

行政院國家科學委員會專題研究計畫 成果報告

介白質 6 號促進蟹足腫細胞增生機轉探討：MAPKs 訊息活化
與 PGE2 在增生機轉中之角色

計畫類別：個別型計畫

計畫編號：NSC91-2314-B-038-047-

執行期間：91 年 08 月 01 日至 92 年 07 月 31 日

執行單位：臺北醫學大學醫學系

計畫主持人：沈杏娟

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中文摘要

蟹足腫 (keloid) 常見於手術後傷口癒合處，其最主要的特徵在於皮下膠質組成的過分增生與沉積。其真正的發生原因至今仍不十分清楚，但一般認為其發生機轉可能與細胞激素 (transforming growth factor- β 和 platelet-derived growth factor) 的慢性刺激，導致皮膚纖維細胞的過分增生以及大量膠質被製造有關。除了上述的因子之外，蟹足腫形成的過程中，被認為可能有尚有其他細胞激素參與在其中。介白質六號 (interleukin-6/IL-6) 是一種無論是在皮膚的角皮細胞或是白血球細胞中均能被製造與分泌的細胞激素，它本身具有多種的生物功能，如在人體組織受損或發炎反應時，IL-6 就扮演相當重要的媒介角色。近期的研究報告顯示，transforming growth factor- β 與 IL-6 之間有密切的關聯性，TGF- β 所誘導的纖維細胞增生需透過 IL-6 的媒介才能達成。因此，探討 IL-6 對人類蟹足腫或纖維細胞之細胞增生或膠質形成的影響確實有其必要性。

關鍵詞：蟹足腫, 介白質 6, 細胞增生, ERK

Abstract

Keloids are characterized by an overabundant deposition of collagen, and they recur frequently following excision. It is thought that normal fibrotic repair progress to dermal fibrosis when fibroblasts are activated persistently by chronic exposure to cytokines such as transforming growth factor- β or platelet-derived growth factor. However, additional cytokines and mechanisms

may play a role in the development of keloidal fibrosis. Interleukin-6 (IL-6), a cytokine that is a major mediator of the host response to tissue injury and infection, is produced by both keratinocytes and leukocytes. Recently, evidences support that TGF- β induced fibroblast cell proliferation was though the mediator of IL-6. In an effort to define the role of interleukin-6 on keloid fibroblasts, cells isolated from keloid tissue or normal tissue were maintained in culture medium. The potential of exogenous interleukin-6 to differentially affect DNA synthesis and collagen expression in cultured human fibroblasts derived from keloid or normal dermis were investigated.

Keywords :

keloid, interleukin-6, cell proliferation, ERK

前言

Keloids and hypertrophic scars are skin abnormalities that are unique to humans and are characterized by excessive deposition of collagen in the dermis and subcutaneous tissues secondary to traumatic or surgical injuries. Contrary to the asymptomatic fine-line scar characteristics of normal wound repair, the exuberant scarring of keloid and hypertrophic scar results typically in disfigurement, contractures, pruritis and pain. Clinically, keloids are defined as scars growing beyond the confines of original wounds, which rarely regress over time. It has been estimated that 15-20% of Blacks, Hispanics and Orientals suffered from keloids and there appears to be a genetic predisposition to keloid formation. Genetic approaches, such as linkage analysis, are being taken to identify the chromosomal location of the predisposing gene for the ultimate purpose of cloning and characterizing the genetic lesion.

Keloid lesions are characterized by an excess deposition of several connective-tissue extracellular matrix components, including elastin, chondroitin sulfate proteoglycan, fibronectin, and especially collagen. It represents an example of pathological scarring from overhealing and are characterized by an abnormal proliferation of fibroblasts. Along with an inflammatory response and neovascularization, there is an excessive extracellular matrix (ECM) accumulation of collagen, resulting in dense scar formation. The collagen is thick and disorganized with a decreased level of crosslinkage^{1,2}. Although its disease pathogenesis is still unknown, it is thought that growth factors and mast cell products, such as histamine, may be involved in the process of keloid formation. Previous studies have focused on primary alterations in the metabolism of fibroblasts as the key in the etiology of this condition. Yet alterations in the production of various cytokines which may alter fibroblast responses secondarily have received little attention. 1992 McCauley et al showed that the production of IFN- α , INF- γ , and TNF- β were markedly depressed in keloid patients compared to normal controls. However, IL-6, TNF- α , and IFN- β production were significantly increased in keloid patients³. Numerous in vitro and in vivo studies have suggested that fibroblast-activating cytokines such as transforming growth factor (TGF)- α and - β , platelet-derived growth factor, epidermal growth factor, interleukin (IL) -1 α and β , tumor necrosis factor- α and IL-4 regulate both the connective tissue formation and remodeling phases of normal dermal fibrotic repair^{4,5}. Especially, transforming growth factor β 1 (TGF- β 1) play an important role in the process of wound healing. Unlike adult wounds, fetal wounds also heal without scarring. One reason for this is the small amount of TGF- β 1. The addition of transforming growth factor β to fetal wounds results in scarring^{6,7}. Recently, TGF- β was found that it can induce extracellular matrix deposition and proliferation of mesenchymal cells and IL-6 is an essential mediator of growth factor-induced proliferation of lung fibroblasts⁸. This finding indicates that a cross-communication between IL-6 and TGF- β 1.

