行政院國家科學委員會專題研究計劃成果報告
計劃編號:NSC88-2314-B-038-126
執行期限:**87** 限 **8** 限 **1** 日至 **88** 限 **7** 限 **31** 日**(**申請延報至 **88** 限 **12** 限 **31** 日**)**
主持人:李婉若 副教授
執行機構:台北醫學院
執行單位:醫學研究位

Title:
Involvement of the G1/S checkpoint genes in the pathogenesis of keloid

Abstract
Keloid represents one of the most common skin proliferation disorders in humans. The keloid formation usually occurs as a result of hyperproliferation of fibroblasts after skin injury. Even though it is not considered a mailgnant skin disorders. However, the keloid fibroblasts can be successfully implanted and grown in Nude mice. The results suggest an uncontrolled cellular proliferation process which escape the normal cell cycle control. The cells from progressing into S phase which regulated by CdkIs such as: p21 and p27 genes. p21 gene was an downstream regulator of p53 which can also control cell cycle progression. p27 gene was the inhibitor of cyclin/CDK complex which also controlled the cell cycle progression. We were investigated the molecular mechanisms of keloid formation. When compare the keloid with normal control skin tissue, We found the p21(2/6) and p27(2/6) genes mRNA was expression in keloid skin tissue. This result may show the p21 and p27 were involved in controlling G1/S checkpoint play a role in the pathogenesis of keloid.
Keywords: keloid, G1/S checkpoint, p21, p27.

中文摘要:
蟹足腫是在人體皮膚上常見的皮膚增生不正常,蟹足腫常因爲皮膚傷害而引起纖維母細胞過度增生,雖然蟹足腫並非惡性皮膚疾病,然而蟹足腫的纖維母細胞可以在裸鼠身上生身,由此推論不受控制的增生可能是因爲由正常細胞週期所逃脫 而在脫細胞週期中是由週期素依賴性激素抑制者所調控,其中包括有 p21 有p27. P21 是p53 下游的基因,p27 是在脫細胞週期推動中伴演週期素/週期素依賴性激素的抑制者. 我們研究蟹足腫形成的分子機制,當比較蟹足腫與正常皮膚組織時,我們發現在蟹足腫的皮膚組織中有 p21 以現 p27 的 mRNA 現現,由這結果可以得知控制 G1/S 的控制點的基因在蟹足腫的致病病因扮演重要的角色.
關鍵詞:蟹足腫, G1/S 控制點, p21, p27.

Introduction
keloid is dermal fibroproliferative disorder presumably resulting from an abnormal response to wound healing following trauma, inflammation surgery, or burns. However, spontanous

occurrence of keloid has also been reported. It is clinically defined as scars growing beyond the boundries of original wounds, occurs primarily in dark-skinned races and in genetically susceptible individuals. Though patients suffering from keloids presenting to clinics mainly for cosmetic problems, these lesions are often pruritic and tender.

Despite of numerous studies on the pathogenesis of keloid, the actual mechanism remains inconcludusive. It is well known that uncontrolled cellular proliferation is the hallmarks of malignant tumors as well as other proliferative disorders. The uncontrolled growth is in part due to the alteration of gene expressions, either by overexpression of oncogenes or by down regulations of tumor suppressor genes that allow the cells to evade the checkpoints of the cell cycle.

Over expression of cyclin D1 and cyclin-dependent kinase 4(Cdk4) has been implicated in human maligant tumors. Concurrent overexpression of cyclin D1 and Cdk4 in intestinal adenomas from multiple intestinal neoplasia(Min) mice and human familial adenomatous polyposis patients. Cyclin D1 overexpression vs. retinoblastoma inactivation: implications for growth control evasion in non-small cell and small cell lung cancer.

