行政院國家科學委員會專題研究計畫　成果報告

探討基底膜調節胰島素訊息傳遞的機轉(3/3)

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計畫編號：NSC92-2320-B-040-040-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：中山醫學大學免疫學研究所

計畫主持人：李宜儒

計畫參與人員：吳東逸

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中　華　民　國 93 年 10 月 28 日

行政院國家科學委員會補助專題研究計畫成果報告

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※ Investigation into the mechanisms for basement ※
※ membrane-dependent modulation of insulin signaling ※
※ 探討基底膜調節胰島素訊息傳遞的機轉 ※
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計畫主持人：李宜儒
共同主持人：

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□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

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基底膜調節胰島素訊息傳遞的機轉

Investigation into the mechanisms for basement membrane-dependent modulation of insulin signaling

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一、中文摘要

細胞外間質可經由影響生長因子的訊息傳遞而改變細胞的生理反應。以前的結果顯示若將乳腺上皮細胞培養在類似基底膜的間質上，胰島素的訊息傳遞則較強，尤其是這些在胰島素受體以下的訊號。本計劃主要在探討細胞外間質調控胰島素訊息傳遞的機轉。先前的結果顯示當細胞培養在塑膠盤上時，β1 integrin、paxillin、p130cas 和 c-Src 的表現量較高，且 FAK、paxillin 和 p130cas 酪胺酸磷酸化的程度也較強。在此，我們更進一步證實細胞因接觸不同的基質而導致 paxillin、p130cas 和 c-Src 的表現量不同的情形是在細胞貼附後不久後即發生，且是經由調控其蛋白質降解所造成。Calpains 是最可能參與此反應之蛋白水解胸，它曾被證實與細胞的展開與移動有關。相反地，β1 integrin 的表現量則是調控在基因轉錄的階段。因此，細胞採用不同的機制以調節其與週遭環境的互動。

關鍵詞：基底膜、胰島素、integrin、微矩陣分析

Abstract

Extracellular matrix (ECM) influences cellular responses by affecting growth factor-activated signaling. Our previous work has shown that mammary cell adhesion to basement membrane (BM) confers better signal propagation downstream of insulin receptor. Investigation into the mechanisms for BM-dependent modulation of insulin signaling was the major focus of this study. We have found that cells cultured on plastic led to enhanced expression of β1 integrin, paxillin and p130cas and c-Src, as well as elevated levels of tyrosine phosphorylation of FAK, paxillin, p130cas. Here we further demonstrated that this differential expression of paxillin, p130cas and c-Src in response to different substrata occurs shortly after their adhesion and is regulated by a mechanism involving protein turnover. The likely proteases responsible for their degradation are calpains which have been shown to affect the cell spreading and migration. By contrast, expression of β1 integrin is controlled at the transcriptional level. Thus, cells adopt different mechanisms to control their interaction with the surrounding ECM. Expression of certain cell surface receptor is modulated at the transcriptional level, whereas levels of the downstream signal/cytoskeletal molecules are regulated at the posttranscriptional level.

Keywords: basement membrane, insulin, integrin, microarray analysis

二、緣由與目的

Cells in multicellular organisms are in contact with an intricate meshwork of extracellular macromolecules composed largely of glycoproteins and proteoglycans. This meshwork is collectively known as the extracellular matrix (ECM). Basement membrane (BM) is one type of ECM; it is a dense, sheet-like structure that is mainly made up of laminin, collagen IV, nidogen and proteoglycans. ECM provides a mechanical framework for tissue architecture with supportive, adhesive and barrier functions. Moreover, it affects cell proliferation, differentiation, migration and

survival by interacting with cell surface receptors. One well-studied receptor is integrin, which are transmembrane heterodimers composed of α and β subunits. Adhesive interactions between the ECM and integrin result in occupancy and clustering of integrins, leading to cytoskeleton reorganization and signal transduction. Numerous proteins have been shown to target to focal contact regions, including structural proteins (tesin, talin, vinculin, α-actinin, actin, etc.) and signaling molecules (FAK, c-Src, PI3K, paxillin, Grb2, p130cas, etc). Accompanied with these events is the activation of the Rho family proteins (Cdc42, Rac and Rho) and several downstream signals such as Erk and JNK. Thus, cell adhesion to ECM promotes actin filament reorganization and initiates cascades of signals, leading to a wide spectrum of cell behavior and gene expression (1).