Interleukin-6 (IL-6) is a cytokine that displays several biological activities. It is produced by a broad range of cells, namely B and T cells, monocytes, keratinocytes, fibroblasts, endothelial cells, and some tumor cells⁹. Cell-specific expression of IL-6 results in multitude of biological responses, including stimulation of B lymphocyte maturation into plasma cells, increase in the production of immunoglobulins, and stimulation of T cell growth and differentiation¹⁰⁻¹⁵. In fact, levels of IL-6 have been reported to be elevated during infection, malignancy, autoimmunity, injury, and inflammation¹⁶⁻¹⁹. Because of its central role in modulating immunity, IL-6 production is normally tightly regulated. On exposure to an inflammatory stimulus (e.g., infectious agent, endotoxin, chemical and physical stresses), IL-6 levels transiently rise in vivo and then return to reference range on resolution of the insult²⁰⁻²². Dysfunction of IL-6 turnover can lead to chronic elevations of the cytokine level and may have pathologic consequences. For example, synovial fluids from patients with rheumatoid arthritis, a chronic inflammatory disease, contain high levels of IL-6²⁴ that may be responsible for some of the systemic abnormalities common in arthritic patients. Similarly, IL-6 has been implicated in the pathogenesis of psoriatic plaque²⁵⁻²⁶, which is an idiopathic skin disease that is histopathologically characterized by accelerated epidermal proliferation and infiltration of inflammatory cells. In addition, IL-6 has been shown to function as growth factor in several human tumors, such as plasmacytoma, melanoma, gastric cancer, prostate cancer, and renal cell carcinoma²⁷⁻²⁹. Here, we seek to study the effects of IL-6 on human dermal fibroblasts and keloid cells,

particularly with respect to cell proliferation and the production of collagen. We established fibroblast stains from keloid fibroblasts by measuring the amount of procollagen type I carboxyterminal propeptide (PICP) in the conditioned media, which reflects the amount of newly synthesized type I collagen³⁰⁻³¹.

結果與討論

IL-6 誘導纖維母細胞及蟹足腫纖維母細胞增生

利用 MTT test 及 [³H]-TdR incorporation test 測得當 IL-6(介白質 6 號)濃度為 10 ng/mL 時, NIH/ 3T3(Mouse NIH/Swiss embryo fibroblast cell line)有最好的促進增生能力(Fig. 1、Fig. 2)。同樣再利用 MTT test 觀察 IL-6 對 Hs68 (Human foreskin fibroblast cell line)、KEL-FIB (Human skin keloid fibroblast)及人類蟹足腫纖維母細胞 (primary human keloid fibroblast cell) 等三株細胞的影響, 發現 IL-6 亦可促使此三種細胞的增生(Fig. 3 Fig. 4 Fig. 5)。其中當 IL-6 濃度為 25 ng/mL 時, Hs68 cell 與 KEL-FIB cell 有最大的增生速率, 而人類蟹足腫纖維母細胞則是在 30 ng/mL 時, 增生速率最高。

IL-6 誘導纖維母細胞及蟹足腫纖維母細胞增生, 是經由活化 MAPK/ERK 作用

Protein tyrosine kinases(PTKs)家族在調節細胞的生長及分化扮演很重要的角色³²⁻³³。因此一般學者認為藉由細胞激素或生長因子的刺激造成 PTKs 磷酸化, 是調節細胞增生、分化及其他生物功能中相當重要的路徑, 其中, 又以 tyrosine kinase-Ras-MAPK cascades 的活化, 進而活化各種 transcription factor ; 而誘導細胞增生是最重要的路徑之一。上述實驗證實 IL-6 在不同濃度下會刺激不同種類的纖維母細胞增生, 且活化 IL-6 接受體 (gp130) 後, 誘導細胞活化使得細胞增生速率增加, 最主要信號傳遞路徑為 MEK/MAPK 磷酸化³⁴。利用西方墨點法證實人類蟹足腫纖維母細胞以及 NIH /3T3 cells 在加入 IL-6 5 至 15 分鐘間 ERK 會明顯的被活化, 而 c-jun 與 c-fos 此二蛋白質則沒

有變化(Fig. 6、Fig. 7)。另外, Hs68 則在 5 至 30 分鐘可以看到 ERK 明顯被磷酸化(Fig. 8、Fig. 9)。由此可證實 IL-6 刺激纖維母細胞增生的機制與 PTKs 家族中的 MAPK/ERK signaling cascade 之間應該存有密切的關係。

