行政院國家科學委員會專題研究計畫成果報告

中文計畫名稱 ： 檳榔萃取物在纖維母細胞內之訊息傳遞
英文計畫名稱 ： The signal transduction of betel nut extracts on fibroblasts
計畫編號 ： NSC 87-2314-B-039-014
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一 中文摘要

檳榔所致之口腔黏下纖維化之纖維母細胞因口腔黏膜下纖維化被認為是癌前期病變，已被研究有十年之久。然而，目前已有的研究仍無法對檳榔所致之及口腔鱗狀細胞癌有最終的結論。本研究證明p44/p42 MAP(Mitogen-Activation- Protein) 動力媒路徑是檳榔鹼刺激黏膜纖維母細胞的訊息傳導主要路徑。檳榔素及檳榔鹼為兩種檳榔最主要的生物鹼，因此用來刺激正常的人類口腔黏膜纖維母細胞，NIH 3T3纖維母細胞，及口腔黏膜下纖維化之纖維母細胞，p44/p42 MAP動力媒在生理濃度下均顯示了活化，且檳榔鹼較檳榔素有較強的活化效應。而JNK動力媒在相同測試狀態下並無顯著的活化現象。此研究顯示p44/p42 MAP動力媒的活化再口腔黏膜下纖維化的過程中扮演了一主要角色，同時造成此活化的上游因子尚待去發掘。

關鍵詞 ： 口腔黏膜下纖維化，檳榔，MAP動力媒，JNK動力媒

二 英文摘要

Fibroblasts from oral submucous fibrosis (OSF) has been studied for more than ten years [1] [2], due to betel nuts related OSF was recognized as a premalignant condition [3]. Unfortunately, the previous studies still haven't reached the conclusion as how betel nuts induce OSF and oral squamous cell carcinoma (SCC). This study demonstrates MAP (Mitogen-Activation- Protein) kinase pathway is the main signal transduction pathway for mucosal fibroblasts under the stimulation of betel nuts derived alkaloids. Arecoline and arecaidine are two major betel nuts derived alkaloids, were used to stimulate normal human oral mucosal fibroblasts, NIH 3T3 fibroblasts and OSF fibroblasts, p44/42 MAP kinase activation were shown at their physiological concentrations and arecaidine had stronger effects. JNK (c-Jun N-terminal Kinase) didn't show significant activation at the same testing condition. The results shown here suggest p44/42 MAP kinase activation might play a role on OSF process and the upstream elements responsible for the activation were waiting for us to find out.

Keywords: OSF, betel nuts, MAP kinase, JNK

三 計畫緣由與目的

Betel nuts chewing is a popular habit in certain areas among the world, Taiwan is one of the prevalent area. According to epidemiology study, betel nutchewing is related to submucous fibrosis which is considered as a premalignant lesion, and oral squamous cell carcinoma.

The arguments of the nature of OSF fibroblasts existed for a long time. The earlier study showed arecoline and arecidine can stimulate collagen synthesis and fibroblasts proliferation [2], no significant difference between OSF and normal

fibroblasts in the rates of proliferation in cell culture, nor in the rate at which they hydrolysed arecoline to arecaidine [4]. Recently, Jeng et. al. showed genotoxic and non-genotoxic effects of betel quit ingredients on human oral mucosal fibroblasts [5]. It was known that exposure of cells to genotoxic agents evokes a series of phosphorylation events leading to the modification of transcription factors and gene expression [6]. Therefore, the phosphorylation events hypothesis was tested in this current study in the case of OSF. At least two different phosphorylation cascades might be involved. The first involves activation of cell membrane-associated tyrosine kinases followed by the sequential activation of Ras and Raf. Raf phosphorylates the mitogen-activated protein (MAP) kinase kinase (MEK), which in turn activates MAP kinase. MAP kinases also called extracellular signal-regulated kinases (ERKs) are members of a ubiquitous family of serine/threonine kinases that are responsible for the phosphorylation and activation of various transcription factors [7]. A second pathway relies on the c-Jun N-terminal kinases (JNK) for gene activation following UV treatment. JNKs can also be activated by a variety of stresses and hence are referred to as stress-activated protein kinases (SAPK) [8]. Once activated, JNK will phosphorylate transcription factors, c-jun and ATF2. Therefore, MAP kinase and JNK activation can be regarded as two major indicators of genotoxic-induced signals, our main purpose is to test which pathway is involved in betel nuts induced signal transduction.