Mammary epithelial cells reside on basement membrane (BM) *in vivo*. Expression of the milk protein β-casein, the hallmark of mammary cell differentiation, requires the presence of lactogenic hormones (prolactin, insulin and hydrocortisone) and BM (2, 3). Moreover, both insulin and BM confers mammary cell survival (4). One explanation for duel requirement of growth factor and BM for mammary functions is that cell adhesion to BM renders these cells full responsiveness to growth factors (5). Indeed, we found that insulin signaling propagates better in cells cultured on BM than in those cultured on collagen I or tissue culture plastic (6). Although tyrosine phosphorylation of insulin receptor (IR) takes place irrespective to substrata, greater extents of tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and its association with PI3K as well as PKB activation occur in cells cultured on BM (4, 6). In the absence of BM, insulin-induced the recruitment of IRS-1 to the receptor was impaired. Moreover, higher extent of PTP is associated with the receptor. These might explain why insulin signaling is disrupted in mammary cells cultured on plastic.

The crosstalk between ECM- and growth factor-triggered signaling has been previously demonstrated to lead to the ultimate cellular responses (1). Several lines of evidence have shown that ECM regulates insulin/IGF-I signaling at different levels. It affects IR endocytosis, IRS-1 expression and tyrosine phosphorylation of signaling molecules, thereby altering the intensity or duration of signaling. Regarding receptor endocytosis, IR internalization has been shown to be decreased in cells adhered on galectin-8 compared with those cultured on fibronectin, collagen or laminin, and this difference is probably due to the distinct organization of actin filaments in these cells (7). IRS-1 expression is also modulated by cell adhesion. It occurs at the transcriptional level and is primarily mediated by FAK activation (8). A number of mechanisms have been shown to be responsible for ECM-mediated regulation of insulin signaling at the level of protein tyrosine phosphorylation. One mechanism involves the association of IR with integrin whereby cell adhesion-triggered integrin oligomerization could consequently induce the aggregation and activation of IR. Various integrins have been identified to be associated with IR or IGF-I receptor (IGF-IR). In response to insulin, IR co-immunoprecipitates with αvβ3 integrin in Rat-1 fibroblast cells (9, 10). Crosstalk between α5β1 integrin and IR signaling pathways has also been documented in Chinese hamster ovary cells overexpressing IR (11, 12). A recent study shows that interactions between tyrosine phosphorylated IGF-IR and α6 integrin occurs in developing lens, further demonstrating the compartmentalization and collaboration of integrin and insulin/IGF-I signaling pathways (13). The other mechanism for ECM-provoked control of insulin signaling is mediated by the tyrosine kinases downstream of integrins. It has been shown that FAK directly associates with IRS-1, and activation of FAK and c-Src restores insulin-induced tyrosine phosphorylation of IR and IRS-1 in suspended cells (14-17). In addition to promoting tyrosine phosphorylation, ECM plays a role in preventing the

dephosphorylation of signaling molecules. In smooth muscle cells, αvβ3 integrin is responsible for prolonging tyrosine phosphorylation of IGF-IR through a control on the rate of recruitment of SHP-2 to the activated IGF-IR (18).

We have examined the signaling pathways triggered by cell adhesion to plastic and BM, and found that cells cultured on plastic exhibit higher levels of p130cas, paxillin and β1 integrin. Here we further explore the regulatory mechanisms for it. Moreover, a microarray analysis was performed to compare the gene expression profiles in cells cultured on different substrata in order to identify the critical proteins involved in BM-regulated insulin signaling.