IL-6 誘導纖維母細胞及蟹足腫纖維母細胞, 細胞週期調控蛋白表現增加。

利用西方墨點法來進一步證明 IL-6 是經由何種機制來誘導纖維母細胞及蟹足腫纖維母細胞的增生。首先觀察與細胞在 G1 時期有關的調控蛋白 cyclin D1, 和 cdk4。在 1 至 12 小時間 IL-6 可以刺激 NIH/ 3T3 細胞的 cyclin D1 表現量增加, 而 cdk4 並沒有變化 (Fig. 10)。再利用免疫沉澱法證明 IL-6 可以促進 cyclin D1/cdk4 complex 的形成, 使細胞由 G1 期進入 S 期 (Fig. 11)。

MEK1, 2 抑制劑 PD98059 亦會抑制由 IL-6 所誘導的纖維母細胞及蟹足腫纖維母細胞增生。

利用 MTT test 觀察 PD98059 對於 IL-6 誘導 NIH /3T3 及人類蟹足腫纖維母細胞增生之影響。結果顯示在加入 PD98059 之後, 原本由 IL-6 所引起的細胞增生現象會被抑制, 且與 PD98059 的濃度成正比。(Fig. 12、Fig. 13)。經由上述兩個實驗可以證明 IL-6 誘導纖維母細胞增生的主要路徑, 是經由 MAPK/ERK signaling cascade 的活化。

MEK1, 2 抑制劑 PD98059 可抑制由 IL-6 所誘導的 ERK 磷酸化。

為了再次確認 IL-6 刺激纖維母細胞增生機制與 MAPK/ERK signaling cascade 的磷酸化有關, 利用西方墨點法觀察 MEK1, 2 的抑制劑 PD98059 與細胞 ERK 磷酸化與的關連性。結果發現 PD98059 可抑制 IL-6 所誘導的 ERK 磷酸化 (Fig. 14、Fig. 15)。

Dexamethasone 抑制 ERK 的磷酸化。

臨床上, 經常使用抗發炎作用的類固醇類藥物

dexamethasone 治療蟹足腫，但是其作用機轉目前尚未清楚。過去研究認為 dexamethasone 可以藉由抑制 COX-2 的合成，進而抑制 COX2-mediated PGE₂ 的反應，來達到減少 PGE₂ 的分泌，降低發炎的反应³⁵。那 dexamethasone 是否會影響 MAK/ERK signaling cascade？利用西方墨點法將 NIH 3T3 及人類蟹足腫纖維母細胞處理同濃度的 dexamethasone 及 IL-6，發現 dexamethasone 的確可以抑制由 IL-6 所誘導的 ERK 磷酸化(Fig. 16、Fig. 17)。

Dexamethasone 抑制 IL-6 誘導纖維母細胞及蟹足腫纖維母細胞增生。

再利用 MTT test 觀察 dexamethasone 對於 IL-6 作用於 NIH 3T3 細胞及人類蟹足腫纖維母細胞增生之影響。結果發現 dexamethasone 會抑制由 IL-6 所誘導的細胞增生作用(Fig. 18、Fig. 19)。即證實在臨床上用來治療蟹足腫的藥物-dexamethasone，其主要支藥理作用為抑制 MAPK/ERK signaling cascade 磷酸化，進而抑制纖維母細胞增生。

圖與表

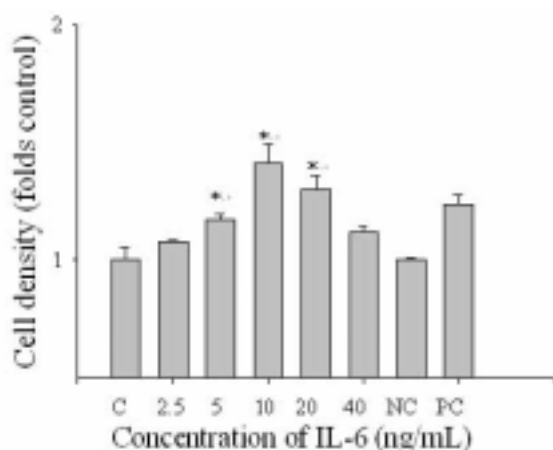


Figure 1. Dose-dependent proliferative response of IL-6 in NIH/ 3T3 cells. 預處理 0.5%血清的 NIH/ 3T3 細胞 16 小時後，分別加入不同劑量之 IL-6 (2.5、5、

10、20 及 40 ng/mL) 小時，再利用 MTT test，觀察細胞生長的情形。實驗發現 IL-6 可以使細胞增生速率增加，並且依劑量之高低產生不同的增生促進作用，IL-6 濃度為 10 ng/mL 時，有最好的促進增生能力。利用 student t-test 計算 p 值。* $p \leq 0.05$ ；** $p \leq 0.01$ 。

