了乙醛經由 IκB、JNK/β-TrCP 及 p38 路徑活

化 NF-κB 與 AP-1 而使 MMP-9 表現增加。

綜合以上結果，我們證實乙醛與腫瘤的轉移有關。而機轉為乙醛可以經由 IκB、JNK 及 p38 活化轉錄因子 NF-κB 與 AP-1 進入細胞核中活化 MMP-9 基因表現(圖 14)。由於我們提供了乙醛與腫瘤的轉移的相關性以及作用之訊息傳導路徑，這可能可以成為抗腫瘤轉移藥物的新標的。

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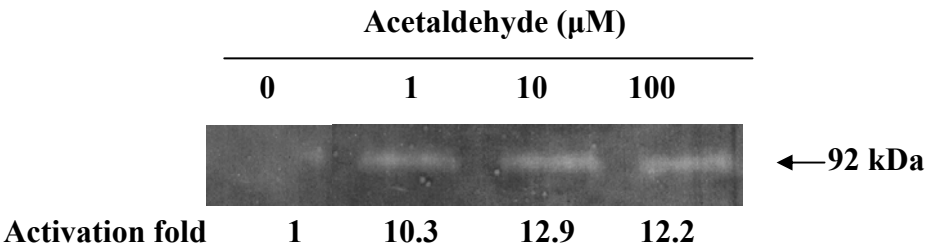
(A) HepG2 cells



(B) Chang liver cells

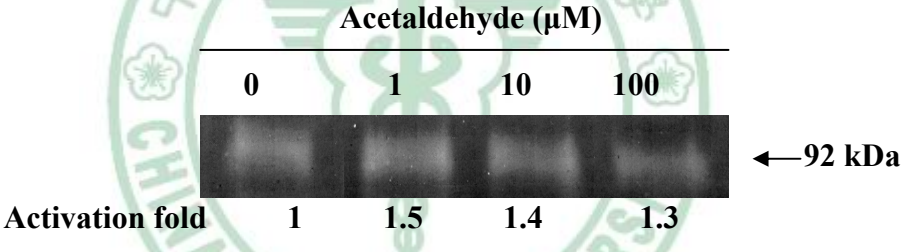


圖 5. 利用 gelatin zymography 證實乙醛在 HepG2 及 Chang liver cells 中可誘發 MMP-9 的活性

HepG2 與 Chang liver cells 培養於含 10% FBS 的 DMEM 中，之後移去培養液加入以 DMEM 稀釋不同濃度的乙醛。 24 小時後收集培養液以 gelatin zymography 分析 MMP 的活性。箭頭所指的是 92 kDa 的 MMP-9，活化倍率的計算方式是比較未處理藥物與處理藥物後 MMP-9 band 的密度。

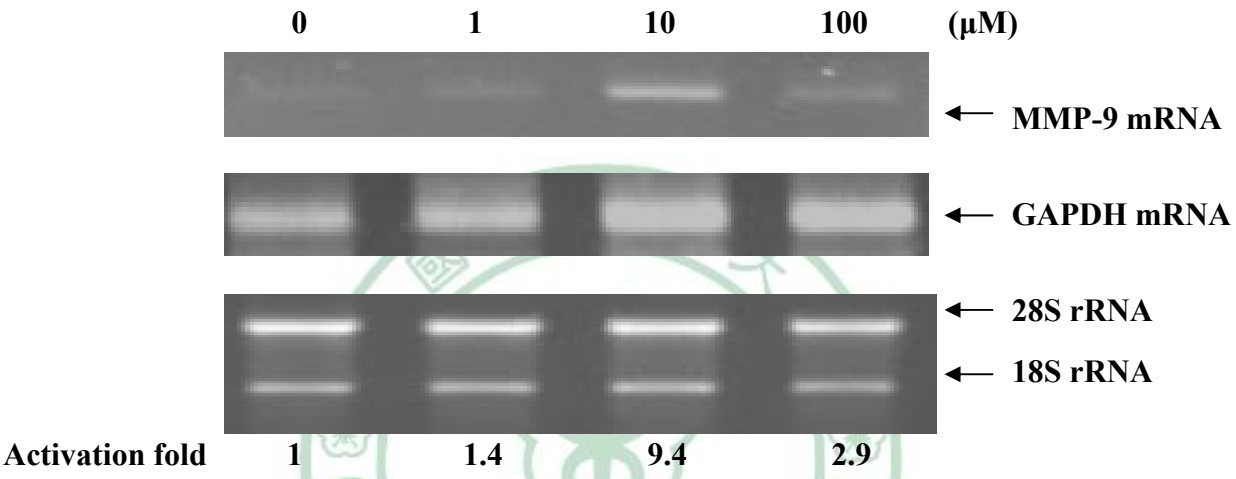


圖 6. 利用 RT-PCR 證實乙醛可以增加 MMP-9 mRNA 的表現量

HepG2 於 25-cm^2 flasks 中培養，加入不同濃度的乙醛 16 小時後，萃取其 RNA，取 1 μg 的 RNA 進行反轉錄。經反轉錄產生的 cDNAs 產物以人類 MMP-9 或 GAPDH 基因專一性的引子進行 PCR。PCR 的結果以 1% agarose gels 偵測後以 ethidium bromide 染色。活化倍率的計算方式是比較未處理藥物與處理藥物後 MMP-9 band 的密度。

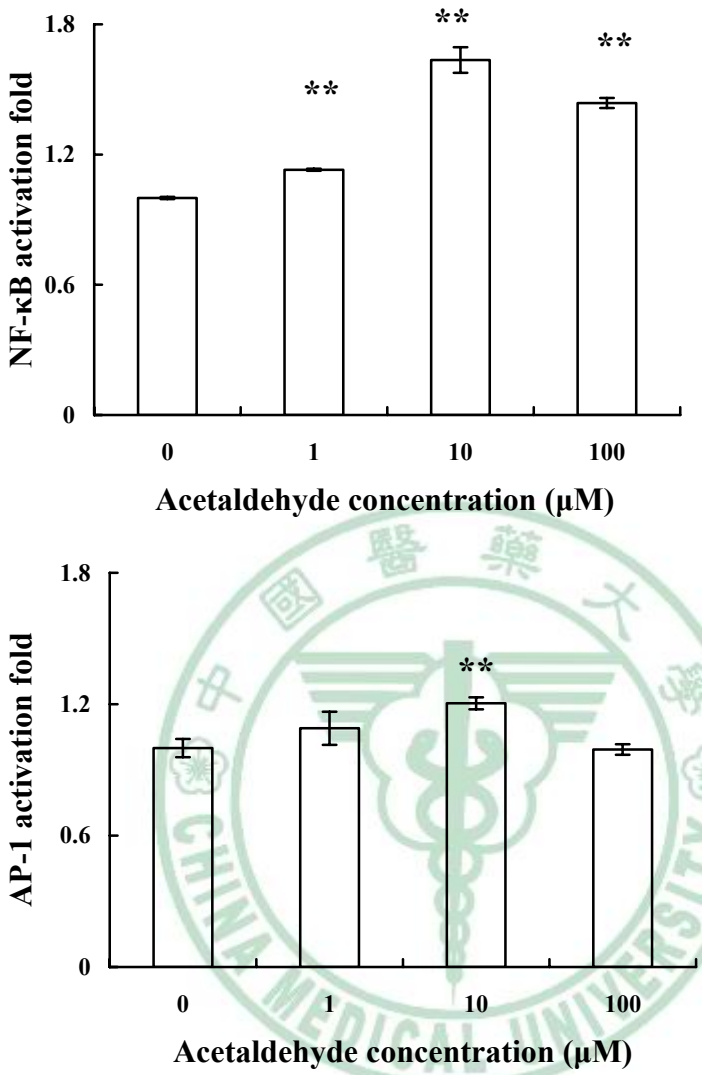


圖 7. 乙醛可以活化 NF-κB、AP-1

轉錄因子的活性。HepG2 cells 穩定性轉染 pNF-κB-Luc 或 pAP-1-Luc，加入不同濃度的乙醛，8 小時後測量其冷光活性。單位以 relative luciferase unit (RLU) 表示。活化倍率的計算方式是以乙醛處理細胞的 RLU 除以未受乙醛處理細胞的 RLU。以上實驗為三次實驗的平均值±標準差。**表示 $p<0.01$。

