

行政院國家科學委員會專題研究計畫成果報告

以抗體阻斷纖維芽細胞生長激素受體活化之研究

Antibody blocking of fibroblast growth factor receptor 3

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中文摘要

纖維芽細胞生長激素 (fibroblast growth factor, FGF) 及其受體是生物體中很重要得的一套訊息傳遞系統，然而纖維芽細胞生長激素受體 (FGFR) 和人類遺傳疾病之關係，直到最近才被發現。由基因連鎖分析建立基因圖譜後，發現軟骨形成不全侏儒症 (achondroplasia)，一種常見顯性遺傳侏儒，在圖譜上的位置和 FGFR3 基因相同。後來果然找到了軟骨形成不全侏儒症的突變點，在台灣我們也證實了軟骨形成不全侏儒症，Apert，Cruzon，hypochondroplasia, thanatophoric dysplasia 等骨骼疾病都和這一類受體 (FGFR1, FGFR2, and FGFR3) 有關。

這些疾病多半是顯性遺傳，軟骨形成不全侏儒的突變後來竟然被證實是激發性的。因此我們計畫以抗體去阻斷受體的激發，以期達到治療的酵素。我們首先自 Dr. Hayman 處得到了 FGFR3 的 cDNA (Keegan 1991)。我們接著把 cDNA 中決定細胞外受體的一段，放入 pRSET 載體中，並以表現出的蛋白質，注射兔子得到了 FGFR3 抗體。這個抗體可以在人類皮膚纖維芽細胞及一些細胞株的 Western blot 分析中，偵測到約 130kDa 的 FGFR3 蛋白。然而這個抗體無法在免疫沉澱實驗中抓下 FGFR3 蛋白。由於免疫沉澱實驗是研究細胞表面受體表現、功能及活化不可缺少的工具，因此後續的實驗就無法進行。

我們試圖將 pRSET 表現出的 FGFR3 蛋白質，注射小鼠製造單株抗體。我們可以挑選到不少可以和 FGFR3 蛋白質作用的抗體株，但是其效價在培養的過程中很快的降低。我們試圖表現其他位置的 FGFR3 蛋白質來製造抗體，FGFR3 蛋白質細胞外的部分表現量都很低，細胞內部分則順利表現

出來。利用這個蛋白，我們製造了一個新的抗體，不幸的，這個抗體在 Western blot 分析中，偵測到不只一個蛋白質，所以也沒有幫助。

本實驗的困難應該是因為 FGFR3 是一個細胞膜蛋白質，而且有大量糖化 (glycosylation) 的現象。這樣的蛋白質由細菌表現較難，所製造出之抗體也未必能有效偵測細胞膜蛋白質，尤其是做免疫沉澱實驗時。用 FGFR3 細胞內的部分做抗體就簡單得多，可是 tyrosine kinase domain 相似性太高，抗體的特異性就不夠了。

關鍵詞：纖維芽細胞生長激素受體，軟骨形成不全侏儒，抗體

Abstract

This Fibroblast growth factors and their receptors are an important set of signal transduction system in animals. the association between this system and human diseases was understood recently. Achondroplasia, a common type of dominantly inherited dwarfism, was found to be linked to FGFR3, and patients were found to have the FGFR3 mutations. Initiated by this finding, the FGF receptors (FGFR1, FGFR2 & FGFR3) was found to be related to several human bone dysplastic diseases including hypochondroplasia, thanatophoric dysplasia, Apert and Cruzon syndromes.

The mutations causing achondroplasia and thanatophoric dysplasia were stimulating mutations. Therefore, it is possible to modify or to block the activation of the mutated receptors by antibody or other methods. We got FGFR3 cDNA from Dr. Hayman. We cloned a portion of the cDNA responsive for the extracellular domain of the receptor into

pRSET vector. The expressed recombinant protein was used to immunized rabbit for FGFR3 antibody. This antibody detected a 130 kDa protein in both human skin fibroblasts lysate and in many cell lines by western blot analysis. However, this antibody was unable to capture FGFR3 protein in immunoprecipitation (IP) experiment. Since IP is the indispensable tools in the study of membranous receptors, either for the expression, function or activation. We tried to raise monoclonal antibody with this pRSET protein. We got some clones at the initial stage of screening, but the potencies of these clones dropped rapidly during continuing culture.

In order to solve the problem, we tried to express other fragments of FGFR3. It is pretty difficult to express any of the extra-membranous domains of FGFR3, but the intracellular domain was smoothly expressed. With the expressed intracellular domain, a second antibody was raised. However, this antibody detected more than one protein in western blot analysis.

The difficulty in this study may arise from the character of FGFR3. It is a membranous receptor with intense glycosylation. E coli expressed protein may not reflect the structure of FGFR3, and thus difficult to capture the receptor in IP study. The intracellular domain, the tyrosine kinase domain, in the way is homologous to other tyrosine kinase proteins. However, we got a lot of experience in this study which will help us in future studies.