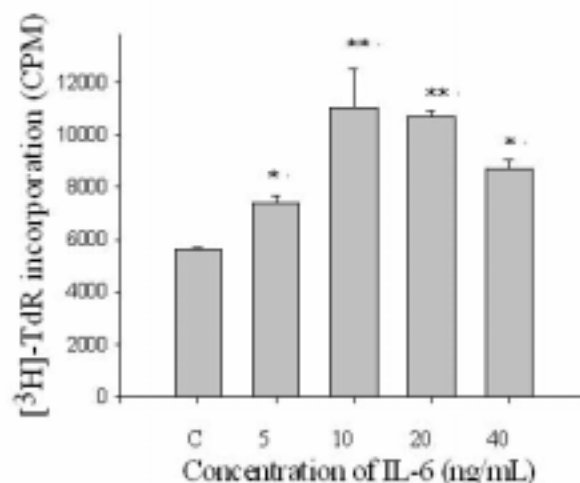


Figure 2. Dose-dependent proliferative response of IL-6 in NIH/ 3T3 cells. 預處理 0.5%血清的 NIH/ 3T3 細胞 16 小時後，分別加入不同劑量之 IL-6 (5、10、20 及 40 ng/mL) 24 小時，再利用 [3H]-TdR incorporation tests，觀察細胞生長的情形。實驗發現 IL-6 可以使細胞增生速率增加，並且依劑量之高低產生不同的增生促進作用，IL-6 濃度為 10 ng/mL 時，有最好的促進增生能力。利用 student t-test 計算 p 值。* $p \leq 0.05$ ；** $p \leq 0.01$ 。

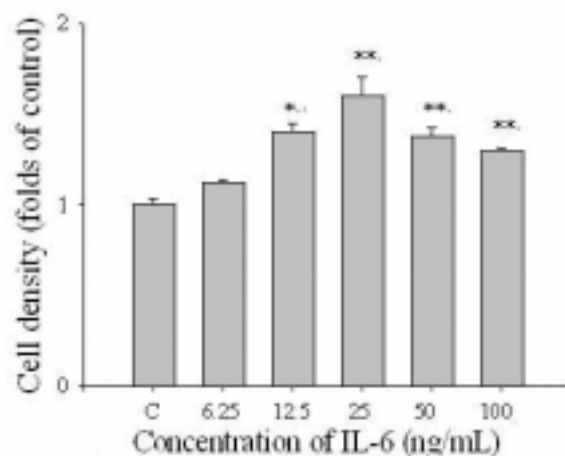


Figure 3. Dose-dependent proliferative response of

IL-6 in Hs68. 預處理 0.5%血清的 Hs68 細胞 16 小時後，分別加入不同劑量之 IL-6 (12.5、25、50、100 及 200 ng/mL) 72 小時後利用 MTT test，觀察細胞生長的情形。實驗發現 IL-6 可以使細胞增生速率增加，並且依劑量之高低產生不同的增生促進作用，IL-6 濃度為 25 ng/mL 時，有最好的促進增生能力。利用 student *t*-test 計算 *p* 值。* $p \leq 0.05$ 。

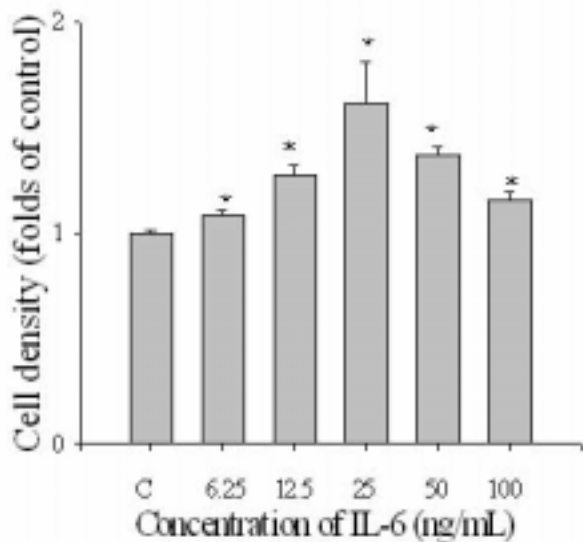


Figure 4. Dose-dependent proliferative response of IL-6 in KEL-FIB. 預處理 0.5%血清的 KEL-FIB 16 小時後，更，再加入不同劑量之 IL-6 (6.25、12.5、25、50 及 100 ng/mL) 72 小時，利用 MTT test，觀察細胞生長的情形。實驗發現 IL-6 可以使細胞增生速率增加，並且依劑量之高低產生不同的增生促進作用，IL-6 濃度為 25 ng/mL 時，有最好的促進增生能力。利用 student *t*-test 計算 *p* 值。* $p \leq 0.05$ ；** $p \leq 0.01$ 。