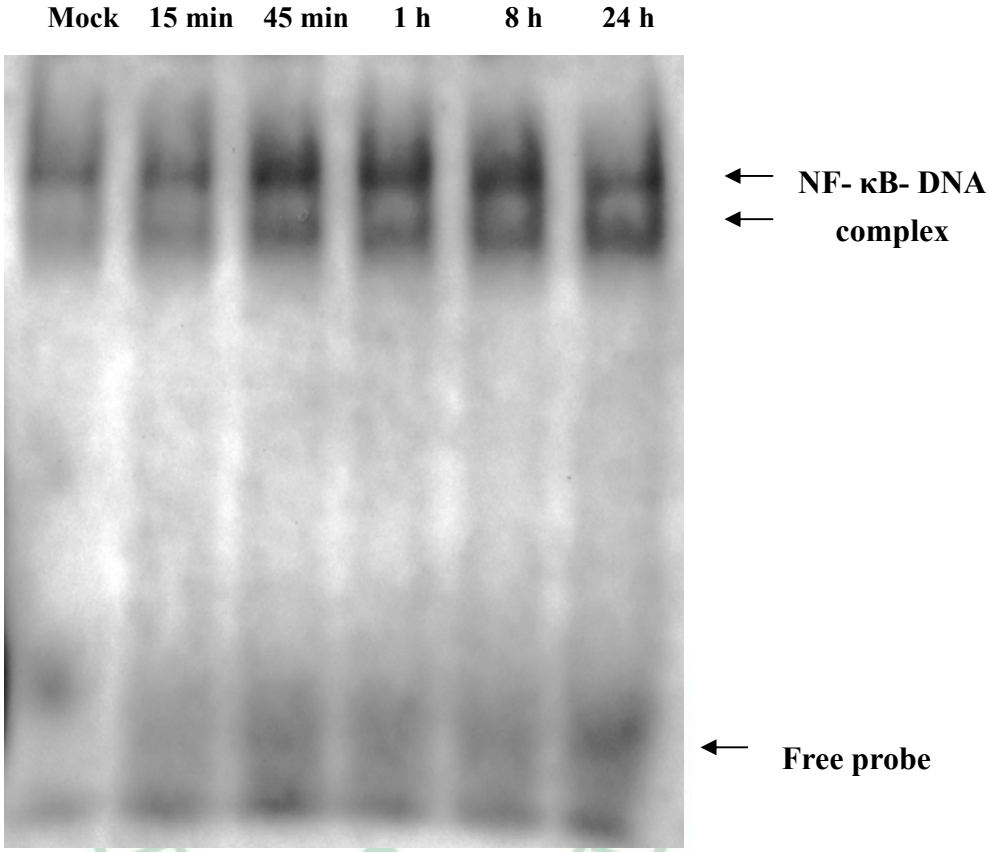


圖 8. 乙醛可促進 NF-κB 的活性

HepG2 cells 經乙醛處理不同時間後，收集細胞核的萃取液。之後加入會與 NF-κB 結合的 biotin 標定的雙股核苷酸序列進行反應。

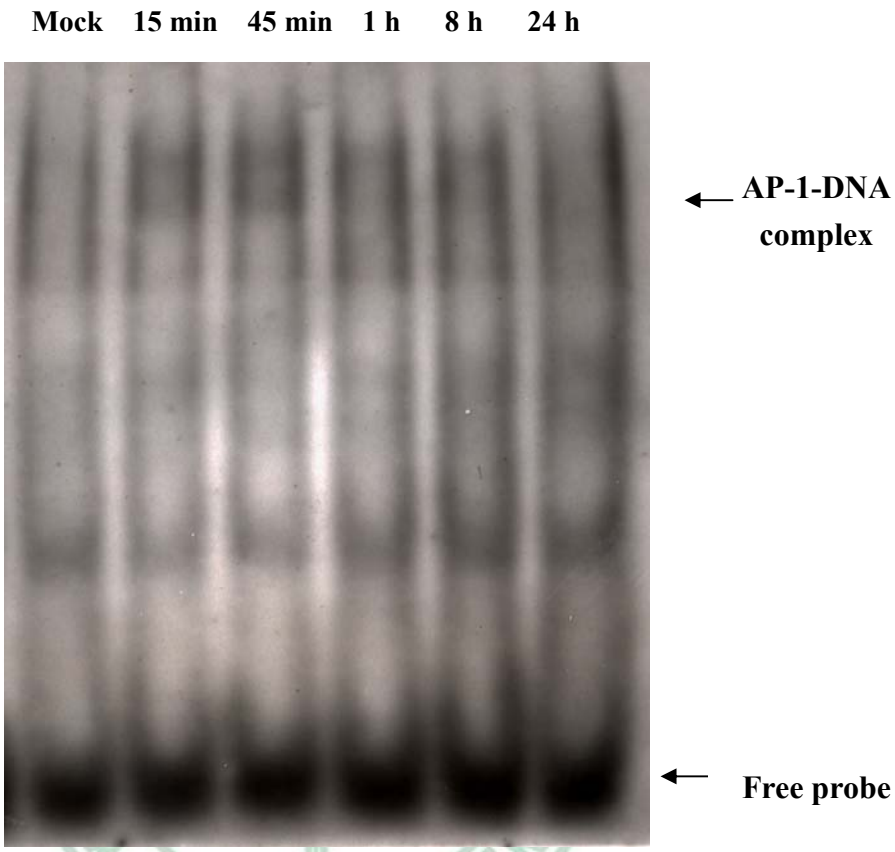
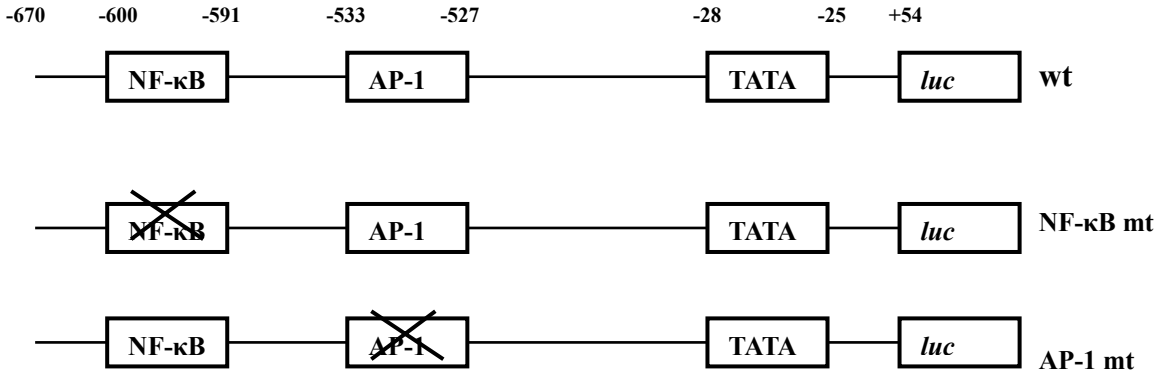


圖 9. 乙醛可促進 AP-1 的活性

HepG2 cells 經乙醛處理不同時間後，收集細胞核的萃取液。之後加入會與 AP-1 結合的 biotin 標定的雙股核苷酸序列進行反應。

(A)



(B)

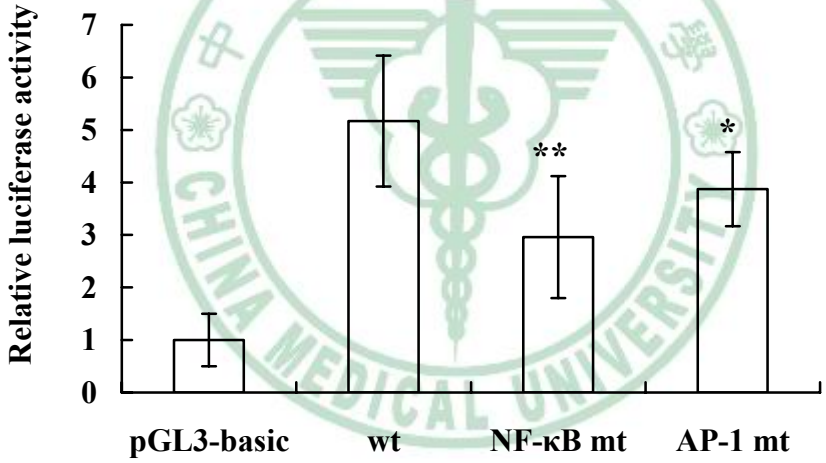


圖 10. 乙醛可以活化 MMP-9 啟動子的轉錄

(A) Wild-type (wt) 或 NF-κB 結合位 (NF-κB mt) 或 AP-1 結合位 (AP-1 mt)突變的 MMP-9 啟動子的示意圖，轉錄因子的結合位如圖所示。(B) MMP-9 啟動子活性。HepG2 cells 暫時性轉染 pGL3-basic 或 MMP-9 報導質體後，加入 10 μM 的乙醛。單位以 relative luciferase unit (RLU)表示。活化倍率的計算方式是以 MMP-9 報導質體的 RLU 除以 pGL3-basic 的 RLU。以上實驗為三次實驗的平均值±標準差。*表示 $p< 0.05$，**表示 $p< 0.01$。