三、結果與討論

Differential expression of paxillin, p130cas and c-Src in response to different substrata occurs shortly after their adhesion. Due to the critical role of ECM in the regulation of insulin signaling, we were especially interested in the delineation of signaling pathways elicited by cell adhesion on different substrata. We started with the examination of the expression of a number of molecules involved in cell adhesion-activated signaling and found that except for FAK whose expression was not influenced by the substrata that cell resided on, expression levels of β1 integrin, paxillin, p130cas and c-Src were elevated in cells cultured on plastic. Moreover, tyrosine phosphorylation of FAK, paxillin and p130cas were higher in cells cultured on plastic.

A long-term culture of mammary epithelial cells has been used in this study whereby cells had established stable cell-matrix interactions over several days, thus mimicking the types of interactions they would experience *in vivo*. Here we performed a short-term culture in order to find out how soon the differential expression of paxillin, p130cas and c-Src in response to different substrata could be observed. Primary mouse mammary epithelial cells cultured on collagen I were trypsinized and replated on either collagen I- or BM-coated dishes for varying periods of time. Cell lysates were then collected and subjected to immunoblotting. Levels of paxillin, p130cas and c-Src in cells cultured on collagen I remained fairly constant throughout the time course of examination, whereas those in cells cultured on BM began to drop 4-8 h after initial plating (Fig.1). Thus, cells alter their protein expression to adjust their immediate environment. Regarding these proteins involved in the formation of focal contact, it does not take too long to make such changes.

Differential regulation of the expression of paxillin, p130cas and c-Src by ECM does not occur at the transcriptional level. In order to know whether altering the expression of these molecules is controlled at the transcriptional level, RT-PCR was performed. Levels of FAK, paxillin and p130cas mRNA were indiscernible in cells cultured on plastic, collagen I and BM (Fig. 2A), suggesting that ECM-mediated regulation of these molecules takes place at the post-transcriptional level. By contrast, expression of β1 integrin mRNA is higher in cells cultured on plastic and collagen I (Fig. 2B), which is in consistent with other observation (19). We have also examined other receptors for BM, α3 integrin, α6 integrin and dystroglycan, and found that their mRNA expression is comparable irrespective of the substrata (Fig. 2B). Another critical cell surface receptor involved in interaction with BM is β4 integrin which dimerizes with α6 integrin to form hemidesmosome. Examination its expression under different culture conditions is undergoing now.

Turnover of paxillin, p130cas and c-Src is more rapidly in cells cultured on BM. Based on the observation that the expression of paxillin, p130cas and c-Src was not regulated at the transcriptional level, we then examined their turnover in cells cultured on different substrata. Mammary cells cultured on plastic and BM were treated with cycloheximide and harvested after varying periods of time. As shown in

Figure 3, levels of these proteins decreased more rapidly in cells cultured on BM, suggesting that some proteases might be involved in the degradation of these proteins.

Calpains are a large family of intracellular proteases which have been implicated in enabling cells spreading by modifying adhesion sites and in promoting cell migration by facilitating rear-end detachment (20). Thus, the possibility that it is responsible for the degradation of paxillin, p130cas and c-Src in mammary cells cultured on BM was examined. Inclusion of ALLN, a calpain inhibitor, in cultures resulted in an increase of expression of these proteins, although its effectiveness was varying among different proteins (Fig.4). Similar results were obtained when another calpain inhibitor calpeptin was used (data not shown). The involvement of other proteases such as proteasome, caspases and MMP were also examined, but no obvious effect was observed (data not shown). Taken together, expression of paxillin, p130cas and c-Src in response to different substrata is, at least in part, controlled by protein turnover, although the possibility that translational control may have a role in it is not excluded.