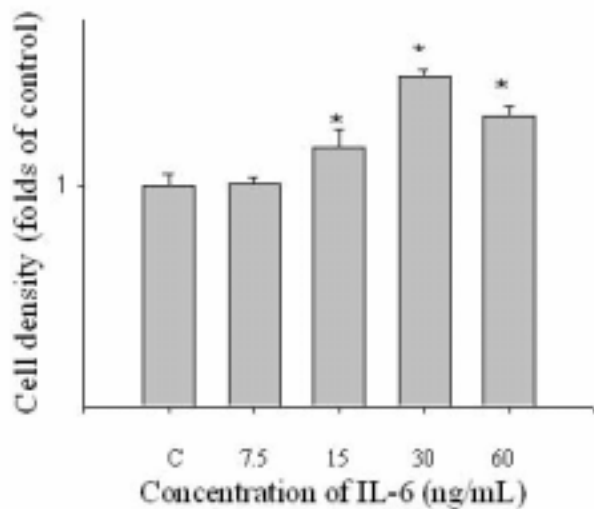


Figure 5. Dose-dependent proliferative response of IL-6 in primary human keloid fibroblast cell. 預處理 0.5%血清的人類蟹足腫纖維母細胞 16 小時後，再加入不同劑量之 IL-6 (7.5、15、30 及 60 ng/mL) 48 小時，利用 MTT test，觀察細胞生長的情形。實驗發現 IL-6 可以使細胞增生速率增加，並且依劑量之高低產生不同的增生促進作用，IL-6 濃度為 30 ng/mL 時，有最好的促進增生能力。利用 student *t*-test 計算 *p* 值。* $p \leq 0.05$ ；** $p \leq 0.01$ 。

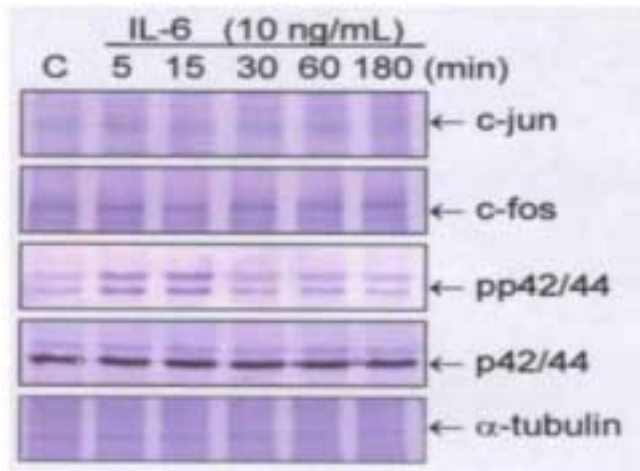


Figure 6. IL-6 induced ERK activation in NIH/3T3. 預處理 0.5%血清的 NIH/3T3 16 小時後，加入 10 ng/mL 之 IL-6，再以西方墨點法分析 c-jun、c-fos、pp42/44、p42/44 和 α-tubulin 在不同時間點 (0、5、15、30、60 及 180 分鐘) 蛋白質的表現。結果 5 至 15 分鐘間 ERK 會明顯的被活化，而 c-jun 與 c-fos 此二蛋白質則沒有變化。

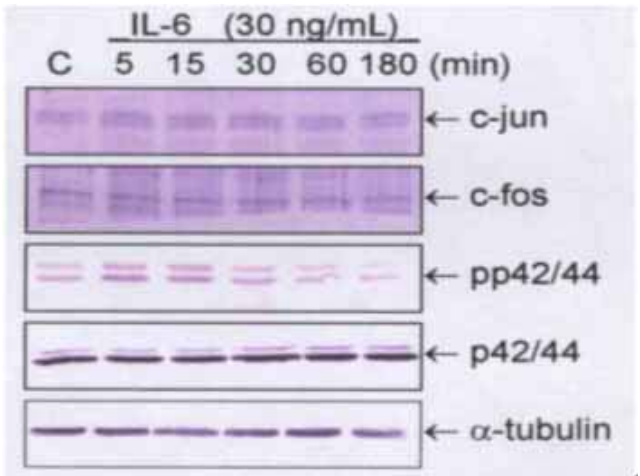


Figure 7. IL-6 induced ERK activation in primary

human keloid fibroblast cell. 預處理 0.5%血清的人類蟹足腫纖維母細胞 16 小時後，加入 30 ng/mL 之 IL-6，再以西方墨點法分析不同時間點（0、5、15、30、60 及 180 分鐘）蛋白質 c-jun、c-fos、pp42/44、p42/44 和 α -tubulin 的表現。結果 5 至 15 分鐘間 ERK 會明顯的被活化，而 c-jun 與 c-fos 此二蛋白質則沒有變化。

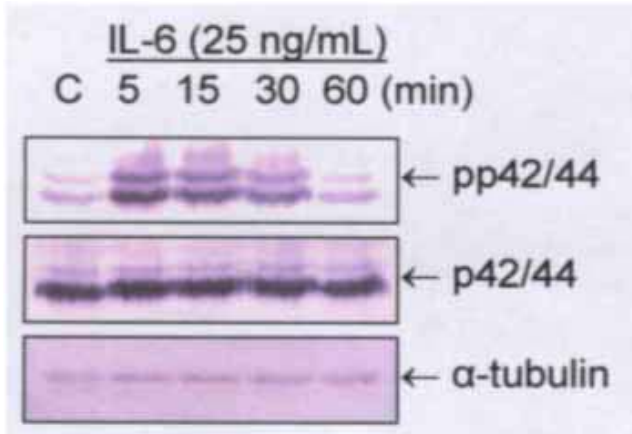


Figure 8. IL-6 induced ERK activation in Hs68 cells. 預處理 0.5%血清的 Hs68 16 小時後，加入 25 ng/mL 之 IL-6，再以西方墨點法分析不同時間點（0、5、15、30 及 60 分鐘）pp42/44、p42/44 和 α -tubulin 等蛋白質的表現。結果 5 至 30 分鐘間 ERK 會明顯的被活化。