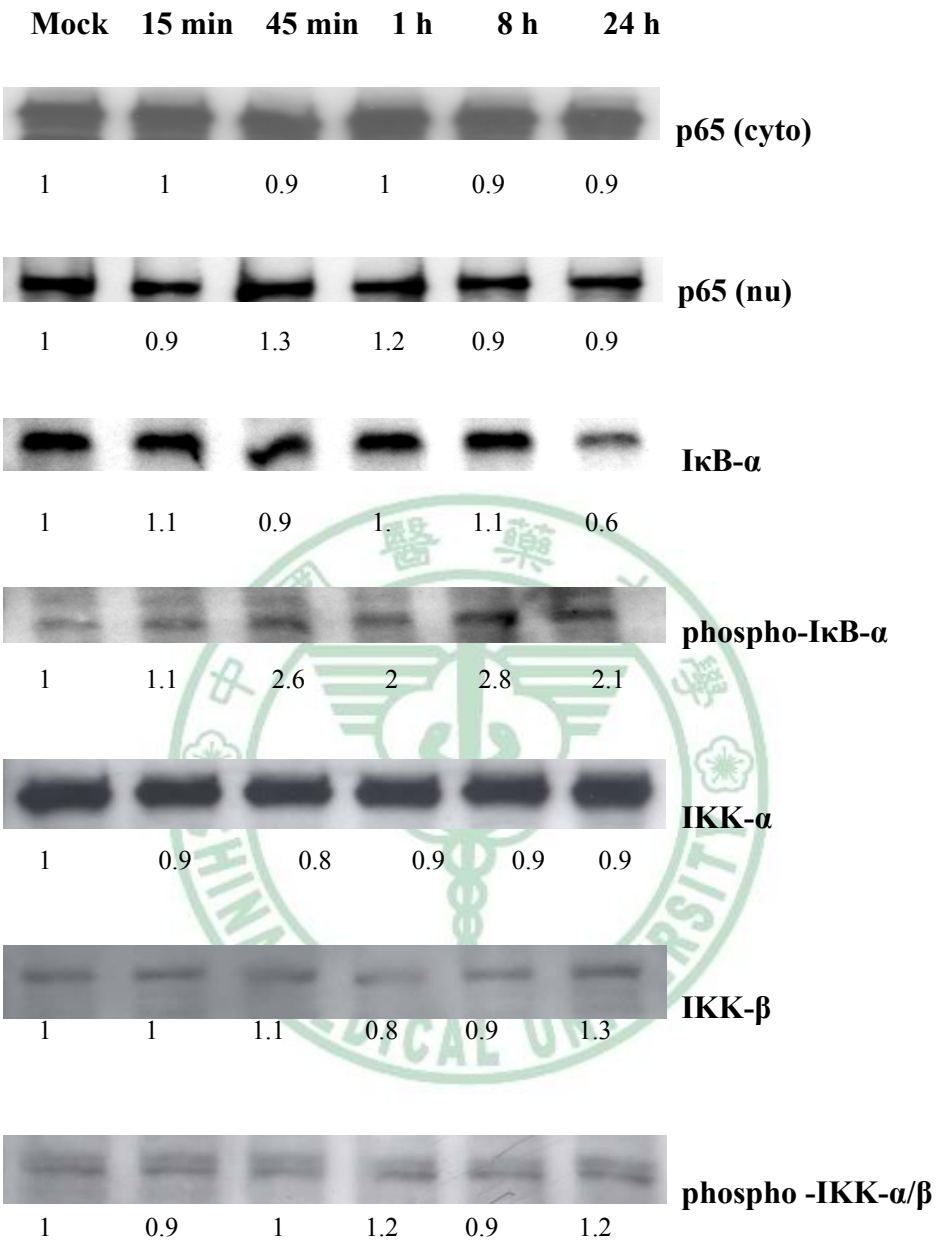


圖 11. 乙醛將 IκB 磷酸化後使得 NF-κB 進入到細胞核中

HepG2 cells 經 10 μM 的乙醛處理不同時間後，以蛋白質轉漬法偵測細胞萃取液中 p65、IκB、IκB 磷酸化、IKK 及 IKK 磷酸化的表現量。以上為三次實驗呈現相似的結果。

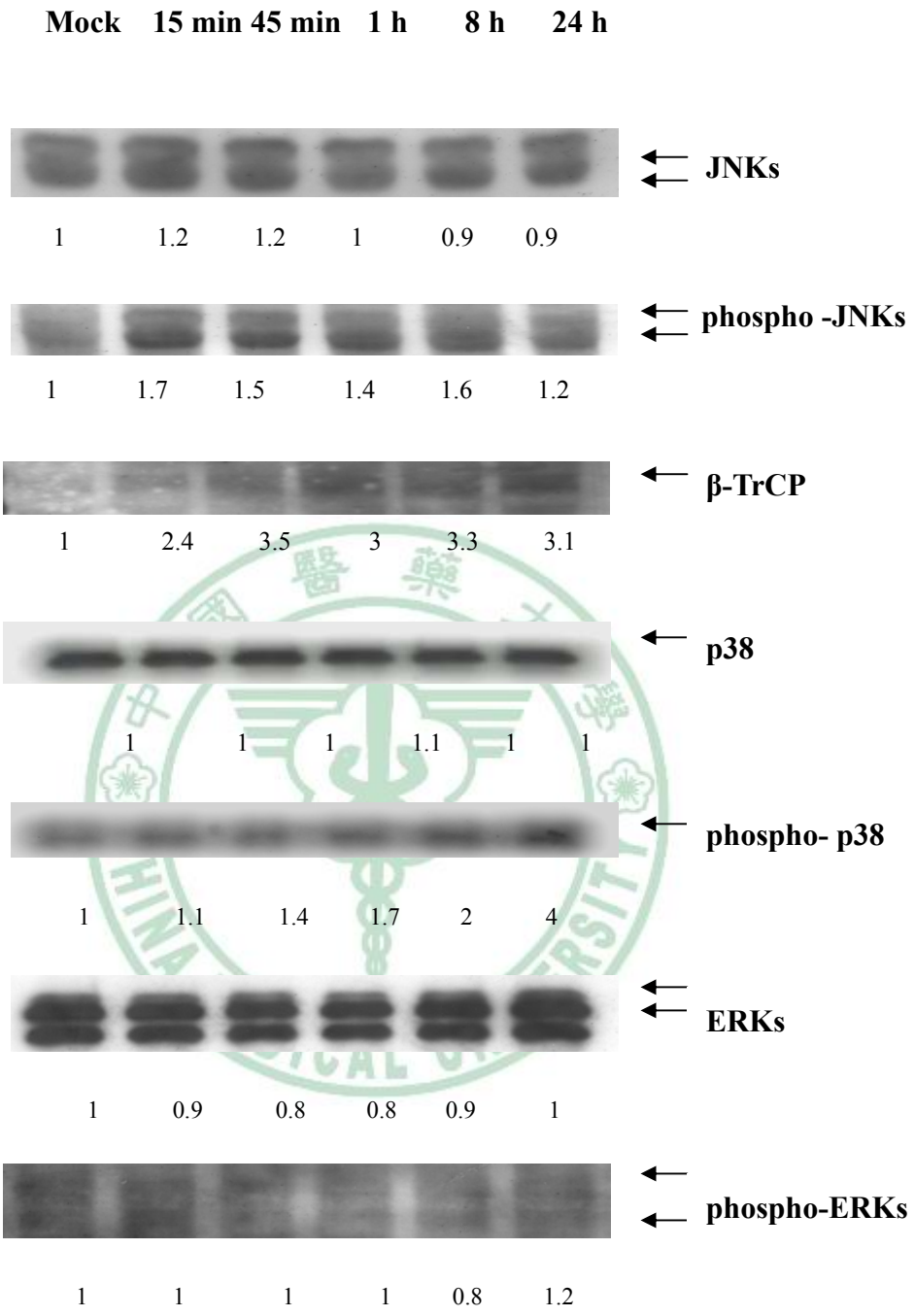


圖 12. 乙醛可以經由 JNK/β-TrCP 和 p38 訊息傳導路徑活化 NF-κB 和 AP-1 的活性

HepG2 cells 經 10 μM 的乙醛處理不同時間後，以蛋白質轉漬法偵測細胞萃取液中 JNKs、JNKs 磷酸化、β-TrCP、p38、p38 磷酸化、ERKs 及 ERKs 磷酸化的表現量。以上為三次實驗呈現相似的結果。

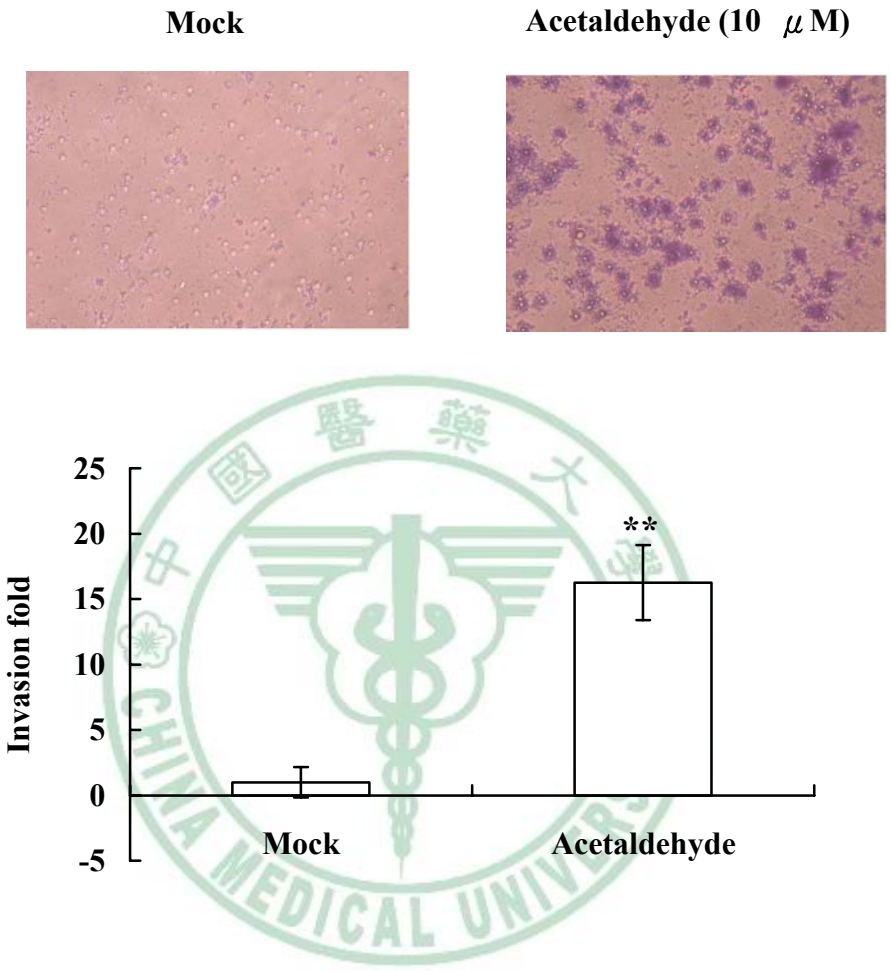


圖 13. 乙醛可造成腫瘤細胞轉移

HepG2 cells (5×10^4) 與含或不含乙醛的 200 μl DMEM 混合後加到 Matrigel invasion chambers 的上層。培養 24 小時後, 利用棉棒刮去 Insert chamber 上層細胞，下層的細胞染色後利用 200x 顯微鏡計數細胞數目。(A) 轉移的細胞顯微鏡下的型態 (B) Invasion assay 的柱狀圖。細胞轉移倍率的計算方式是以加入乙醛的細胞轉移數目除以未加乙醛的細胞轉移數目。以上實驗為三次實驗的平均值± 標準差。**表示 $p<0.01$。