Thus, cells adopt different mechanisms to control their interaction with the surrounding ECM. Expression of certain cell surface receptor is modulated at the transcriptional level, whereas levels of the downstream signal/cytoskeletal molecules are regulated at the posttranscriptional level. It is not clear why cells develop different strategies to cope with the alteration of environment. Since cell surface receptors are the molecule directly interacting with the ECM and this engagement then triggers the subsequent formation of focal contact, cut-down their expression by a transcriptional mechanism is probably more efficient and economic.

In this study, cells cultured on plastic seemed to behave similarly to those cultured on collagen I. Since most experiments were conducted in a long-term culture, we believe that cells cultured on plastic synthesize and deposit matrices on dishes. This speculation is supported by our results of microarray analysis in that expression of fibronectin mRNA is greatly upregulated in cells cultured on plastic (Table 1). We wonder whether the differential regulation of paxillin, p130cas and c-Src in mammary cells is due to the types of ECM or the physical nature of the matrix that cells are interacting with. To be noted that the most dramatic feature between cells cultured on plastic/collagen I and those cultured on BM is their morphology. The former forms monolayer, while the latter adopts an alveolar shape. Dimension (2D vs 3D) or rigidity or the matrix probably account for this difference. Indeed, a recent paper studying the rigidity of matrix has demonstrated that cells cultured on malleable collagen I gel have decreased expression of the protein involved in the formation of focal contact (21). We are currently investigating into this mechanism in our system.

Gene expression profile in mammary cells cultured on plastic and BM. Our previous data have shown that the intensity of insulin-stimulated IRS-1 tyrosine phosphorylation increases as the time of cell adhesion to BM persists. Maximal induction is reached when cells are in contact with BM for one to two days. Since two days is such a long period, we suspect that *de novo* protein synthesis is required for optimal insulin signaling.

In order to unravel the gene products involved in promotion of insulin signaling, DNA microarray analysis was performed. Primary mammary cells cultured on plastic or BM for 4 days were harvested for total RNA, and then subjected to microarray analysis using Agilent mouse oligo chips. Around four hundread genes were found to be upregulated in cells cultured on BM, including caseins, prolactin receptor, and proteins involved in redox reactions (Table 1). Fewer genes were upregulated in cells cultured on plastic. Among them the most notable ones are MMPs and some matrix proteins such as fibronectin and tenascin C (Table 1). Protein tyrosine kinases or phosphatases that have been reported to be involved in modulation of insulin signaling

were not revealed by this analysis, neither were the known regulatory proteins controlling the recruitment of IRS-1 to IR/IGF-IR. This could be due to that the time chosen for harvesting cells is not adequate, or the proteins truly responsible for regulation of insulin signaling in mammary cells are not those previously reported. However, the results of microarray analysis are consistent with our RT-PCR data whereby regulation of paxillin, p130cas and c-Src in response to different substrata does not occur at the transcriptional level. Moreover, our previous observation that MMP-9 is upregulated in cells cultured on plastic is also confirmed by this analysis.

四、計畫成果自評

We have accomplished almost 90% of the experiments proposed in this project. Preparation of two manuscripts are undergoing at the moment.