The p21 $^{WAF1/Cip1}$ and p27 ^{kip1} have been confirmed to be tumor suppressor genes, lost of functions of these gene have been reported in a wide variety of human cancers. Furthermore, p21 $^{WAF1/Cip1}$ knockout mice showed the cells lacking p21 $^{WAF1/Cip1}$ or can not undergo G1 arrest in cells after DNA damage. Mice lacking p21 $^{WAF1/Cip1}$ undergo normal development, but are defective in G1 checkpoint control. Radiation-induced cell cycle arrest compromised by p21 $^{WAF1/Cip1}$ deficiency. By pass of senescence after disruption of p21 $^{WAF1/Cip1}$ gene in normal diploid human fibroblasts. P27 ^{kip1} knockout mice showed increased body size, multiple organ hyperplasia, and pituitary tumors. A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27 ^{kip1}-deficient mice. Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27 ^{kip1}. Mice lacking p27 ^{kip1} display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. The abnormalities of these cyclins, cyclin kinase and cyclin kinase inhibitors all point to the uncontrolled cellular proliferation, a marjor observation found in keloid.

Material and method

1. Sample collection:

   Six patients with typical clinic diagnosed keloid and four healthy control subjects will recruit in this study. An incision biopsy from keloid lesions and normal skin of healthy control subjects will be taken in the OPD. Histological studies will be done after the keloid and normal skin tissue are harvested and fix in 10% formalin and acetic acid. The fixed tissue will be subjected to paraffin embedding, thin section (5mm) cutting and stained with

hematoxklin and eosin. H&E sections will be examined by histopathologists in a blinded fashion to confirm the morphological natural of the keloid versus normal control skin tissue.

2. RNA extraction:

RNA from keloid skin samples or normal control skin samples was extracted using the TriPure isolation reagent (Boehringer mannheim). Briefly, for each 50mg of tissue to be processed. The skin sample was washed two times in 1XPBS. The washed tissue was homogenized by Polytron with 1ml TriPure reagent. Incubate the sample in room temperature for five minutes and add 0.2ml coloroform to the sample. After centrifugation, take the upper aqueous phase for RNA extraction. Precipitate the RNA by ethanol precipitation. After washing once with 70% ethanol the pellet will be air dried for 10 minutes. Total RNA isolated from skin tissue will be subjected to further cDNA synthesis.

3. cDNA synthesis:

cDNA was synthesized using MMLV reverse transcriptase(Amersham). 28μl of total RNA (up to 20μg) will be mixed with 1.6μl of oligo (dT)12-18(0.5μg/μl) and incubated at 65oC for 5 minutes. The mixture will then be incubated on ice for 1 minutes. 10.4 μl of reaction mixture(8μl of MMLV reverse transcriptase, 1.6μl 10mMdNTP, 0.8μl MMLV reverse transcriptase) was added and incubated at 37oC for 60 minutes. The reaction will be terminated at 95oC for 10 minutes. The synthesis cDNA will then be used for PCR amplification.

4.PCR amplification:

PCR was performed with equal amounts of cDNA to amplify messages specific for GADPH, $p16^{INK4}$, $p21^{WAF1/Cip1}$, $p27^{kip1}$, p53, Rb, Cyclin D and Cyclin-dependent kinase 4. Sense and antisense primers for GADPH (sense:GTCCACTGGCGTCTTCACCA-CC; anitsense:AGGCATTGCTGATGATCT-TGAGGC), P16 (sense:CGGGAGG-GCTTCCTGGACAC; antisense:CGGGGATGTCTGAGGGACCTTTC), P21 (sense:CGTGAGCGATGGAACTTCGAC;antisense:CGTTTTCGACCCTGAGAGTCTTC), P27 (sense:TCAAACGTGCGAGTGTCTAACG;antisense:ACGGATC-AGTCTTTGGGTCCAC) ,P53(sense:CTTGCATTCTGGGACAGCCAAGTC;antisense:TCGGATAAGATGCTGAGG AGGGG), Rb(sense:AAAGCTGTTGGGACAGGG-TTGTGTC;antisense:TGGTCCTTCTCGGTCCTT TGATTG) ,Cyclin D(sense:CAA-ATGTGTGCAGAAGGAGCTCC;antisense:AAATCGTGCGGGGTCATTG), Cyclin-dependent kinase 4(sense:GCCGAAACGATCAAGGATCTGAATG;antisense:TCG-CTTGTGT-GGGTTAAA ACTCAGC). Were based on the published sequences of GADPH, $p16^{INK4}$, $p21^{WAF1/Cip1}$, $p27^{kip1}$, p53, Rb, Cyclin D and Cyclin-dependent kinase 4. PCR products were visualized by