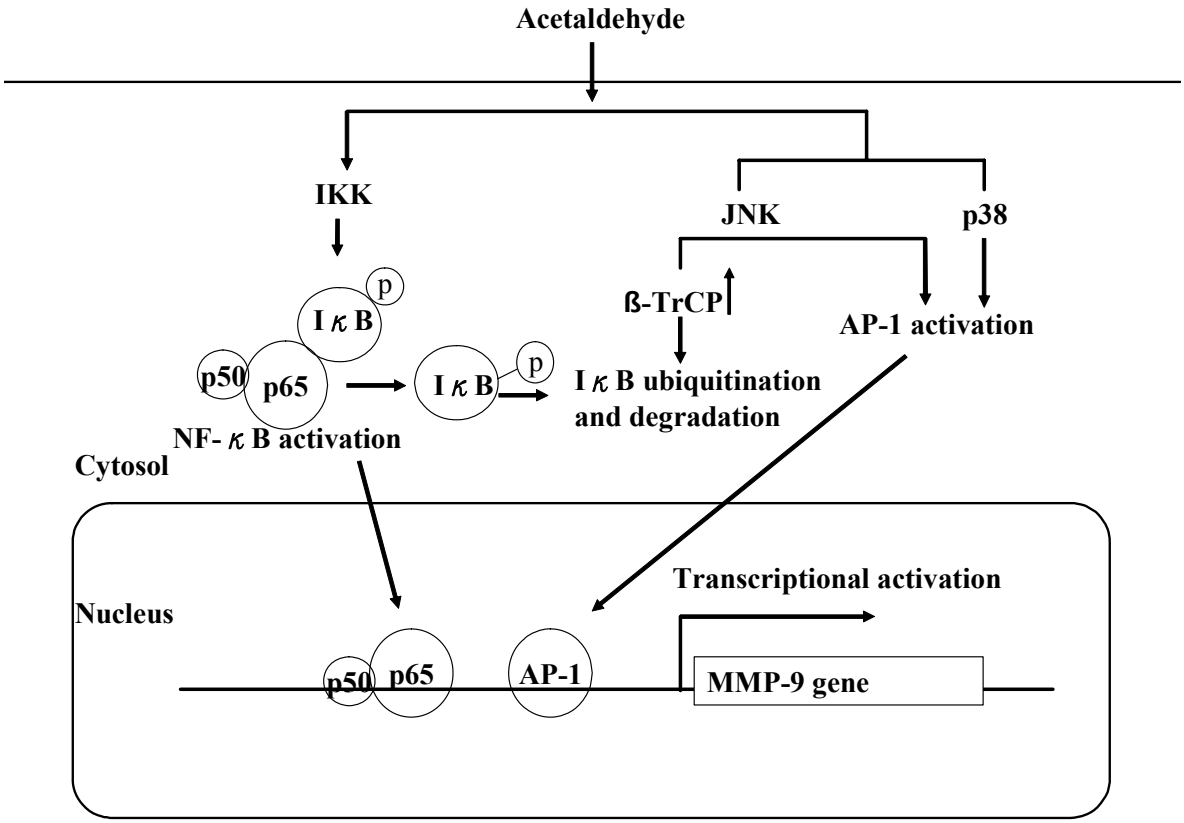


圖 14. 乙醛增加 MMP-9 基因表現之分子機轉

乙醛可以經由 IκB、JNK 及 p38 訊息傳導路徑同時活化轉錄因子 NF-κB 與 AP-1 進入細胞核中與 MMP-9 基因上游的啟動子結合而增加 MMP-9 基因表現。

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附錄一

Acetaldehyde induces matrix metalloproteinase-9 gene expression and promotes hepatocarcinoma cells invasion through nuclear factor-κB and activator protein 1 signaling pathways

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Abstract

Acetaldehyde, the very reactive intermediate of oxidative metabolism of ethanol, is potentially associated with alcohol-induced liver diseases. Matrix metalloproteinase-9 (MMP-9) is directly involved in human hepatic tumorigenesis and metastasis. However, the relationship between acetaldehyde and MMP-9 expression in liver diseases is currently poorly understood. Herein we demonstrated that acetaldehyde increased MMP-9 gelatinolytic activity and promoted cell invasion through the up-regulation of MMP-9 gene transcription in HepG2 cells. The transcription of MMP-9 gene was regulated by acetaldehyde via inductions of nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) activities. Western blot analysis indicated that acetaldehyde stimulated the translocation of NF-κB into nucleus through inhibitory κB-α (IκB-α) and c-Jun N-terminal kinase (JNK)/β-transducin repeat-containing protein (β-TrCP) signaling pathways. Acetaldehyde also induced AP-1 activity via the phosphorylation of p38 kinase. In conclusion, our findings demonstrated that acetaldehyde activated NF-κB and AP-1 activities via IκB, JNK/β-TrCP, and p38 signaling pathways, resulting in the induction of MMP-9 gene expression and the increase of cell invasion. On the basis of these data, we suggested that acetaldehyde plays an important role in tumor invasion and metastasis.

Introduction

Acetaldehyde, the product of oxidative metabolism of ethanol, is potentially associated with the alcohol-induced liver diseases, such as hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) (Morgan et al., 2004). Previous *in vivo* and *in vitro* studies have revealed that acetaldehyde is able to form covalent adducts with various proteins, leading to alter the liver function and structure (Lieber, 1994; Pares et al., 1994; Carter and Wands, 1988). In addition to these effects, it has been shown that acetaldehyde induces the activations of transcription factors, such as nuclear factor-κB (NF-κB) and activator protein 1 (AP-1), in HepG2 cells (Roman et al, 2000). Since NF-κB and AP-1 have recently been implicated in the control of tumor invasion and metastasis (Huber et al., 2004), whether the acetaldehyde induced NF-κB and AP-1 activities and resulted in the tumor metastasis was the question we'd like to address in this study..

Tumor invasion and metastasis require increased expressions of matrix metalloproteinases (MMPs) (Stamenkovic, 2000). MMP family is involved in the degradation of extracellular membrane, and members of MMPs have been implicated in malignancy and metastasis (Stamenkovic, 2000; Westermarck and Kahari, 1999). Previous study indicated that MMPs might serve as new serum markers of HCC in patients with chronic liver disease (Paradis et al., 2005). Especially, MMP-9 gene (also termed gelatinase B or 92 kDa type IV collagenase) was found to express highly in HCC with invasive potential (Arii et al., 1996). MMP-9 degrades basement membrane type IV collagen and expresses during cellular invasion and metastasis (Nelson et al., 2000). In addition, elevated serum levels of MMP-9 were reported in patients with HCC (Hayasaka et al., 1996). The activity of MMP-9 is tightly controlled, with regulation occurring mainly at the transcription level (Stamenkovic,

2000). The promoter of MMP-9 is highly conserved and shown to contain multiple functional elements, including NF-κB and AP-1 elements (Sato and Seiki, 1993; Sato et al., 1993). In the previous study, we demonstrated that acetaldehyde is capable of activating NF-κB activity and, in turn, inducing cytokine expression (Hsiang et al., 2005a). We are now interested in whether acetaldehyde activated MMP-9 expression via the induction of NF-κB or AP-1 activity and played a role in tumor metastasis.

NF-κB is an inducible transcription factor, which is involved in inflammation, immune response, and malignant transformation (Karin and Ben-Neriah, 2000). NF-κB is a dimeric transcription factor that consists of p50, p52, p65 (RelA), RelB, and c-Rel (Siebenlist et al., 1994). Under normal condition, NF-κB is sequestered in the cytoplasm by inhibitory protein, IκB. When cells are exposed to stress, IκB is phosphorylated by IκB kinase (IKK), conjugated with β-transducin repeat-containing protein (β-TrCP) ubiquitin ligase, and degraded by 26S proteasome. Following IκB release, NF-κB translocates into the nucleus and binds to a unique decameric nucleotide sequence, leading to gene expression (Baldwin et al., 1996; Barnes et al., 1997). AP-1 is a nuclear transcription factor, which is involved in cell proliferation, differentiation, apoptosis, and neoplastic transformation (Angel and Karin, 1991; Shaulian and Karin, 2001). AP-1 consists of homodimers and heterodimers of members from Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) families (Angel and Karin, 1991). AP-1 activity is regulated at transcriptional and post-translational levels mainly by mitogen-activated protein (MAP) kinases, such as c-Jun N-terminal kinases (JNKs), p38, and extracellular signal-regulated kinases (ERKs) (Karin et al., 1997). Herein we demonstrated that acetaldehyde activated NF-κB and AP-1 activities through IκB, JNK/β-TrCP, and p38 signaling pathways. The activations of NF-κB and AP-1 by acetaldehyde resulted in the induction of

MMP-9 expression and, in turn, the promotion of cell invasion. Our findings suggested a role of acetaldehyde in the late steps of tumor development and metastasis.

Material and methods

Cell culture, transfection, and acetaldehyde treatment

HepG2 cells were maintained in Dulbecco modified Eagle medium (DMEM) (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, Utah). HepG2 cells were transiently transfected with 5 μg plasmid DNAs by SuperFect® transfection reagent (Qiagen, Valencia, CA) and treated with acetaldehyde. Acetaldehyde (Sigma, St. Louis, MO) was prepared freshly in phosphate-buffered saline (137 mM NaCl, 1.4 mM KH_2PO_4, 4.3 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2). HepG2 cells were cultured in 25-cm^2 flasks at 37^0C. After a 24-h incubation, cells were treated with various amounts of acetaldehyde in DMEM. To control evaporation, flasks were capped immediately and sealed with parafilm.