五、參考文獻

1. Aplin, A. E., Howe, A., Alhari, S. K., and Juliano, R. L. (1998) *Pharmacol. Rev.* **50**, 197-263
2. Streuli, C. H., Bailey, N., and Bissell, M. J. (1991) *J. Cell Biol.* **115**, 1383-1395
3. Aggeler J. Ward J. Blackie LM. Barcellos-Hoff MH. Streuli CH. Bissell MJ. (1991) *J. Cell Sci.* **99**, 407-417
4. Farrelly, N., Lee, Y.-J., Oliver, J., Dive, C., and Streuli, C. H. (1999) *J. Cell Biol.* **144**, 1337-1348
5. Edwards, G. M., Wilford, F. H., Liu, X., Hennighausen, L., Djiane, J., and Streuli, C. H. (1998) *J. Biol. Chem.* **273**, 9495-9500
6. Lee, Y.-J. and Streuli, C. H. (1999) *J. Biol. Chem.* **274**, 22401-22408
7. Boura-Halfon, S., Voliovitch, H., Feinstein, R., Paz, K. and Zick, Y. (2003) *J. Biol. Chem.* **278**, 16397-16404
8. Yujiri, T., Nawata, R., Takahashi, T., Sato, Y., Tanizawa, Y., Kitamura, T. and Oka, Y. (2003) *J. Biol. Chem.* **278**, 3846-3851.
9. Vuori, K., and Ruoslahti, E. (1994) *Science* **266**, 1576-1578
10. Schneller, M., Vuori, K., and Ruoslahti, E. (1997) *EMBO J.* **16**, 5600-5607
11. Guilherme, A., and Czech, M. P. (1998) *J. Biol. Chem.* **273**, 33119-33122
12. Guilherme, A., Torres, K., and Czech, M. P. (1998) *J. Biol. Chem.* **273**, 22899-22903
13. Walker, J. L., Zhang, L., Zhou, J., Woolkalis, M. J. and Menko A. S. (2002) *Dev. Dyn.* **223**, 273-284
14. Lebrun, P., Mothe-Satney, I., Delahaye, L., Van Obberghen, M., and Baron, V. (1998) *J. Biol. Chem.* **273**, 32244-32253
15. Arbet-Engels, C., Janknecht, R. and Eckhart, W. (1999) *FEBS Letters* **454**, 252-256
16. El Annabi, S., Gautier, N. and Baron, V. (2001) *FEBS Letters* **507**, 247-252
17. Zheng, B. and Clemmons, D. R. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 112117-112122
18. Maile L. A. and Clemmons, D. R. (2002) *Endocrinology* **143**, 4259-4264
19. Delcommenne, M. and Streuli, C. H. (1995) *J. Biol. Chem.* 270, 26794-26801
20. Glading, A., Lauffenburger, D. A. and Wells, A. (2002) *Trends Cell Biol.* **12**, 46-54
21. Wang, Y. K., Wang, Y. H., Wang, C. Z., Sung, J. M., Chiu, W. T., Lin, S. H., Chang, Y. H. and Tang, M. J. (2003) *J. Biol. Chem.* **278**, 21886-21892

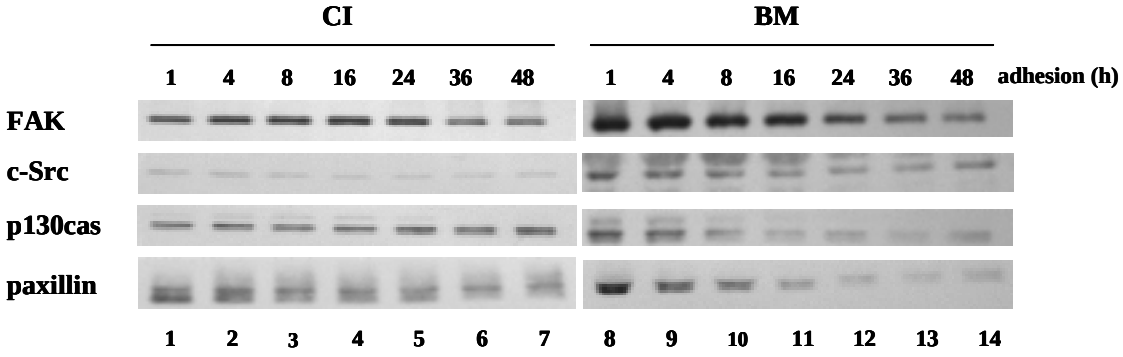

Figure 1. Kinetic analysis of the effect of cell adhesion to ECM on the expression of FAK, c-Src, p130cas and paxillin. Primary mammary cells were trypsinized and plated on collagen I (CI) and BM for 1, 4, 8, 16, 24, 36 and 48 h. Total cell lysates were subjected to immunoblotting and the blots were probed with antibodies to FAK, c-Src, p130cas and paxillin.