electrophoresis on 3% agarose followed by eithidium bromide staining. Size of PCR products were: control GADPH 161 bp, p16 ^{INK4} 208 bp , p21 $^{WAF1/Cip1}$ 290 bp , p27 ^{kip1} 409 bp , p53 250 bp , Rb 453 bp , Cyclin D 345 bp and Cyclin-dependent kinase 4 510 bp.

Result

1. P53, P21 $^{WAF1/Cip1}$ and p27 ^{kip1} mRNA expression in keloid patient's skin tissue:

Two families of CdkI are defined based on the structural homology, cdk targets and mechanism of inhibition. P21 $^{WAF1/Cip1}$ like family of CdkIs includes p27 ^{kip1} and p57 ^{kip2}, which inhibit multiple cyclin-cdk complexes. In our result Fig.1 show that the p53 (1 of 6 patients), p21 $^{WAF1/Cip1}$ (2 of 6 patients) and p27 ^{kip1} (2 of 6 patients) mRNA were expression in keloid skin tissue.

Discussion

Keloids represent a pathological wound-healing response in predisposed individuals. Keloids are characterized grossly as thick scar tissue that has escaped the boundaries of the original wound, producing excess deposition of extracellular matrix molecular such a collagen and fibroblast overgrowth. In this study, we found the p53, p21 and p27 mRNA were expression in the keloid skin tissue, but didn't in the normal skin tissue.

Reference:

1. Ala-Kokko, L., Rintala, A., and Savolainen, E. R. (1987). J Invest Dermatol 89, 238-44.
2. Appleton, I., Brown, N. J., and Willoughby, D. A. (1996). Am J Pathol 149, 1441-7.
3. Babu, M., Diegelmann, R., and Oliver, N. (1989). Mol Cell Biol 9, 1642-50.
4. Babu, M., Diegelmann, R., and Oliver, N. (1992). J Invest Dermatol 99, 650-5.
5. Bernestein, L. J., and Geronemus, R. G. (1997). Arch Dermatol 133, 111-2.
6. Bettinger, D. A., Yager, D. R., Diegelmann, R. F., and Cohen, I. K. (1996). Plast Reconstr Surg 98, 827-33.
7. Borok, T. L., Bray, M., Sinclair, I., Plafker, J., LaBirth, L., and Rollins, C. (1988). Int J Radiat Oncol Biol Phys 15, 865-70.
8. Brown, M. D., Johnson, T., and Swanson, N. A. (1990). J Dermatol Surg Oncol 16, 867-9.
9. Calderon, M., Lawrence, W. T., and Banes, A. J. (1996). J Surg Res 61, 343-7.
10. Craig, S. S., DeBlois, G., and Schwartz, L. B. (1986). Am J Pathol 124, 427-35.
11. Datubo-Brown, D. D. (1990). Br J Plast Surg 43, 70-7.
12. Duncan, M. R., Hasan, A., and Berman, B. (1995). J Invest Dermatol 104, 128-33.
13. Ehrlich, H. P., Desmouliere, A., Diegelmann, R. F., Cohen, I. K., Compton, C. C., Garner, W. L., Kapanci, Y., and Gabbiani, G. (1994). Am J Pathol 145, 105-13.
14. Escarmant, P., Zimmermann, S., Amar, A., Ratoanina, J. L., Moris, A., Azaloux, H., Francois, H., Gosserez, O., Michel, M., and G'Baguidi, R. (1993). Int J Radiat Oncol Biol

