行政院國家科學委員會專題研究計畫　成果報告

開發可應用於化妝品的抗皮膚老化之成份及研究其分子作用機轉

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執行單位：嘉南藥理科技大學藥學系

計畫主持人：施美份

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研究計畫之背景及目的

皮膚及眼睛是人類唯一暴露到日光紫外線的部位。日光紫外線的暴露引起的皮膚細胞的傷害，進而影響到皮膚的緊縮性及彈性，並且誘發皮膚提前老化現象：包括有皮膚粗糙、皺紋、斑點、皮膚鬆弛、及色澤暗沉等（Gilchrest &Yaar, 1992）。紫外線的波長可分四段：UVC (<290nm), UVB (290-320nm), UVA2 (320-340nm), and UVA1 (340-400nm)。其中 UVB 所引起的皮膚機能的傷害主要是皮膚老化及癌症，UVA2 的穿透能力較 UV 強因此也是引起皮膚老化的主要波長之一。陽光紫外線中的 UVC 及大部分的 UVB 在穿過大氣層時，會被臭氧層所吸收，因此抵達地球表面的量有限（除在臭氧層有破洞的南半球）。

正常的皮膚是靠 extracellular matrix (ECM)生合成及分解的平衡維持，而 Matrix metalloproteinases (MMPs)則在維護 ECM remodeling 上扮演著重要角色。Interstitial collagenase (MMP-1) 是一種 Zinc-dependent 蛋白質水解酵素 type I 及 III 膠原蛋白(collagens)的細胞外纖維。而 MMP-3 (stromelysin-1)則是負責分解醣蛋白 proteoglycans、fibronectin 和 type III 膠原(Giambernardi et al, 1998; Kuroda & Shinkai 1997)，可以與 MMPs 的活性相抗衡的物質則是 tissue inhibitor of metalloproteinase (TIMPs)。膠原蛋白約為真皮乾重的 70% 之多。其中 type I 膠原約佔 85% ，type III 膠原只佔 10% 。因此任何的因素會造成 MMPs 的活性或 TIMPs 的活性不足皆會引起皮膚的變化，如失去彈性、皺紋的產生(Uitto & Bernstein, 1998)。實驗證實抽煙也會促進皮膚的老化，其發生原因也與紫外線引起的皮膚改變相似。當皮膚細胞的培養液中添加有煙草的萃取物時，即可發現 MMP-1 及 MMP-3 的產量及其 mRNA 會比沒有添加煙草萃取物的細胞多(Yin *et al.*, 2000)。但相同的處理卻對 TIMP-1 及 TIMP-3 mRNA 無影響，顯示皮膚的細胞中的 MMPs 及 TIMPs 的活性只要失去了之間的平衡，皮膚老化現象即可被引發出來。

體外試驗發現：皮膚細胞來源若來自於提前老化的細胞其生命期（lifespan）則比同年齡未有提前老化的細胞的短(Hayflick 1965)，而這種現象則與皮膚細胞來源事來自於年紀較老的皮膚細胞相似(Gilchrest & Yaar, 1992)。紫外線引起的皮膚改變，已知是一種氧化性傷害進而去活化及增加細胞內的 PKC 的活性，PKC 的活性增加的結果便促進 MMPs 生合及造成膠原蛋白的分解，此一現象可藉由 Vitamin E 的抗氧化作用而達到改善(Berneburg *et al*, 1999)。其他的研究團體則是證實出單一次的紫外線照射便會增加 MMP 基因的表現，而且這種現象可持續長達 24 小時（Fisher et al., 1999），但 48-72 小時之後即可恢復至基本值。如果進行多次的紫外線照射則會引起持續性的增加 MMP 基因的表現（此種增加可長達至 7 天之久）。紫外線照射雖然也同時增加了 TIMPs 的產量，但是增加的比率比 MMPs 的產量少而不足以阻遏 MMPs 的活性。因此，造成長期的紫外線照射易促進皮膚老化的現象。

