行政院國家科學委員會專題研究計畫成果報告

計畫名稱：寡核酸作為肝癌治療劑之研發

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一、中文摘要

本研究以大白鼠肝癌細胞株及大白鼠為模式探討以反意寡核酸抑制細胞內重要訊息傳遞分子時，是否癌細胞的生長會受抑制，並探討所引發的分子機制。

GP7TB **為似幹細胞的肝癌細胞株，將此細胞種於** F-344 **大白鼠可生成肝腫瘤。我們發現甲型蛋白激脢**(PKC-α)**在癌細胞內有過度表現的現象。以** PKC-α**的反意寡核酸在體外及體內降低** PKC-α**蛋白質的生成時可以導致細胞生長受抑制。隨著** PKC-α**的減少，我們發現** Bcl-2 **的量以及** NF-κB **活化量的減少，細胞週期停滯於** S-**期的初期進而導致細胞凋亡。體內試驗為將腫瘤植入老鼠的肝臟，同時將內盛反意寡核酸的滲透式幫浦置入腹腔。經** 14 **天的治療，結果發現治療組腫瘤的大小僅為控制組的三分之一，在癌組織也見到細胞凋亡的現像。肝組織細胞則不受藥物的影響。**

本研究初步結果顯示 PKC-α**的反意寡核酸在體外及體內均可抑制肝癌細胞的生長，值得一步研究成為藥劑的可行性。**

關鍵詞: **甲型蛋白激脢，反意寡核酸，似幹細胞肝癌細胞，細胞凋亡，原位腫瘤模式**

Abstract

In this study, rat hepatoma cell line and rat were used as model system to study whether antisense oligonucleotides of signal transduction component would result in cell growth inhibition? The molecular mechanism underlying was also studied.

GP7TB, a rat liver epithelial tumor cell line, possesses characteristics of liver stem-like cell and can develop into tumor in syngeneic Fischer 344 (F-344) rat. We found that the protein kinase C- α (PKC- α) is overexpressed in the cells as compared with liver cells of the rats. Antisense ODN (oligodeoxynucleotide) was used to inhibit the synthesis of PKC- α *in vitro* and *in vivo*. The suppression of PKC- α resulted in a reduction in the levels of nuclear NF- κ B and Bcl-2. FACS analysis showed that the cells were arrested at early S-phase. These cells in turn went into apoptosis without completing a cell cycle. The *in vivo* experiment was conducted by implanting tumor mass of GP7TB in the liver of the rat. At the same time, an osmotic pump, which delivered antisense or control ODN, was embedded in peritoneum. It was found that the growth of tumor was suppressed efficiently; average tumor size was one third of the blank or

control group after a 14-day treatment. Apoptosis was found in the orthotopic tumor treated with antisense ODN against PKC-α. The normal liver cells were not affected.

The success of suppressing orthotopic tumor growth *in vivo* suggests that PKC-α antisense ODN would be a promising therapeutic agent for liver cancer.

Keywords: Protein Kinase C-α, Antisense Oligonucleotide, Stem-like Liver Cancer Cell, Apoptosis, Orthotopic Tumor Model

二、計畫緣由與目的

The protein kinase C (PKC) family is a group of serine and threonine kinase involved in signal transduction for processes such as proliferation, angiogenesis, inflamation and the immune response. The gene family represents at least ten related genes that are subdivided into three types, the classic PKCs (α, βI, βII and γ), new PKCs (δ, ε, η and θ) and atypical PKCs (λ and ζ). Although the specific functions of the individual isozymes are unclear, it is evident that one or more PKC isoforms may play a role in cell cycle progression and transformation. When mice were cutaneously treated with phorbol ester, PKC is chronically activated leading to a selective outgrowth of initiated keratinocytes. This results in the growth of multiple benign squamous cell carcinomas. The stable overexpression of different isoforms of PKC in cell lines results in deregulated positive or negative growth. For example, overexpression of PKC-α in the MCF-7 breast cancer cells led to a more aggressive neoplastic phenotype, which exhibited increased proliferative rate, anchorage-independent growth and tumorigenicity in nude mice. Transfection of PKC-βI and PKC-γ into fibroblast cells also led to an increased growth rate and anchorage-independent growth. However, overexpression of PKC-βI in colon cells caused growth inhibition. These studies demonstrated the effects of abnormal PKC levels on cellular homeostasis. There have also been many clinical findings which indicate elevated PKC levels in malignant tissue.

In this report, we show that PKC-α was overexpressed in GP7TB cells. To study the importance of PKC-α for this tumor cell line, we utilized PKC-α isoform-specific antisense ODN (oligonucleotides) to treat these cells. Antisense ODN, which has been demonstrated as an inhibitor of protein synthesis, can be used to lower the level of specific cellular protein. Using this approach, certain regions of a target mRNA are chosen to be the annealing sites for antisense ODN leading to an abolishment of protein synthesis by blocking the translation or mediating degradation of the targeted mRNA through RNase H. Antisense ODN of PKC-α has been reported to suppress the growth of different tissue types of cancer cell lines *in vitro* and in nude mice. However, antisense ODN of PKC-α against liver tumor cell has not been documented.