Phys 26, 245-51.
15. Estrem, S. A., Domayer, M., Bardach, J., and Cram, A. E. (1987). Laryngoscope 97, 1214-8.
16. Gonzalez-Martinez, R., Marin-Bertolin, S., and Amorrortu-Velayos, J. (1995). Br J Plast Surg 48, 47-8.
17. Granstein, R. D., Rook, A., Flotte, T. J., Haas, A., Gallo, R. L., Jaffe, H. S., and Amento, E. P. (1990). Arch Dermatol 126, 1295-302.
18. Haisa, M., Okochi, H., and Grotendorst, G. R. (1994). J Invest Dermatol 103, 560-3.
19. Harper, R. A. (1989). Cell Biol Int Rep 13, 325-35.
20. Kazeem, A. A. (1988). J Surg Oncol 38, 16-8.
21. Kikuchi, K., Kadono, T., and Takehara, K. (1995). Dermatology 190, 4-8.
22. Kischer, C. W., Sheridan, D., and Pindur, J. (1989). Anat Rec 225, 189-96.
23. Kischer, C. W., Pindur, J., Shetlar, M. R., and Shetlar, C. L. (1989). J Trauma 29, 672-7.
24. Kischer, C. W., Wagner, H. N., Jr., Pindur, J., Holubec, H., Jones, M., Ulreich, J. B., and Scuderi, P. (1989). Connect Tissue Res 23, 279-88.
25. Kischer, C. W. (1992). J Submicrosc Cytol Pathol 24, 281-96.
26. Koch, R. J., Goode, R. L., and Simpson, G. T. (1997). Plast Reconstr Surg 99, 1094-8.
27. Larrabee, W. F., Jr., East, C. A., Jaffe, H. S., Stephenson, C., and Peterson, K. E. (1990). Arch Otolaryngol Head Neck Surg 116, 1159-62.
28. Lawrence, W. T. (1991). Ann Plast Surg 27, 164-78.
29. Lee, K. S., Song, J. Y., and Suh, M. H. (1991). J Dermatol Sci 2, 316-23.
30. Lee, K. S., Jung, J. B., Ro, Y. J., Ryoo, Y. W., Kim, O. B., and Song, J. Y. (1994). J Dermatol Sci 8, 33-7.
31. Lemont, H. (1992). J Am Podiatr Med Assoc 82, 439-41.
32. Lim, T. C., Moochhala, S. M., Tan Walter, T. L., Chhatwal, V. J., Suhumaran, S., Hon, W. M., and Khoo, H. E. (1996). Plast Reconstr Surg 98, 911-2.
33. Ma, S., Chang, W. P., and Fang, R. H. (1996). Plast Reconstr Surg 98, 821-6.
34. Ma, S., Fang, R. H., and Chang, W. P. (1996). Cell Biol Int 20, 289-92.
35. Martin, C. W., and Muir, I. F. (1990). Br J Plast Surg 43, 655-62.
36. Matsuoka, L. Y., Uitto, J., Wortsman, J., Abergel, R. P., and Dietrich, J. (1988). Am J Dermatopathol 10, 505-8.
37. Myles, M. E., Russell, J. D., Trupin, J. S., Smith, J. C., and Russell, S. B. (1992). J Biol Chem 267, 9014-20.
38. Nakajima, H., Kishi, T., Tsuchiya, Y., Yamada, H., and Tajima, S. (1997). Ann Plast Surg 39, 536-41.
39. Nakaoka, H., Miyauchi, S., and Miki, Y. (1995). Acta Derm Venereol 75, 102-4.
40. Nichter, L. S., and Abrams, R. S. (1988). Plast Reconstr Surg 82, 923-4.
41. Nimni, M. E. (1997). Biomaterials 18, 1201-25.