Gelatin zymography

To measure the production and activity of MMPs in the conditioned media from cultures receiving various treatments, gelatin zymography was performed using a previously reported method (Preáux et al., 1999). Briefly, conditioned medium was mixed with equal volume of 2x non-reducing sample buffer (0.5 M Tris-HCl, 20% glycerol, 10% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, pH 6.8). Samples were fractionated in 7.5% polyacrylamide gels containing 1 mg/ml gelatin by electrophoresis at 90 V for 120 min at 4°C. The gels were then soaked in 2.5% Triton X-100 for 10 min at room temperature to remove SDS and sequentially incubated in incubation buffer (50 mM Tris-HCl,10 mM $CaCl_2 \cdot 2H_2O$, 50 mM NaCl, 0.05% Brij35,

pH 7.6) at 37°C for 48 h to allow gelatinases digestion. The gels were stained with 0.25% Coomassie brilliant blue R-250 in 40% methanol for 30 min and destained with 40% methanol and 10% acetic acid. Gelatinolytic activities appeared as clear bands of digested gelatin against a dark blue background of stained gelatin. The intensity of band on the gel was calculated by Gel-Pro® Analyzer (Media Cybernetics, Inc., Silver Spring, MD).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from HepG2 cells according to the acid guanidium-phenol-chloroform method (Chomczynski and Sacchi, 1987). One microgram of RNA was reverse transcribed using oligo$(dT)_{15}$ primer and SuperScript™III (Invitrogen , Carlsbad, CA) in a total volume of 20 μl. Two microliters of reverse transcription mixture were subjected to PCR to measure the mRNAs of MMP-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR amplification was performed with Taq polymerase (Promega, Madison, WI) for 36 cycles at 92°C for 45 sec, 55°C for 45 sec, and 72°C for 2 min. PCR primers for MMP-9 were as follows: sense, 5'-CGATGACGAGTTGTGGTCCCTGGGC-3'; antisense, 5'-AATGATCTAAGCCCAGCGCGTGGC-3'. PCR primers for GAPDH were as follows: sense, 5'-CACCCATGGCAAATTCCATGGCACC-3'; antisense, 5'-CCTCCGACGCCTGCTTCACCACC-3'.

Construction of MMP-9 promoter/reporter plasmids

The MMP-9 promoters, including wild-type and mutants, were provided by Douglas D. Boyd (MD Anderson Cancer Center, University of Texas, Houston) and originally constructed by Hiroshi Sato (Cancer Research Medicine, Kanazawa

University, Kanazawa, Japan) (Sato and Seiki, 1993). The -670 to +54 fragment of the MMP-9 promoter was inserted into the pGL3-basic vector to generate the MMP-9 reporter plasmid. Wild-type and mutant sequences of NF-κB and AP-1 sites are: NF-κB (wild-type) GGAATTCCCC, NF-κB (mutant) TTAATTCCCC; AP-1 (wild-type) TGAGTCA, AP-1 (mutant) TATGTCA. The resulting constructs were confirmed by DNA sequencing and prepared by Qiagen plasmid midi kit (Qiagen, Valencia, CA).

Luciferase assay

HepG2 cells were treated with various amounts of acetaldehyde for 8 h. Luciferase activity was determined as described previously (Hsiang et al., 2005b). Briefly, cells were then lyzed by 350 μl Triton lysis buffer (50 mM Tris-HCl, 1% Triton X-100, 1 mM dithiothreitol, pH 7.8) and centrifuged at 12,000 xg for 2 min at 4^0C. The luciferase activity was measured by mixing 20 μl of cell lysate with 20 μl of luciferase reagent (470 μM luciferin, 33.3 mM dithiothreitol, 270 μM coenzyme A, 530 μM ATP, 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, pH 7.8), and determined with a luminometer (FB15, Zylux Corp., Maryville, TN). Relative luciferase activity was calculated by dividing the relative luciferase unit (RLU) of MMP-9 reporter plasmid-transfected cells by the RLU of pGL3-basic-transfected cells.

Biotinylated electrophoretic mobility shift assay (EMSA)

HepG2 cells were treated with acetaldehyde for various periods of time and nuclear extracts were prepared as previously described (Hsiang et al, 2002). The

biotin-labeled complementary oligonucleotides corresponding to the NF-κB and AP-1-binding sites were annealed by heating to 90^0C for 3 min and cooling slowly to 45^0C. Biotinylated EMSAs were performed as previously described (Hsiang et al., 2005). After electrophoresis, gels were transferred to nylon membranes. Membranes were blocked in blocking solution and detected with alkaline phosphatase-conjugated streptavidin (Chemicon, Australia) followed by chemiluminescence (Roche, Germany).

Western blot analysis

HepG2 cells were treated with acetaldehyde for various periods of time and then lyzed with 250 μl sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% bromophenol blue, pH 6.8). The proteins (10 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and the protein bands were then transferred electrophoretically to nitrocellulose membranes. Membranes were blocked in blocking buffer (20 mM Tris-HCl, 140 mM NaCl, 0.1% Tween-20, 5% skim milk powder, pH 7.6) and probed with polyclonal antibodies against IKK, IκB-α, p65, JNK, β-TrCP, p38, or ERKs (Cell Signaling Technology, Beverly, MA). The bound antibody was detected with peroxidase-conjugated anti-rabbit antibody followed by chemiluminescence (ECL system, Amersham, Buckinghamshire, UK) and exposed by autoradiography.

Invasion assay

Cell invasion was measured using the Matrigel-coated film insert (8 μm pore size)

fitting into 24-well invasion chambers (Becton-Dickinson Bioscience, Franklin Lakes, NJ). HepG2 cells ($5x10^4$), which were resuspended in 200 μl DMEM, were added to the upper compartment of the invasion chamber in the presence or absence of 10 μM acetaldehyde, and 500 μl DMEM was then added to the lower compartment of the invasion chamber. The Matrigel invasion chambers were incubated at 37^0C in 5% CO_2. After 24-h incubation, the filter inserts were removed from the wells, and the cells on the upper side of the filter were removed using cotton swabs. The cells in the lower surface of the filter were stained, and the cell number was counted with a microscope. Values obtained were calculated by averaging the total number of cells from three filters.

Statistical analysis

Data were presented as mean ± standard error. Student's *t* test was used for comparisons between two experiments. A value of $p<0.05$ was considered statistically significant.

Results

1. Acetaldehyde induced MMP-9 activity in HepG2 cells

To study whether the gelatinolytic activity of MMP was activated by acetaldehyde in HepG2 cells, we performed the zymographic analysis. Fig. 1A shows that acetaldehyde significantly stimulated MMP-9 secretion in HepG2 cells, with a maximal activation fold of 12.9 at 10 μM acetaldehyde. These results indicated that acetaldehyde stimulated MMP-9 activation in HepG2 cells. To further determine whether the activation of MMP-9 by acetaldehyde was resulted from the increased MMP-9 mRNA level, we performed RT-PCR analysis with primers specific to human

MMP-9 gene. As shown in Fig. 1B, MMP-9 mRNA level was increased after a 16-h treatment with acetaldehyde in HepG2 cells. The MMP-9 mRNA level reached a maximal induction (9.4-fold) at 10 μM acetaldehyde treatment. These findings indicated that MMP-9 transcripts were increased by acetaldehyde in HepG2 cells.

2. Acetaldehyde activated the transcription of MMP-9 promoter via NF-κB and AP-1 binding sites

To investigate whether the transcriptional activity of MMP-9 was regulated by acetaldehyde, we examined the promoter activity of MMP-9 gene by luciferase assay. A genomic fragment containing the promoter region (-670 to +54) of MMP-9 gene was amplified by PCR and subcloned into pGL3-basic vector (Fig. 2A). The resulting construct was transfected into HepG2 cells, the cells were treated with acetaldehyde for 24 h, and the luciferase activity was measured by luciferase assay. Fig. 2B shows that MMP-9 promoter was activated by acetaldehyde in HepG2 cells, at levels of approximately 5.2-fold over the pGL3-basic-transfected cells. Since NF-κB and AP-1 have been shown to be transactivators of MMP-9 expression (Sato and Seiki, 1993; Sato et al., 1993), the reporter plasmids with single site mutations in the NF-κB or AP-1 binding site of the MMP-9 promoter were constructed (Fig. 2A). The responses of these mutant reporters by acetaldehyde were monitored by transfection into HepG2 cells. Fig. 2B shows that mutation of the NF-κB or AP-1 element significantly reduced the acetaldehyde-induced MMP-9 activation. These results indicated that acetaldehyde activated MMP-9 expression via modulating NF-κB and AP-1 activities.

3. Acetaldehyde enhanced DNA-binding abilities of NF-κB and AP-1 in HepG2 cells

To further determine the molecular mechanism of acetaldehyde on the inductions of NF-κB and AP-1 activities, we examined the effect of acetaldehyde on the DNA-binding abilities of NF-κB and AP-1 by biotinylated EMSA. Fig. 3(A) shows that acetaldehyde caused a significant increase of NF-κB DNA binding. The time course study showed that NF-κB activity reached a maximal level at 8 h when cells were exposed to 10 μM acetaldehyde. Acetaldehyde also increased the DNA-binding ability of AP-1 (Fig. 3B). The time course study showed that AP-1 activity reached a maximal level at 45 min when cells were exposed to 10 μM acetaldehyde. These results indicated that acetaldehyde enhanced DNA-binding abilities of NF-κB and AP-1 in HepG2 cells.