三、結果與討論

Evidence suggested that GP7TB cells was a cell lineage from liver stem-like cell. When carcinogenic liver injury occurs, the stem-like cells may be activated and proliferate to replace the lost liver cells. The progeny of this undifferentiated cell was suspected to be the prime culprit of certain liver tumors. This is the significance of choosing GP7TB cells in strategy design for liver cancer therapy. The F-344 rat with the implanted orthotopic tumor was sacrificed. Sections containing normal liver tissue and tumor mass were stained with antibody of PKC-α. The result showed that the orthotopic liver tumor cells had a much higher expression level of PKC-α than the normal liver. This suggests the importance of PKC-α on the growth or tumorigenicity of GP7TB cells.

We used unmodified ODN αm in this study. The efficacy of αm on suppression of PKC-α synthesis in GP7TB cells was revealed in Western-blot experiment. The result disclosed a gradual and prolonged inhibitory effect of αm on PKC-α. It seems that no compensatory production of PKC-α was triggered. At 72 h, PKC-α protein was reduced to one fifth of original level. No inhibition was found in the cells treated with control ODNs and Gö6976. The suppression of the PKC-α level resulted in a growth inhibition of GP7TB cells. Dose–dependent inhibition was found in the range of 0 – 10 μM of αm. An approximately 80% decrease in the number of cumulative viable cells was achieved at the 10 μm concentration. Again, no inhibition was found in the two control groups. Using an ACP assay, the growth inhibition was shown to be about 5%, 52% and 80%, respectively, after 48-, 72- and 96-h treatment of 10 μM αm. It was found that Gö6976, an catalytic inhibitor of PKC-α and βI, also suppressed the growth of GP7TB cells. The IC_{50} values for αm and Gö6976 were 4.0 and 3.3 μm, respectively, in a 96-h treatment.

Next, cell cycle phase distribution of the GP7TB cells was analyzed by FACScan. For the αm-treated group, cell population in the G_1 phase decreased concomitant with an increase of early S-phase cells at the 48-h treatment. A big apoptosis-like peak appeared later at 72 h. It seems that a large population of cells was arrested in the early S-phase and these cells in turn went into apoptosis without completing a cell cycle. The cell cycle phase distributions for the blank, control and even Gö6976 group were similar and apoptosis-like peaks were not prominent. The mechanism of growth inhibition caused by αm and Gö6976 must be different. To verify cell apoptosis after αm treatment, TUNEL assay was conducted. TUNEL assay detects the presence of chromosomal DNA chain breakage, an early onset of cell apoptosis. Apoptosis was clearly found in αm-treated cells, but not in the blank control or the control ODN treated cells.

To elucidate the molecular mechanism underlying cell apoptosis, we analyzed the protein Bcl-2 that has been characterized as an anti-apoptotic protein. Over 90% decrease of Bcl-2 was observed 60 h after αm treatment. This result implies that a decrease

in the level of Bcl-2 is possibly one of the causes of cell apoptosis induced by αm. NF-κB is a down-stream factor, the activity of which may be modulated by PKC-α. In resting state, NF-κB resides in the cytoplasm by binding with I-κB, an inhibitory protein. The phosphorylation of I-κB by protein kinases causes their dissociation and subsequently the translocation of NF-κB into the nucleus. It has been reported that PKC is one of the kinases able to phosphorylate I-κB. NF-κB plays a contradictory role in cell survival in either promoting or suppressing cell apoptosis. Using EMSA, we found that, in the absence of exogenous stimulant, a large amount of NF-κB was present in the nuclei. The amount of NF-κB was apparently decreased in αm-treated group, but not in control group. Specific interaction between NF-κB and its consensus sequence was confirmed by the competition assay. The results imply that PKC-α of GP7TB appears to be a major enzyme involving the activation of NF-κB which mediates a "life signal" to GP7TB cell.

The efficacy of αm *in vivo* was studied by implanting GP7TB cells orthotopically in the liver of syngeneic immuno-competent F-344 rat. A mini-osmotic pump was embedded in the peritoneum. The slow-released ODNs were absorbed by the peritoneum and then circulated directly into liver. By this strategy, a higher concentration of drug could be maintained in the liver. After a 14-day treatment, our result clearly indicates that the growth of tumors was effectively suppressed by αm , about 64% reduction in tumor volume. No suppression was found for the control group. The rats of the αm group showed no difference in body weight gain, feed uptake and locomotive activity as compared with the normal rats. The liver sections containing the tumor cells were H & E-stained and examined by light microscope. In the outer portion of the tumor mass, many apoptotic cells with condensed nuclei were found in αm-treated group but not in the control and blank groups. This result is consistent with the *in vitro* data obtained by FACScan and TUNEL assay. There was no pathological change found in the normal liver portion of ODN-treated groups. These results indicate that suppression of PKC-α level had not affected the normal liver. The possible reason is that normal liver cells are not dividing and are resting in G_0-phase.

Our findings highlight the importance of PKC-α as a target for liver cancer therapy.

五、參考文獻

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