綠藻是一種淡水單胞藻,在日本及台灣食用已有多年的歷史。綠藻含有多種營養成分，如多種胺基酸，礦物質及膳食纖維等(Borowitzka, 1988; Shubert, 1988)。目前已有許多有關綠藻的生理功能的研究，如改善糖尿病鼠的高血糖情形 (Rodriguez-Lopez M. & Lopez-Quijada 1971; 李宏圖等人1977)，降低血脂(Sano & Tanaka, 1987；Okuda *et al.*, 1975；Sano 等人 1988；Yang *et al.*, 2001)提高免疫功能(Singh *et al.*, 1998; Tanaka *et al.*, 1984; Tanaka *et al.*, 1998; Konishi *et al.*, 1996)，抑制腫瘤生長 (Singh *et al.*, 1999; Noda *et al.*, 1996)，預防因壓力引起的潰瘍 (Tanak *et al.*, 1997)，促進動物的生長速率(Ishibashi, 1972) ，及促進因 ethionine 誘發的肝傷害的修復速度(Wang *et al.*, 1979)。

運用綠藻的萃取物當作緩和皮膚老化在上市的化妝品已有先例，雖然產品本身標榜具有此一功效卻無任何相關的研究報告可查證。經我們初步的實驗結果證實綠藻粗萃取物與 MMP inducers 同時加入培養液中可減少 MMP-1 的產量（請見圖一）。 因此，我們將利用已知的促皮膚老化的方式，即以 UVA2 照射人的皮膚的細胞(human skin fibroblasts)或 MMPs 的誘發藥物（如 IL-1β + PDGF-BB 或 PMA）去促進 MMPs 的生成的方式。再比較以純化後的綠藻萃取物處理後的皮膚的細胞的 MMPs 產量來當作初步的證實。

研究方法

Materials:

1. Normal skin fibroblast—966SK (76 years old female skin), 1059SK (20 years old female skin), 1090SK (46 years old female skin)
2. MMP-1 ELISA assay kits
3. TIMP-1 ELISA assay kits

chemicals: phorbol 12 myristate, IL-1β,PDGF-BB, Vit C, Vit E, GM6001, Isolation of extract fraction of Chlorella

UV irradiation: UVA 2 (320-340nm) radiation

Process

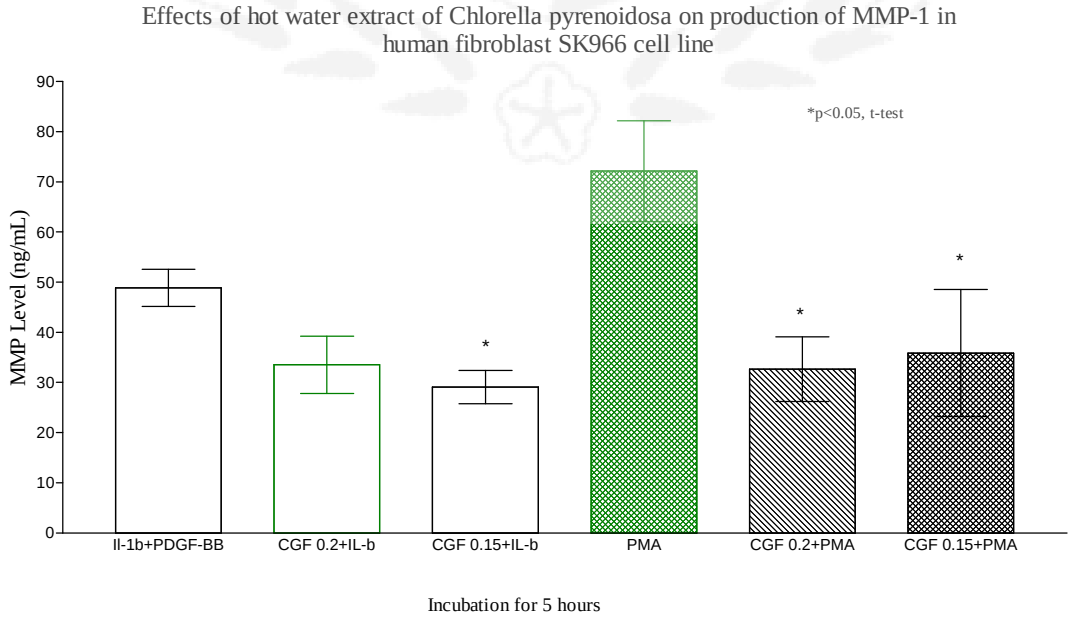
先利用不同年齡的皮膚細胞株先定量其之間 MMP-1, MMP-3, TIMP 的差異，再以不同的 MMP inducers：PKC activator (e.g. 100nM phorbol 12 myristate)或 IL-1β (2ng) + PDGF-BB (10 ng)去誘發 MMP-1 及 MMP-3。定量的方法：以 enzyme-linked immunosorbent assay (ELISA) kits 定量 MMP-1, MMP-3, TIMP。經證實此方法的再現性後，再進行綠藻粗萃取物的有效性的實驗，即綠藻粗萃取物可阻止 MMP inducers 對 MMP-1 及 MMP-3 的誘發的最佳劑量。此可實驗結果與 MMP inhibitors: vitamin E (25 μM)或 C (as positive control)或 GM6001 (0.4nM)對 MMPs 的抑制能力相比較。目前已有測量 MMP-1，MMP-3，TIMP-1 及 TIMP-3 的 ELISA kits （Chemicon Internation）。