4. Acetaldehyde induced IκB-α phosphorylation and NF-κB translocation in HepG2 cells.

The activation of NF-κB is preceded by translocation of NF-κB to the nucleus following phosphorylation and degradation of IκB-α (Baldwin et al., 1996; Barnes et al., 1997). To further investigate how the NF-κB signaling pathway was involved in the activation of MMP-9 expression, we determined the levels of p65, IκB-α, and IKK-α/β in acetaldehyde-treated HepG2 cells. As shown in Fig. 4, p65 translocation was elevated in acetaldehyde-treated cells as measured by Western blot analysis. Since IκB phosphorylation and degradation is a predominant pathway for NF-κB activation (Karin and Greten, 2005), we next determined the levels of IκB in cellular extracts of HepG2 cells exposed to acetaldehyde. Phosphorylation and degradation of IκB-α was stimulated by acetaldehyde (Fig. 4). The reduced IκB-α level was correlated with a constant increase of phosphorylated IκB-α in HepG2 cells. These findings demonstrated that NF-κB activation induced by acetaldehyde was mediated

through IκB-α phosphorylation and degradation.

5. ***Acetaldehyde induced IκB-α degradation by JNK/β-TrCP signaling pathway.***

Our findings indicated that acetaldehyde induced NF-κB activity via IκB-α phosphorylation and degradation. However, the IKK activity was consistent in acetaldehyde-treated cells (Fig. 4). Many signaling pathways upstream of the IKK have been supposed to amplify NF-κB activity, including ras/raf1, MAP kinases, or Akt (Madrid et al., 2001; Kurland et al., 2003; Nawata t al., 2003; Beaupre et al., 1999). Previous study has shown that activation of JNK pathway results in the accumulation of β-TrCP via stabilization of β-TrCP mRNA, and the elevated levels of β-TrCP, in turn, contributes to a rapid IκB degradation and NF-κB nuclear translocation (Spiegelman et al., 2001). Therefore, we determined the levels of β-TrCP and phosphorylated JNK in cellular extracts of HepG2 cells following exposure to acetaldehyde. As shown in Fig. 5, JNK phosphorylation and β-TrCP were markedly elevated in acetaldehyde-treated cells, as measured by Western blot analysis. These results suggested that the degradation of IκB-α by acetaldehyde might be regulated by JNK/β-TrCP signaling pathway.

6. Acetaldehyde modulated AP-1 activity by JNK and p38 signaling pathways

Since AP-1 activity is controlled by signaling through MAP kinases (Karin et al., 1997), we determined the levels of MAP kinases in acetaldehyde-treated HepG2 cells by Western blots. As shown in Fig. 5, the levels of JNK, p38, and ERK proteins were similar in cells treated with acetaldehyde for various periods. Acetaldehyde stimulated the phosphorylations of JNK and p38, and exhibited no effect on the phosphorylation of ERK. These results suggested that the AP-1 activation by acetaldehyde might be

regulated by JNK and p38 signaling pathways.

7. Acetaldehyde promoted HepG2 cells invasion

Tumor invasion requires increased expression of MMP-9 (Nelson et al., 2000). We previously showed that acetaldehyde induced MMP-9 activity in HepG2 cells. Therefore, we examined whether the invasiveness of HepG2 cells was increased by acetaldehyde. As shown in Fig. 6, acetaldehyde induced a 16-fold increase in HepG2 cells migrated through Matrigel-coated filters. These findings indicated that acetaldehyde activated MMP-9 activity and, in turn, increased the potential for invasion.

Discussion

Several stimulators induce an increased expression of MMP-9 via various signaling pathways and result in the invasiveness of cell lines. For examples, hepatitis B virus X protein stimulates the NF-κB and AP-1 activations via phosphatidylinositol 3-kinase (PI3K)/Akt and ERKs pathways, resulting in an increase of MMP-9 expression (Chung et al., 2004). Transforming growth factor-β activates p38 signaling pathway, which, in turn, induces MMP-2 and MMP-9 expressions (Kim et al., 2004). Phorbol ester induces MMP-9 secretion mainly through protein kinase C-dependent activation of the Ras/ERK signaling pathway (Liu et al., 2002). Radiation enhances HCC cell invasiveness by MMP-9 expression through the PI3K/Akt/NF-κB signal transduction pathway (Cheng et al., 2006). In this study, we demonstrated that acetaldehyde stimulates the NF-κB and AP-1 activities via IκB, JNK/β-TrCP, and p38 signaling pathways, resulting in the increase of MMP-9 activity and HepG2 cells invasion. To

our knowledge, this is the first described intracellular signaling pathway of MMP-9 by acetaldehyde.

Most of the reactive oxygen species participate in the carcinogenic process at both the initiation and promotion steps of tumor development (Shi et al., 2004). In addition to the tumor development, H_2O_2 has also been shown to be involved in tumor metastasis by increasing pro-MMPs and active MMPs expression at both the transcriptional and posttranscriptional levels (Siwik et al., 2001). Acetaldehyde is the very reactive intermediate of the oxidative metabolism of ethanol. It has been known that acetaldehyde exhibits mutagenic and carcinogenic properties by forming adducts with DNA, inhibiting the DNA repair, and exchanging the sister chromatid (Garro et al., 1986; Obe and Ristow et al., 1979; Vaca et al., 1998; Clemens et al., 2002). Additionally, there are sufficient evidences to identify that acetaldehyde is a carcinogen in animals and potentially associated with the development of HCC (Boffetta and Hashibe, 2006; Seitz and Stickel, 2006; International Agency for Research on Cancer, 1999). Herein we demonstrated that acetaldehyde was capable of enhancing MMP-9 activity, resulting in the invasiveness of HepG2 cells. From those and these data, we concluded that, in addition to tumor development, acetaldehyde was also associated with the spreading of tumor.

In nonstimulated cells, NF-κB is retained in a latent form in the cytoplasm by a family of IκBs, which binds to the β-TrCP ubiquitin ligase. Upon phosphorylation by an inducible IKK, phosphorylated IκB undergoes ubiquitination and 26S proteasome-dependent degradation. The NF-κB then relieves inhibition and translocates to the nucleus (Karin and Ben-Neriah, 2000). In this study, we demonstrated that acetaldehyde induced IκB phosphorylation and degradation via JNK/β-TrCP pathway and, in turn, contributed to the induction of MMP-9 expression.

In addition to the NF-κB activity, acetaldehyde also activated AP-1 activity. Because MAP kinases, such as JNKs, p38 and ERKs, have has been shown to mediate AP-1 induction in response to extracellular signals (Karin et al., 1997), we speculated that acetaldehyde induced both the NF-κB and AP-1 activities via JNK pathway (Fig. 7). Overall, our findings demonstrated the roles of IκB, JNK/β-TrCP, and p38 pathways in the transcriptional regulations of NF-κB and AP-1 activities and the induction of MMP-9 activity by acetaldehyde.

In this study, we demonstrated that acetaldehyde increased MMP-9 gelatinolytic activity and cells invasion through the up-regulation of MMP-9 gene transcription. The MMP-9 gene transcription was regulated by acetaldehyde via activations of NF-κB and AP-1. Further analysis showed that there was a correlation between the phosphorylation and degradation of IκB, phosphorylations of JNK and p38, and accumulation of β-TrCP in HepG2 cellular extracts treated with acetaldehyde. In conclusion, our results suggested that acetaldehyde is potentially associated with tumor metastasis. The mechanism described herein clarified that acetaldehyde induced IκB, JNK/β-TrCP, and p38 pathways to activate NF-κB and AP-1 activities. The activations of NF-κB and AP-1 by acetaldehyde resulted in the increases of MMP-9 expression and cell invasion. The involvement of acetaldehyde in tumor spreading represents a new finding in the contribution of acetaldehyde to HCC and provides new clues for understanding the signal pathway of acetaldehyde during metastasis.

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Figure legends

Fig. 1. MMP-9 activity was stimulated by acetaldehyde in HepG2 cells

(A) Gelatin zymography analysis. HepG2 cells were cultured in DMEM containing 10% FBS and treated with various amounts of acetaldehyde for 24 h in serum-free DMEM. The conditioned medium (serum-free DMEM) was collected and then analyzed by gelatin zymography. The arrow indicates the 92-kDa MMP-9. Activation fold is presented as comparison with the intensity of MMP-9 band relative to untreated cells. (B) RT-PCR analysis. HepG2 cells were cultured in 25-cm^2 flasks and treated with various amounts of acetaldehyde for 16 h. Total RNAs were extracted and 1 μg of total RNA was reverse transcribed. The resulting cDNAs were then amplified by PCR using primers for human MMP-9 or GAPDH. PCR products were resolved in 1% agarose gels and visualized with ethidium bromide. Activation fold is presented as comparison with the intensity of MMP-9 band relative to untreated cells.

Fig. 2. MMP-9 promoter activity was stimulated by acetaldehyde in HepG2 cells.

(A) Schematic maps of reporter plasmids carrying either wild-type MMP-9 promoter (wt) or specific mutation at NF-κB site (NF-κB mt) or AP-1 site (AP-1 mt). (B) MMP-9 promoter activity. HepG2 cells were transiently transfected with pGL3-basic or MMP-9 reporter plasmids and treated with 10 μM acetaldehyde. After a 24-h treatment, luciferase activities were determined. Results are expressed as relative luciferase activity, which is presented as comparison with the RLU relative to pGL3-basic-transfected cells. Values are mean ± standard error of three independent assays. $^{*}p<0.05$, $^{**}p<0.01$ from pGL3-basic-transfected cells.

Fig. 3 The DNA-binding abilities of NF-κB and AP-1 were activated by

acetaldehyde in HepG2 cells.