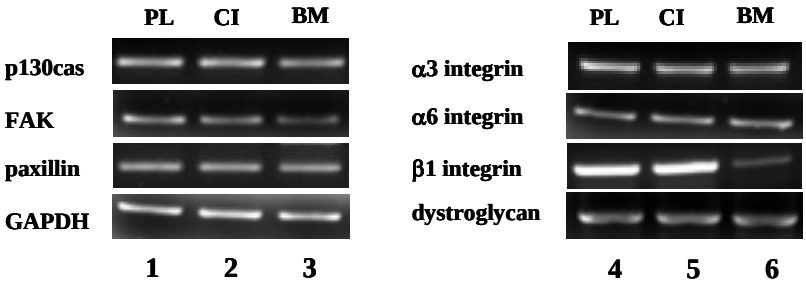

Figure 2. mRNA expression of the protein molecules involved in formation of focal contact in response to different substrata. Primary mammary cells were cultured on plastic (PL), collagen I (CI) and BM, and total RNA were purified and subjected to RT-PCR analysis.

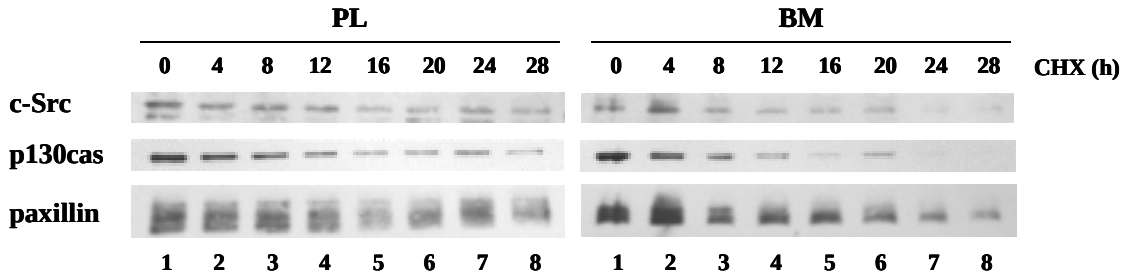

Figure 3. Turnover of c-Src, p130cas and paxillin in cells cultured on plastic and BM. Primary mammary cells cultured on plastic (PL) and BM were treated with 10 μg/ml cycloheximide for 0-28 h, and total cell lysates were then collected for immunoblotting.

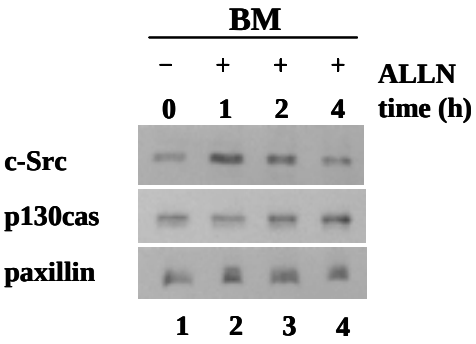

Figure 4. Effect of ALLN on the expression of c-Src, p130cas and paxillin in cells cultured on BM. Primary mammary cells cultured on BM were treated with 10 mM ALLNfor 0-4 h, and total cell lysates were then collected for immunoblotting analysis.

Table 1. Microarray analysis of gene expression in cells cultured on plastic and BM

gene	**fold change** (BM versus plastic)	**gene**	**fold change** (plastic versus BM)
cytochromeP450, 2f2	53.54	MMP10	10.00
beta-casein	37.85	MMP12	7.69
keppa-casein	26.31	fibronectin	7.69
Lactoferrin	15.94	MMP9	5.88
Integrin alpha 8	6.34	tenascin C	4.76
MMP-3	5.89	integrin beta 6	3.23
prolactin receptor	5.24	integrin alpha 2	3.13
nidigen 2	4.61	IGFBP2	3.03
procollagen, type IV alpha 6	3.85	syndecan 1	2.70