Figure 9. IL-6 induced ERK activation in KEL-FIB cells. 預處理 0.5%血清的 KEL-FIB 16 小時後，加入 25 ng/mL 之 IL-6，再以西方墨點法分析不同時間點（0、5、15、30 及 60 分鐘）pp42/44、p42/44 和 α -tubulin 蛋白質的表現。結果 5 至 30 分鐘間 ERK 會明顯的被活化。

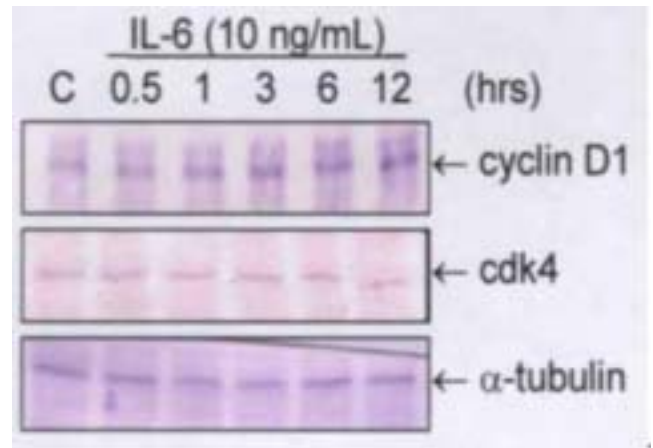


Figure 10. IL-6 induced cyclin D1 expression in NIH/3T3. 預處理 0.5%血清的 NIH/3T3 16 小時後，加入 10 ng/mL 之 IL-6，再以西方墨點法分析不同時間點（0、0.5、1、3、6 及 12 小時）cyclin D1、cdk4 和 α -tubulin 蛋白質的表現。結果 1 至 12 小時間 cyclin D1 的量明顯的增加，而 cdk4 此蛋白質則沒有變化。

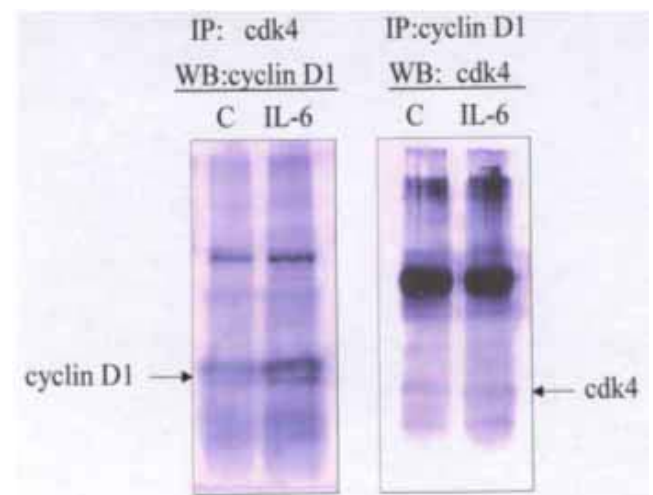


Figure 11. IL-6 induced cyclin D1 expression in NIH/3T3. 預處理 0.5%血清的 NIH/3T3 16 小時後，加入 10 ng/mL IL-6，6 小時後收取蛋白質，並以免疫沉澱法評估 cyclin D1 和 cdk4 蛋白質結合的情形。結果證明 IL-6 可以促進 cyclin D1/ cdk4 complex 的形成，使細胞由 G1 期進入 S 期。