接著利用 UV irradiation: UVA 2 (320-340nm) radiation 定量不同年齡的皮膚細胞株所引發的 MMP-1, MMP-3, TIMP 的差異，再進行綠藻粗萃取物的有效性的實驗，即綠藻粗萃取物可阻止 MMP inducers 對 MMP-1 及 MMP-3 的誘發的最佳劑量。因為不同的 MMP inducers 及 UV irradiation 所引導的 MMP-1, MMP-3, TIMP 的差異性可能不同，因此可能導致所需的綠藻粗萃取物阻止 MMP 的誘發的最佳劑量也不相同。

同時一併進行的尚有使用更精緻、純化的綠藻萃取物來篩選可能的成分及所需的最佳劑量。綠藻精（1 Kg），加 95 %酒精 3 公升冷浸，過濾後所得的殘渣，再用甲醇-氯仿溶液溶出，酒精濾液與甲醇-氯仿溶液溶出部分分別用減壓濃縮得粗萃取物 Fr. A 與 Fr. B。粗萃取物 Fr. A 與 Fr. B 再分別進行減少 MMP 的產量實驗。實驗顯示 Fr. B 的減少 MMP 的產量達近 100 %。Fr. B 將進行各種色層分析，每次色層分析所得的各個分畫便進行減少 MMP 的產量實驗，以實驗的結果來當作實驗的指標，期待分離精製到真正有減少 MMP 的產量的化合物。如 Scheme1 所示，由於粗萃取物 Fr. B 均為高極性物質，故先用 RP-18 open column (H_2O-MeOH 等)分離得到數個分畫後，再進行減少 MMP 的產量實驗，有作用的分畫再用其他的色層分析分法，反覆分離精製，等分離到單一化合物，再利用各種光譜分析法，如 NMR, IR, UV, MS 來決定其構造。