四 結果與討論

MAP kinase assay demonstrates MAP kinase activations are observed when fibroblasts were stimulated with both arecaidine and arecoline. Fig. 1 show MAP kinase activation is an immedate response in normal fibroblasts, the reaction will occur within minutes. Higher concentration of arecaidine (100 ug/ml) will decrease the activation which might be due to the result from cell number decreased if cells were cultured for 48 hours at higher concentration [9], although cells were shown more than 90% vitality during 5 minutes stimulation. NIH 3T3 fibroblasts show similar activation pattern as normal fibroblasats , and MAP kinase activation is not only immediate but also transient (Fig. 2). OSF fibroblasts have higher basal MAP kinase activity (Fig. 3), this might be due to specific cell types or constitutely expressed MAP kinase activity. The activation is also immediate and transient. Among the alkaloids in betel nuts, arecaidine had stronger ability of stimulating fibroblast proliferation than arecoline [9], consistent with the previous observation, MAP kinase assay show stronger MAP kinase activation under arecaidine stimulation on normal fibroblasts(Fig. 4) and OSF fibroblasts (data not shown). Another main stream of genotoxic stress induced JNK activation was not the case of fibroblasts stimulated by arecaidine and arecoline. (Fig. 5,6) Therefore, the conclusion is MAP kinase activation is involved in the signal transduction of arecaidine and arecoline treated fibroblasts. The signal transduction effects could be related to cell proliferation, as suggested by the consistency with data mention above, or can further influence change OSF premalignant condition into malignancy. These are still open questions.

五 計畫成果自評

This current study was following the proposal and reached the main purpose of finding which pathway is important. Quantitative analysis for the activation will be performed soon, once an analysis source is available. Combined alkaloid and other betel nut component effects will be tested in latter experiment.

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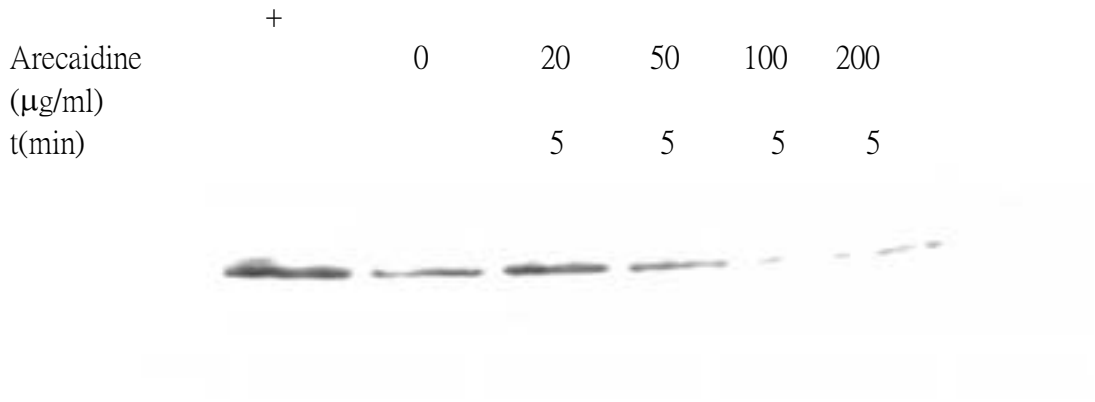

Fig.1 Normal fibroblasts show MAP kinase activation in 5 minutes on arecoline stimulation at differention concentration. Lane one is positive control, 2ug phospho MAP kinase was added in the cell extract.