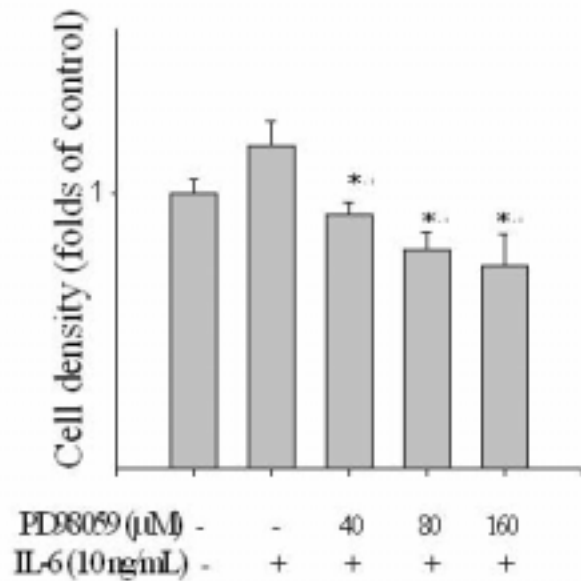


Figure 12. A MEK specific inhibitor PD98059 inhibited IL-6-induced cell proliferation in NIH/ 3T3 cells. 預處理 0.5%血清的 NIH/ 3T3 16 小時後，加入不同濃度之 PD98059 (40、80 及 160 μM) 30 分鐘後，再加入 10 ng/mL 之 IL-6，24 小時，利用 MTT test，觀察細胞生長的情形。實驗發現細胞增殖情形明顯被抑制，且與 PD98059 的濃度呈正比。利用 student *t*-test 計算 *p* 值。* $p \leq 0.05$ 。

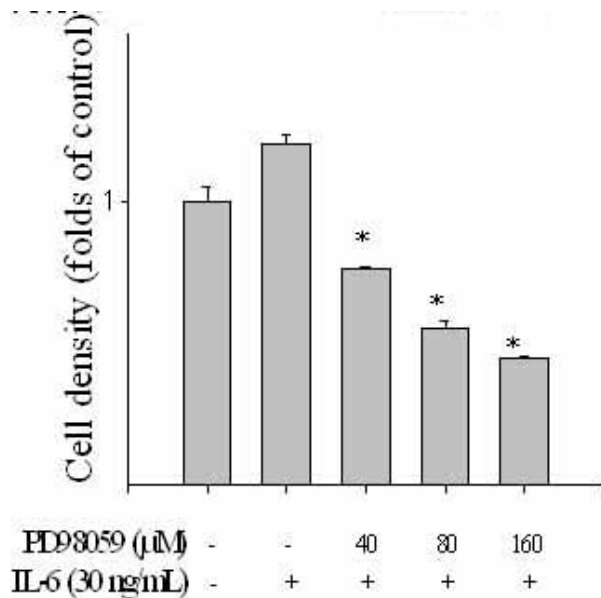


Figure 13. A MEK specific inhibitor PD98059 inhibited IL-6-induced cell proliferation in primary human keloid fibroblast cell. 預處理 0.5%血清的蟹足腫纖維母細胞 16 小時後，加入不同濃度之 PD98059 (40、80 及 160 μM) 30 分鐘，分別加入 10 ng/mL 之 IL-6 24 小時後，利用 MTT test，觀察細胞生長的情形。實驗發現細胞增殖情形明顯被抑制，且與 PD98059 的濃度呈正比。利用 student *t*-test 計算 *p* 值。** $p \leq 0.01$ 。

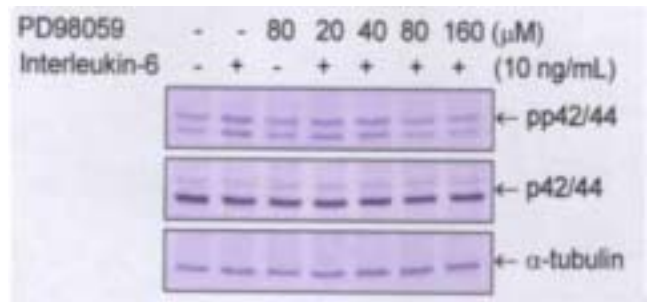


Figure 14 Inhibition of MEK antagonist (PD98059) on IL-6 induced ERK phosphorylation in NIH/3T3. 預處理 0.5%血清的 NIH/3T3 16 小時後，加入不同濃度之 PD98059 (0、20、40、80 及 160 μM) 30 分鐘，再加入 10 ng/mL 之 IL-6，5 分鐘後，利用西方墨點法觀察 pp42/44、p42/44 和 α-tubulin 蛋白質的表現。結果發現當 20 μM PD98059，即可部分抑制 IL-6 所誘導的 ERK 磷酸化，而 80 μM PD98059，則可完全抑制。

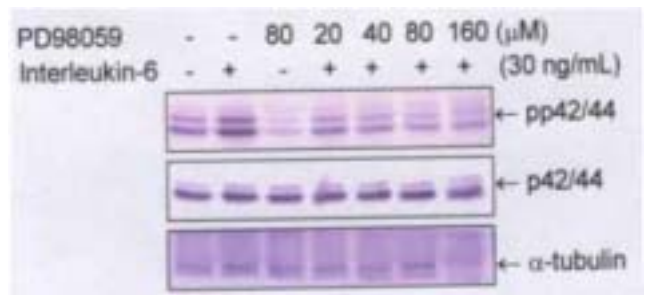


Figure 15. Inhibition of MEK antagonist (PD98059) on IL-6 induced ERK phosphorylation in primary human keloid fibroblast cell. 預處理 0.5%血清的人類蟹足腫纖維母細胞 16 小時後，加入不同濃度之 PD98059 (0、20、40、80 及 160 μM) 30 分鐘，再加入 30 ng/mL 之 IL-6 5 分鐘後，利用西方墨點法觀察 pp42/44、p42/44 和 α-tubulin 蛋白質的表現。結果發現當加入 20 μM PD98059，即可部分抑制 IL-6 所誘導的 ERK 磷酸化，而 80 μM PD98059 則可完全抑制。