HepG2 cells were treated with 10 μM acetaldehyde for various time points as indicated. The nuclear extracts were then prepared and incubated with biotin-labeled double-stranded oligonucleotides corresponding to NF-κB (A) or AP-1 (B) sequence as described in Material and Methods. The arrowheads point to the location of NF-κB/DNA or AP-1/DNA complex.

Fig. 4 Acetaldehyde induced IκB-α phosphorylation and NF-κB translocation in HepG2 cells.

HepG2 cells were treated with 10 μM acetaldehyde for various periods of time. The phosphorylated (phospho-IKK-α/β, phospho-IκB-α) and non-phosphorylated proteins (IKK-α, IKK-β, IκB-α) in cellular extracts were detected by Western blot. The levels of p65 in cytoplasm (cy) and nucleus (nu) were also determined by Western blot. Similar results were obtained in three different experiments.

Fig. 5. Acetaldehyde modulated NF-κB and AP-1 activations by **JNK/β-TrCP and p38 signaling pathways.**

HepG2 cells were treated with 10 μM acetaldehyde for various periods of time. The phosphorylated (phospho-JNK, phospho-p38, phospho-ERKs), non-phosphorylated proteins (JNK, p38, ERKs), and β-TrCP in cellular extracts were detected by Western blot. Similar results were obtained in three different experiments.

Fig. 6. Cell invasion was promoted by acetaldehyde.

HepG2 cells (5×10^4) were resuspended in 200 μl DMEM and added to the upper compartments of Matrigel invasion chambers supplemented with or without

acetaldehyde. After 24-h incubation, the total number of cells on the lower surface of the insert chamber was stained and counted under microscope with 200x magnifications. (A) Microscopic photos of stained migration cells. (B) Histogram of invasion assay. Results are expressed as invasion fold, which is presented as comparison with the total number of invasive cells relative to untreated cells. Values are the means ± SD of three independent experiments. $^{**}p<0.01$ from untreated cells.

Fig. 7. Schematic diagram illustrate the molecular mechanism of acetaldehyde -induced MMP-9 expression.

Acetaldehyde modulates JNK/β-TrCP and p38 pathways. Stimulation of these signaling pathways by acetaldehyde leads to the activations of NF-κB and AP-1. The activations of NF-κB and AP-1 result in the increases of MMP-9 expression and HepG2 cells invasion.

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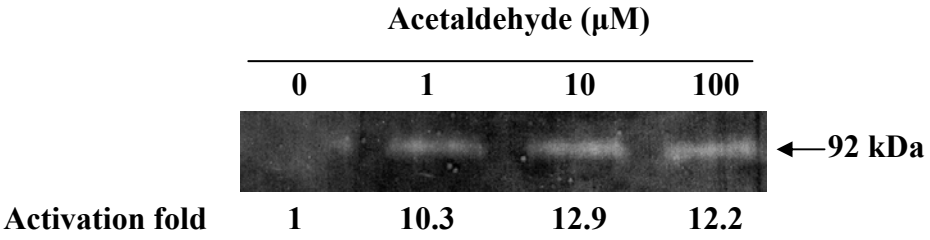
Figure 1

(A)


Acetaldehyde (μM)
0
1
10
100
92 kDa
Activation fold
1
10.3
12.9
12.2

(B)

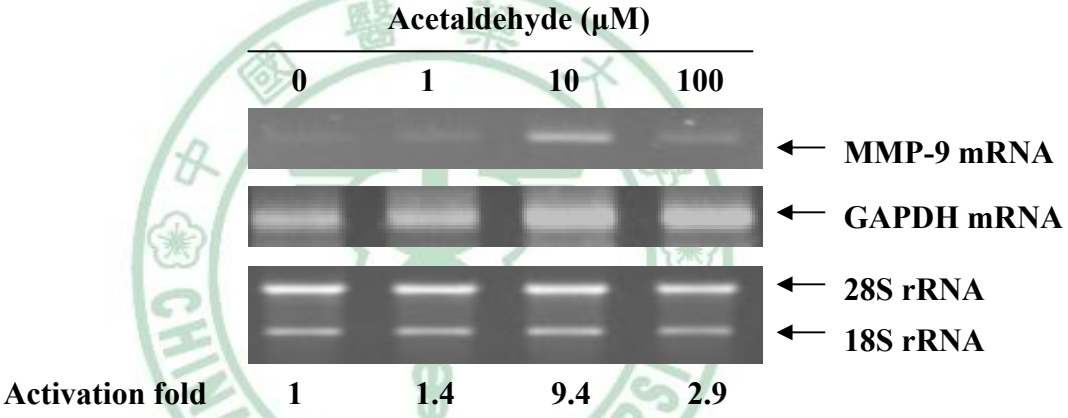
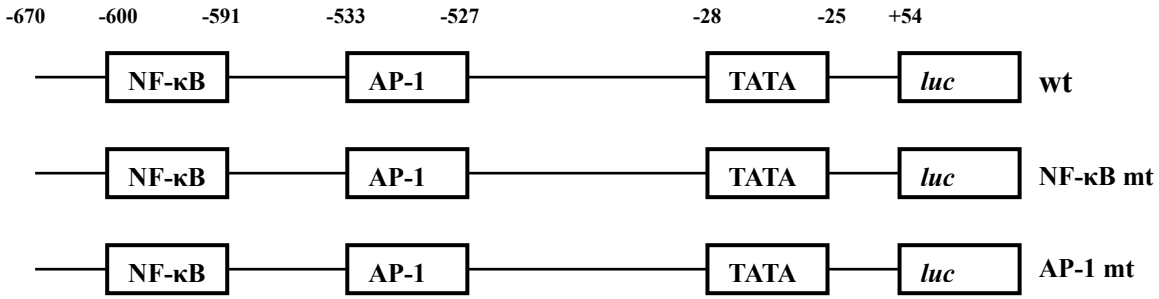

Acetaldehyde (μM)
0
1
10
100
MMP-9 mRNA
GAPDH mRNA
28S rRNA
18S rRNA
Activation fold
1
1.4
9.4
2.9

Figure 2

(A)


-670
-600
-591
-533
-527
-28
-25
+54
NF-κB
AP-1
TATA
luc
wt
NF-κB
AP-1
TATA
luc
NF-κB mt
NF-κB
AP-1
TATA
luc
AP-1 mt

(B)

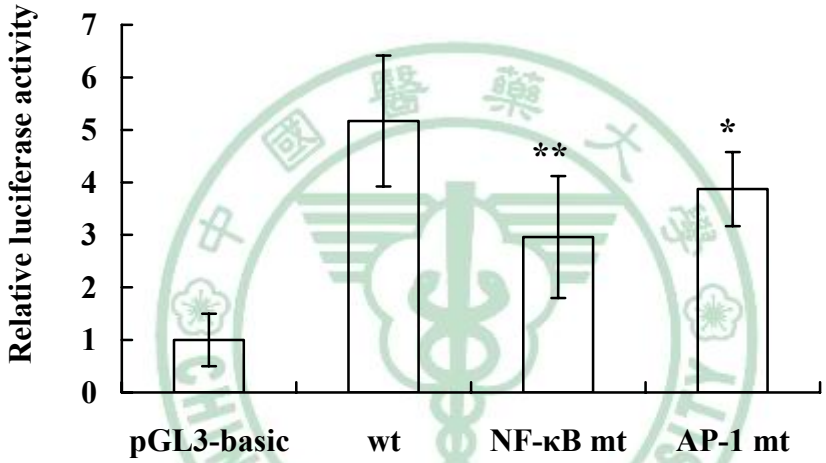
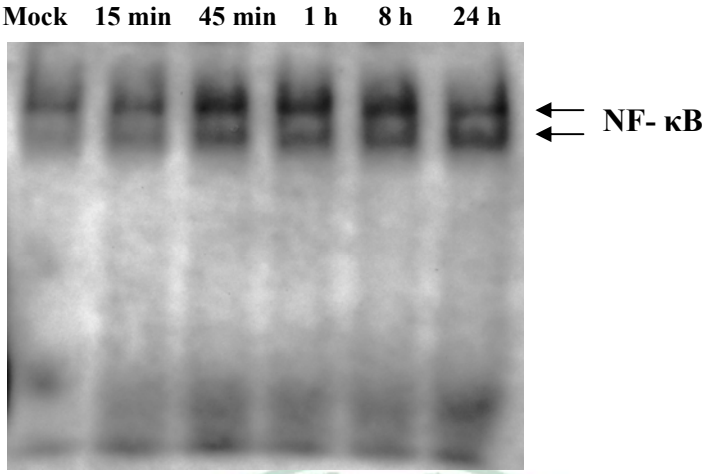

Relative luciferase activity
7
6
5
4
3
2
1
0
**
*
pGL3-basic
wt
NF-κB mt
AP-1 mt

Figure 3

(A)



(B)