42. Oliver, N., Babu, M., and Diegelmann, R. (1992). J Invest Dermatol 99, 579-86.
43. Panabiere-Castaings, M. H. (1988). J Dermatol Surg Oncol 14, 1275-6.
44. Peltonen, J., Hsiao, L. L., Jaakkola, S., Sollberg, S., Aumailley, M., Timpl, R., Chu, M. L., and Uitto, J. (1991). J Invest Dermatol 97, 240-8.
45. Pinol, G., Rueda, F., Marti, F., Puig, L., and De Moragas, J. M. (1990). Med Cutan Ibero Lat Am 18, 13-7.
46. Placik, O. J., and Lewis, V. L., Jr. (1992). Surg Gynecol Obstet 175, 185-93.
47. Requena, L., Martin, L., Farina, M. C., Pique, E., and Escalonilla, P. (1996). Br J Dermatol 134, 953-7.
48. Russell, S. B., Trupin, K. M., Rodriguez-Eaton, S., Russell, J. D., and Trupin, J. S. (1988). Proc Natl Acad Sci U S A 85, 587-91.
49. Russell, S. B., Trupin, J. S., Myers, J. C., Broquist, A. H., Smith, J. C., Myles, M. E., and Russell, J. D. (1989). J Biol Chem 264, 13730-5.
50. Sammartino, A., Cerbella, R., Lembo, G., Federico, A., and Loffredo, L. (1986). J Fr Ophtalmol 9, 725-9.
51. Shetlar, M. R., Shetlar, C. L., Hendricks, L., and Kischer, C. W. (1985). Proc Soc Exp Biol Med 179, 549-52.
52. Shetlar, M. R., Shetlar, C. L., Kischer, C. W., and Pindur, J. (1991). Connect Tissue Res 26, 23-36.
53. Shirakami, A., Shigekiyo, T., Hirai, Y., Takeichi, T., Kawauchi, S., Saito, S., and Miyoshi, K. (1986). Lancet 1, 473-4.
54. Sible, J. C., Eriksson, E., and Oliver, N. (1996). Gene Expr 5, 269-83.
55. Siraganian, P. A., Rubinstein, J. H., and Miller, R. W. (1989). Med Pediatr Oncol 17, 485-91.
56. Sit, K. H., Lau, Y. K., and Aw, S. E. (1991). J Dermatol 18, 572-9.
57. Sugiura, K., Abe, M., Inasaka, H., Ueda, H., Hirano, K., and Adachi, T. (1986). Nippon Hifuka Gakkai Zasshi 96, 171-4.
58. Suzawa, H., Kikuchi, S., Arai, N., and Koda, A. (1992). Jpn J Pharmacol 60, 91-6.
59. Tan, E. M., and Peltonen, J. (1991). Biochem J 278, 863-9.
60. Tan, E. M., Hoffren, J., Rouda, S., Greenbaum, S., Fox, J. W. t., Moore, J. H., Jr., and Dodge, G. R. (1993). Exp Cell Res 209, 200-7.
61. Tan, E. M., Rouda, S., Greenbaum, S. S., Moore, J. H., Jr., Fox, J. W. t., and Sollberg, S. (1993). Am J Pathol 142, 463-70.
62. Tredget, E. E., Nedelec, B., Scott, P. G., and Ghahary, A. (1997). Surg Clin North Am 77, 701-30.
63. Wakelin, S. H., and Marren, P. (1996). Ann R Coll Surg Engl 78, 558.
64. Waki, E. Y., Crumley, R. L., and Jakowatz, J. G. (1991). Arch Otolaryngol Head Neck Surg 117, 1177-81.

65. Weiner, M. J., and Albert, D. M. (1989). Acta Ophthalmol Suppl 192, 188-96.
66. Wong, T. W., Chiu, H. C., and Yip, K. M. (1994). Br J Dermatol 130, 683-5.
67. Yamada, H., Tajima, S., and Nishikawa, T. (1995). J Dermatol Sci 9, 45-7.
68. Younai, S., Nichter, L. S., Wellisz, T., Reinisch, J., Nimni, M. E., and Tuan, T. L. (1994). Ann Plast Surg 33, 148-51.