結果：

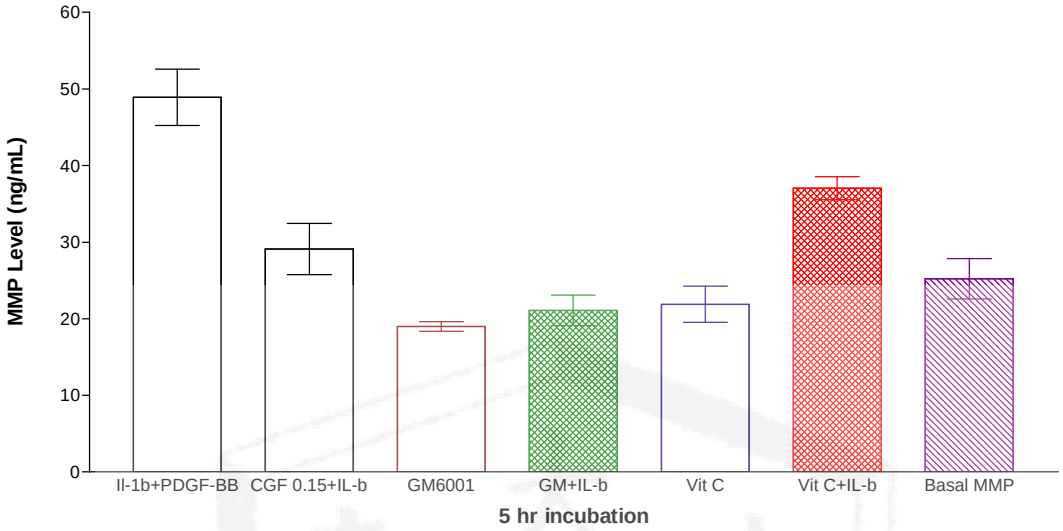
Figure 1: Effects of MMP1 level of Extract of Chlorella after 5 hr incubation with MMP activators



MMP1 is a type of secreted proteins and its levels in cell culture medium were measured induced after incubating with its promoters (10ng PDGF-BB/2ng Il-1b and PMA). The increase was prevented by co-incubation of 0.15mg and 0.2 mg of

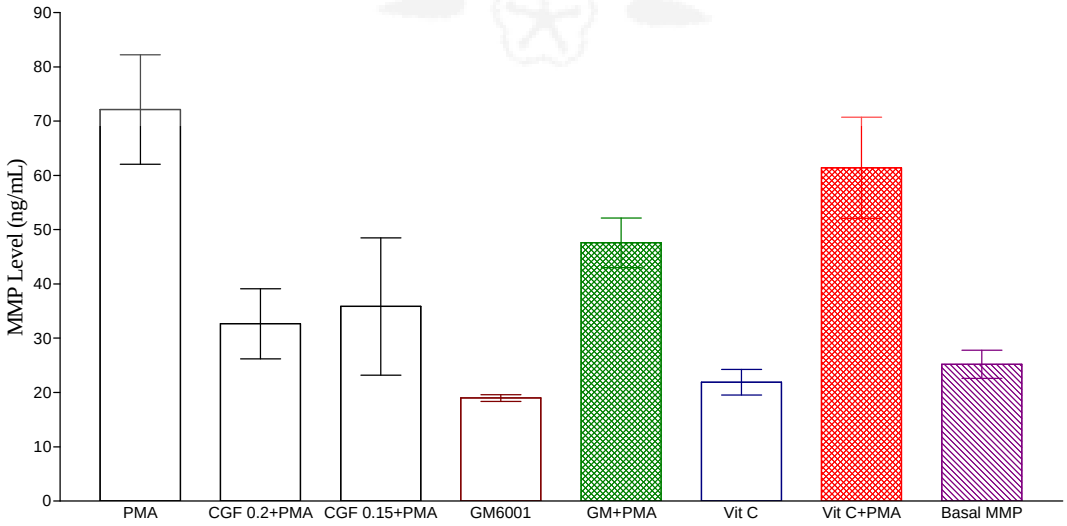
Chlorella extract (named CGF) 5 hours after the incubation.

Figure 2: Effects of MMP1 level of Extract of Chlorella compared to Vit C after 5 hr incubation with PDGF-BB/Il1b



Effects of CGF and vit C and GM6001 (a specific MMP inhibitor) on induced MMP1 levels were compared. Vit C (125μM) had a less effect compared to 0.15 mg of CGF or GM6001 (0.4nM). GM6001 and Vit C themselves did not affect basal MMP1 level after 5 hr incubation.