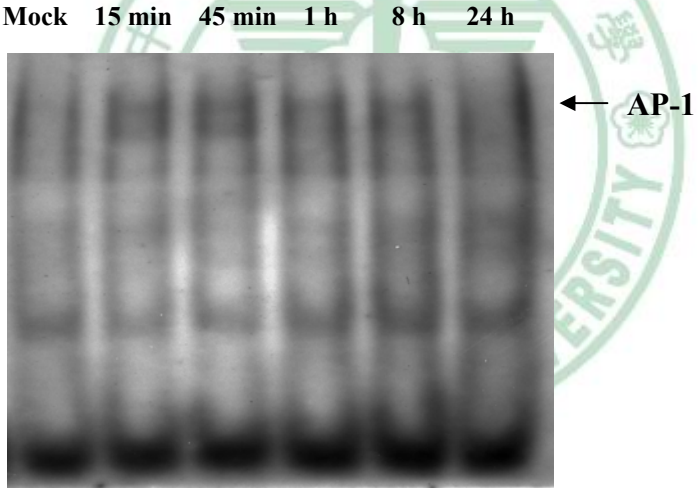


Figure 4

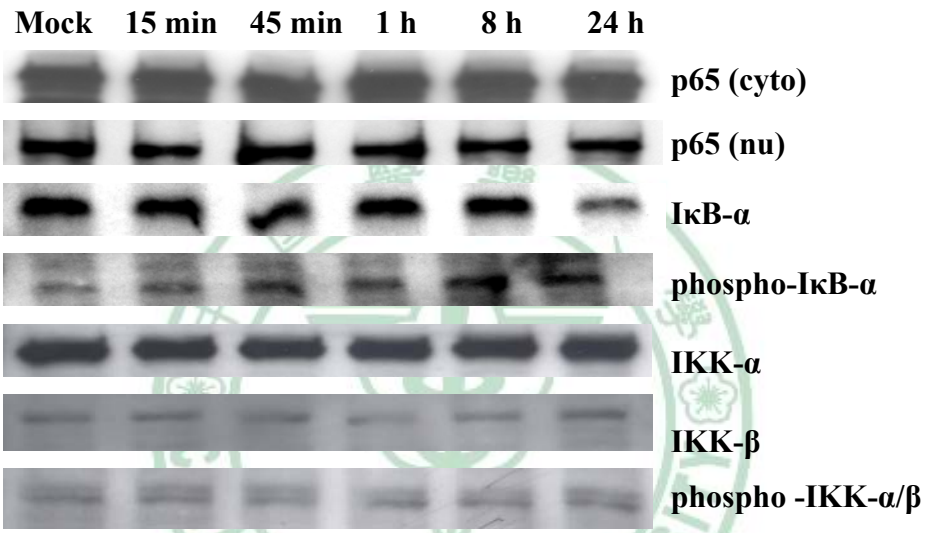

Mock
15 min
45 min
1 h
8 h
24 h
p65 (cyto)
p65 (nu)
IκB-α
phospho-IκB-α
IKK-α
IKK-β
phospho -IKK-α/β

Figure 5

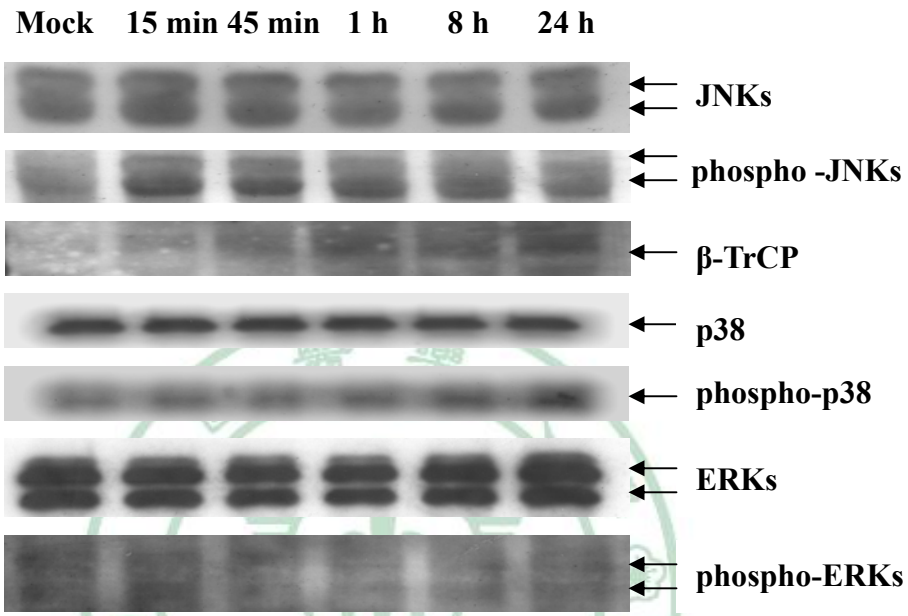


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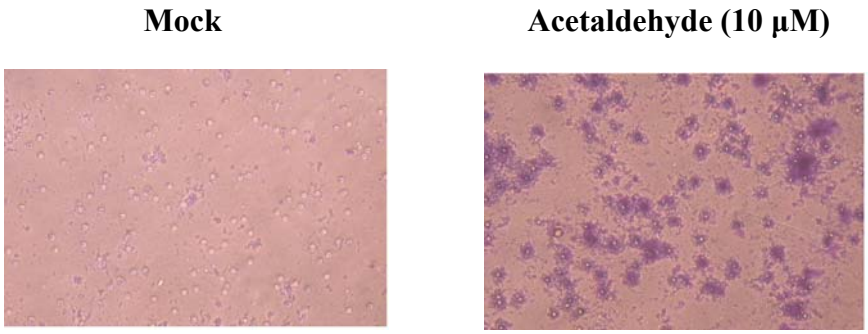
Figure 6

(A)


Mock
Acetaldehyde (10 μM)

(B)

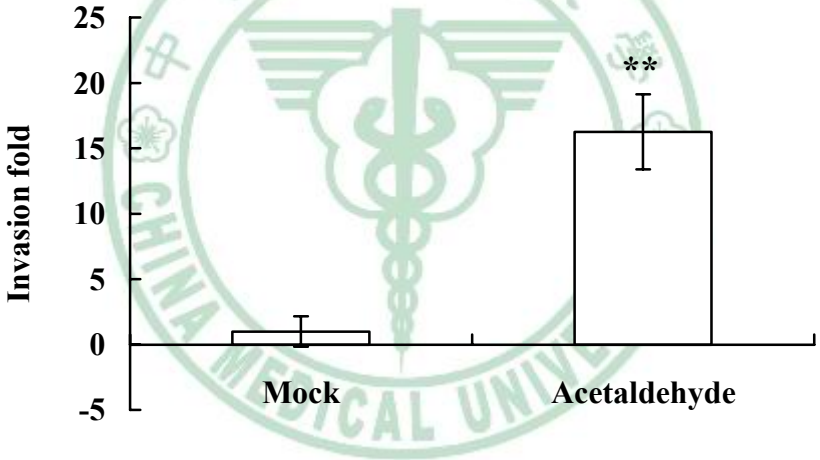
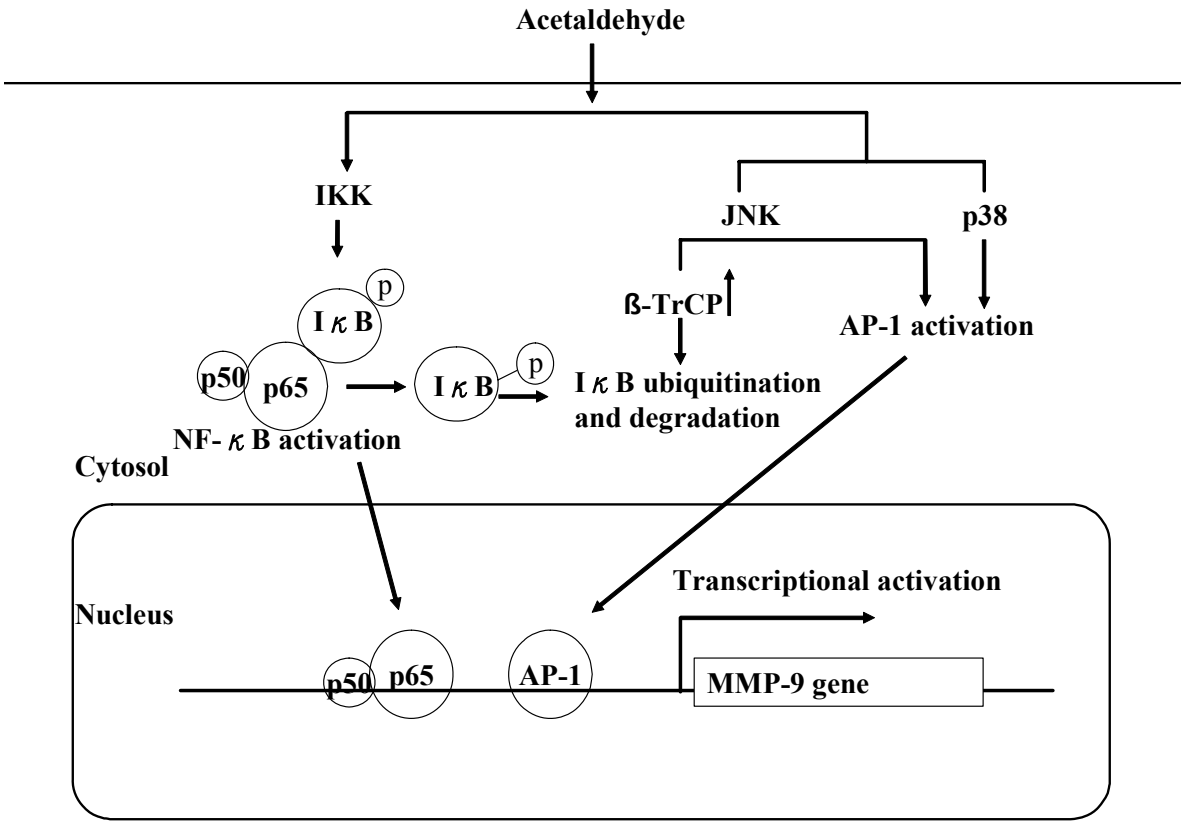

Invasion fold
25
20
15
10
5
0
-5
**
Mock
Acetaldehyde

Figure 7


Acetaldehyde
IKK
JNK
p38
p
I κ B
ß-TrCP
AP-1 activation
p50
p65
I κ B
p
I κ B ubiquitination
and degradation
NF- κ B activation
Cytosol
Nucleus
Transcriptional activation
p50
p65
AP-1
MMP-9 gene

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中台科技大學 醫技系 二技部

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