Normal fibroblasts were primary culture from mucosal tissue of a healthy donor during his wisdom tooth removal. Passage 3 to 6 were used in these experiments. Confluent quiescent cells were stimulated with arecoline and MAP kinase assay were performed as following: cell extracts were immunoprecipitated (IP) with phospho MAPK antibody, then incubate IP pellets in kinase buffer containing Elk-1 fusion protein and cold ATP. Analyzing Elk-1 phosphorylation at Ser383 using phospho- antibodies by Western blotting and chemiluminescent detection. Cell vitality tests were performed at the same time by trypan blue exclusion assay, and cells showed more than 90% vitality.

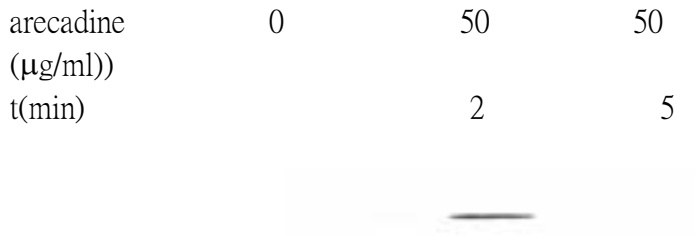

Fig. 2 MAP kinase activation on NIH 3T3 fibroblasts after 50ug/ml arecaidine stimulation.

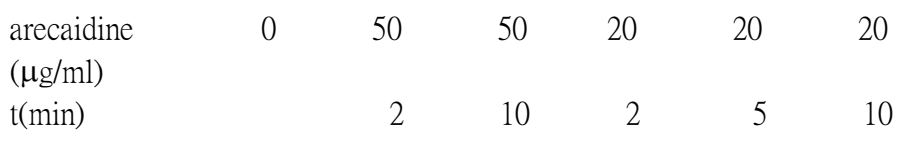

Fig. 3 Arecaidine induced MAP kinase activation on OSF fibroblasts.
OSF fibroblasts were primary culture from one OSF patient. Passage 3 to 6 were used in these experiments.

	+	arecaidine						arecoline				
(μg/ml)		0	20	20	100	100	200	20	20	50	50	50
t(min)			2	10	5	10	10	5	10	2	5	10

Fig. 4 Arecaidine induced higher MAP kinase activation than arecoline on normal fibroblasts.
Lane one is positive control, 2ug phospho MAP kinase was added in the cell extract.

	+	Normal fibrolast						3T3					
arecaidine (μg/ml)		0	20	20	50	50	100	20	20	20	50	50	100
t(min)			2	10	2	10	10	0	2	10	2	10	10

Fig. 5 JNK activities were shown by JNK assay. Only basal JNK activity were shown on Normal

fibroblasts and NIH 3T3 fibroblasts under arecaidine stimulation. Lane one is positive control, from cell extract of NIH 3T3 fibroblasts under UV exposurefor 20 minutes.
JNK assay is similar to MAP kinase assay, except cell extracts were IP with c-jun fusion protein beads and perform kinase reaction using c-jun fusion protein as substrate and add cold ATP for reaction.

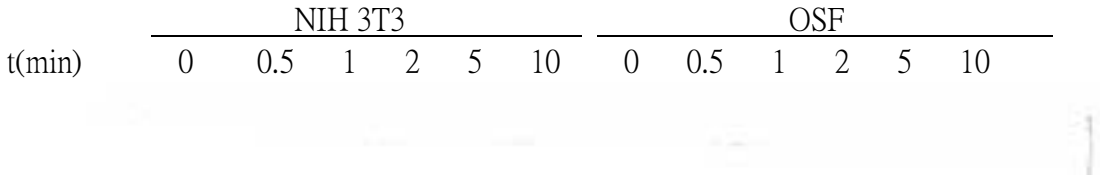

Fig. 6 JNK activities were not detected on NIH 3T3 and OSF fibroblasts under arecoline(20 ug/ml) stimulation.