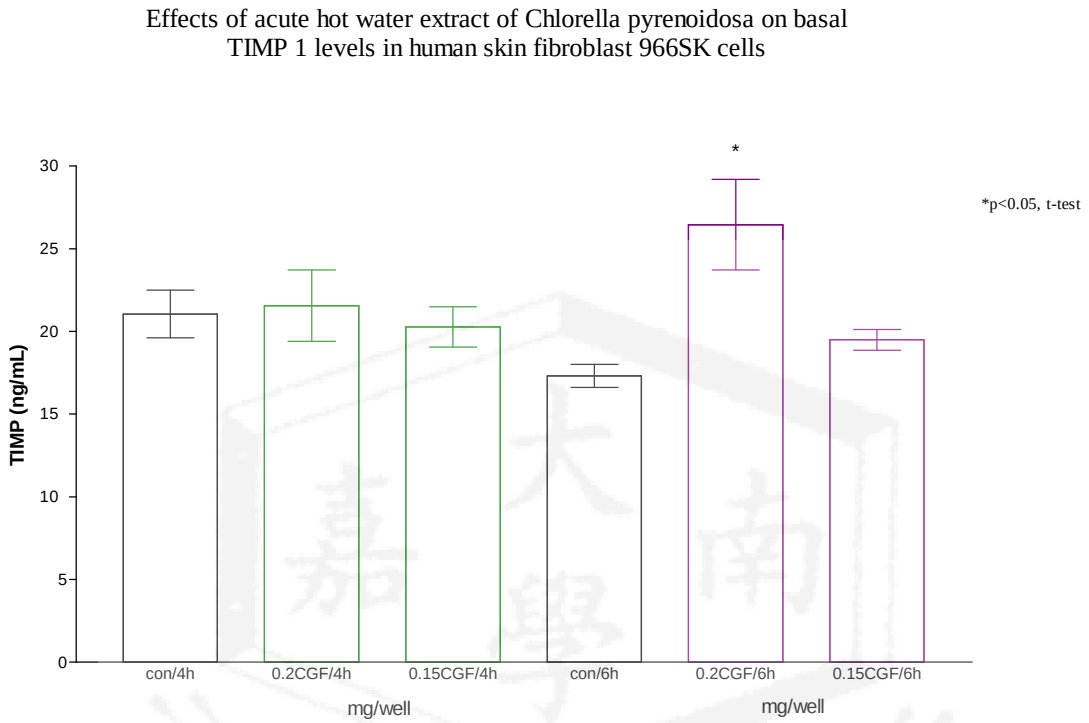
Figure 3: Effects of MMP level of Extract of Chlorella compared to Vit C after 5 hr incubation with PMA



MMP1 is a type of secreted proteins and its levels in cell culture medium were measured induced after incubating with PMA (100nM). The increase was prevented

by co-incubation of 0.15mg and 0.2 mg of CGF 5 hours after the incubation. GM6001 also inhibited increased MMP1 under the same stimulation, whereas Vit C had a less effect on PMA-induced MMP1 level.

Figure 4: Effects of extract of Chlorella on basal TIMP1 level



Basal TIMP1 levels were significantly higher in the cells co-culture with 0.2mg CGF for 6 hours compared to controls.

討論：

The extract of Chlorella (CGF) showed the capacities in preventing PDGF/Il-1b and PMA-induced MMP1 production in skin fibroblast (see figure 1). The preventing effects were compared with GM6001, an inhibitor of MMP, and Vit C, an anti-oxidant and a new component in cosmetic product. CGF had a similar effect as GM6001 in preventing MMP production, whereas Vit C showed a less effect. Neither CGF nor GM 6001 affected basal MMP1 levels. In addition, CGF increased basal TIMP-1 production after 6 hr incubation (see figure 4). The results show that CGF may provide a new and useful ingredient in preventing skin ageing in cosmetic industrial.

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