Figure 16. Dexamethasone inhibition of IL-6 induced ERK phosphorylation in NIH/3T3 cells. 預處理 0.5%血清的 NIH/3T3 16 小時後，加入不同濃度之

dexamethasone (0、2.5、5.0、10 及 20 μM) 30 分鐘，再加入 10 ng/mL 之 IL-6，5 分鐘後，利用西方墨點法觀察 pp42/44、p42/44 和 α -tubulin 蛋白質的表現。結果發現當 dexamethasone 在濃度 5.0 μM 即可部分抑制 IL-6 所誘導的 ERK 磷酸化，而 10 μM PD98059 則可完全抑制。



Figure 17. Dexamethasone inhibition of IL-6 induced ERK phosphorylation in primary human keloid fibroblast cell. 預處理 0.5%血清的人類蟹足腫纖維母細胞 16 小時後，加入不同濃度之 dexamethasone (0、2.5、5.0、10 及 20 μM) 30 分鐘，再加入 30 ng/mL 之 IL-6 5 分鐘後利用西方墨點法觀察 pp42/44、p42/44 和 α -tubulin 蛋白質的表現。結果發現當 dexamethasone 的濃度在 5.0 μM ，即可部分抑制 IL-6 所誘導的 ERK 磷酸化，而 10 μM dexamethasone，則可完全抑制。

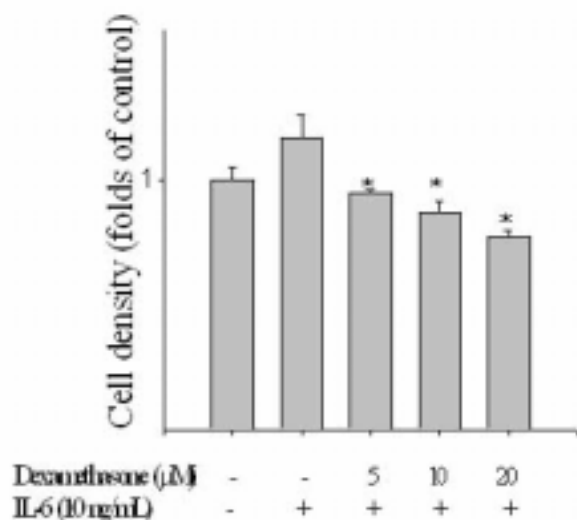


Figure 18. Dexamethasone inhibited IL-6-induced cell proliferation in NIH/3T3 cells. 預處理 0.5%血清的 NIH/3T3 16 小時後，加入不同濃度之 dexamethasone (0、5、10 及 20 μM) 30 分鐘後，再加入 10 ng/mL 之 IL-6 24 小時，最後利用 MTT test，觀察細胞生長的情形。實驗發現細胞增殖情形明顯被抑制，而細胞增殖被抑制的程度與 dexamethasone 的濃度呈正比。利用 student *t*-test

計算 *p* 值。** $p \leq 0.01$ 。

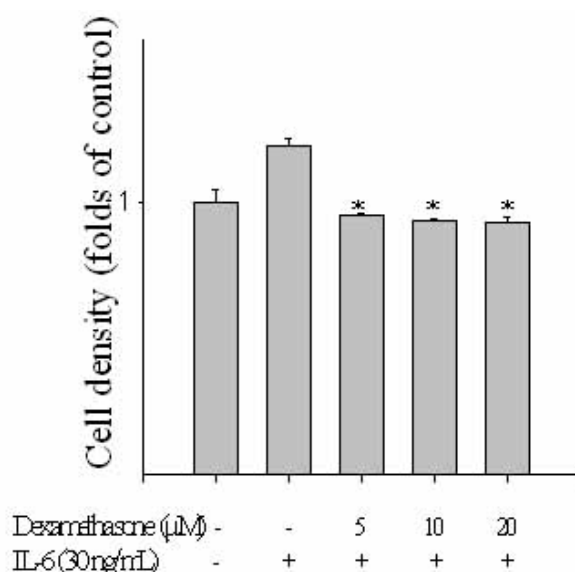


Figure 19. Dexamethasone inhibited IL-6-induced cell proliferation in primary human keloid fibroblast cell. 預處理 0.5%血清的人類蟹足腫纖維母細胞 16 小時後，加入不同濃度之 dexamethasone (0、5、10 及 20 μM) 30 分鐘，再加入 30 ng/mL 之 IL-6 24 小時，最後利用 MTT test，觀察細胞生長的情形。實驗發現細胞增殖情形明顯被抑制，而細胞增殖被抑制的程度與 dexamethasone 的濃度呈正比。利用 student *t*-test 計算 *p* 值。** $p \leq 0.01$ 。

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