Keywords: Fibroblast growth factor receptor 3 (FGFR3), achondroplasia, antibody

Introduction

Fibroblast growth factors (FGFs) comprise a family of polypeptide ligands which mediate biological responses in many kind differentiated cells (Burgess 1989). FGFs can serve as chemoattractants for vascular endothelial cells and as survival

factors for postmitotic neurons; as promoters or inhibitors of cellular differentiation; and stimulate proliferation of a wide range of ectoderm- and mesoderm-derived cell types.

FRF responses are triggered by the activation of FGF receptors (FGFRs). FGFRs are a family of structurally related transmembrane tyrosine kinases. There are at least 9 fibroblast growth factor (FGFs) and 4 FGFRs with similar molecular anatomy and closely related structural elements.

Human FGFR3 was isolated by Keegan et al. (Keegan 1991). FGFR3 contains three immunoglobulin domains with cycteine residues, a transmembrane domain and a intracellular kinase domain. The Ig III domain is encoded by two separated exons: exons IIIa encodes the N-terminal half, and the C-terminal half is encoded by either exon IIIb or IIIc. Human FGFR3 IIIb, which binds only aFGF, is more prevalent than FGFR3 IIIc in most organs except brain (Chellaiah 1994).

However, the association between FGFRs and human diseases was known much later than the receptors being found. The relationship between achondroplasia, a common type of dominantly inherited drawfism, and FGFR3 was established by linkage study (Velinov 1994, Le Merrer 1994). Soon after the disease gene being located at the same location as FGFR3, FGFR3 G380R mutation was found in most of the patients (Shiang et al. 1994; Rousseau et al. 1994, Wang 1996). Initiated by this finding, the FGF receptors (FGFR1, FGFR2 & FGFR3) was found to be related to human bone dysplastic diseases including hypochondroplasia, thanatophoric dysplasia, Apert and Crouzon syndromes (Muenke 1995).

High level of FGFR3 expression was found in the cartilage rudiments of developing bone, and during endochondral ossification FGFR3 was expressed exclusively in resting cartilage (Peters 1992), thus proved biochemically that FGFR3 is the

gene for achondroplasia. The mutations were initially thought to knock out the receptors through a "dominant negative" effect, because most of the diseases are dominantly inherited. However, pretty unexpected, later study indicated that FGFR3 is a negative regulator of bone growth, and mutations causing achondroplasia and thanatophoric dysplasia actually constitutively activate the receptor. In the paper of Deng et al., severe and progressive bone dysplasia with enhanced and prolonged endochondral bone growth was identified in mice with disrupted FGFR3 (Deng 1996). This growth is accompanied by expansion of proliferating and hypertrophic chondrocytes within the cartilaginous growth plate. Similar results was reported by Colvin et al. Contrasts between the skeletal phenotype and achondroplasia suggest that activation of FGFR3 causes achondroplasia (Colvin 1996).

The work of Webster and Donoghue gave more direct evidence (Webster 1996). By substituting the transmembrane domain of the Neu receptor tyrosine kinase with the transmembrane domains of wild-type and mutant FGFR3, the Arg380 mutation in FGFR3 is shown to activate both the kinase and transforming activities of this chimeric receptor. Compared with achondroplasia, thanatophoric dysplasia, a disease characterized by neonatal lethality and profound dwarfism, is caused by FGFR3 mutations (R284C, K650E) more strongly activating the receptor (Naski 1996). Therefore, graded activation of FGFR3 induces diseases increasing in severity.

Achondroplasia is also commonly seen in Chinese (Hwu and Wang 1991). Patients with achondroplasia are extremely short, and complicated by progressive bone deformity and cervical and lumbar spinal cord compressions after childhood. Therefore achondroplasia, and other related skeletal disorders, certainly need an active treatment. In this proposal, antibody is used as an

approach to the treatment of genetic disease involving FGFRs.

Materials and Methods:

The major work of this study is the establishment of an assay system for FGFR3 phosphorylation. This includes western blot analysis and immunoprecipitation using rabbit antiserum or monoclonal antibody.

FGFR3 full length cDNA

FGFR3 full length cDNA (clone 17B) was obtained from Dr. Hayman (Keegan 1991). It is a pBluescript vector containing a 4kb insert. When expressed in COS cells, this clone gave a 125 kDa transmembrane glycoprotein. If N-linked glycosylation inhibitor tunicamycin were added, then a 97-kDa protein could be seen (Keegan 1991). The whole insert was then cloned into an eukaryotic cell expression vector pSVneo.1 which contains SV40 early promoter, SV40 splicing and poly A site (pSV-FGFR3). The direction of insert was checked by Xho I cutting. For recombinant protein expression in E coli, a 500 bp Pst I fragment of the cDNA was cloned into pRSET-C vector (pRSETC-FGFR3). Pst I cut at nt 324 and 807, thus this DNA encompasses part of the Ig I domain and the whole Ig II domain. Because pRSET vector (Invitrogen) contains a histidine tag, the expressed DNA could be purified by Nickel column (according to the operation manual).

Antiserum production

pRSETC-FGFR3 will be transformed into DE3 cells. Protein expression will be induced with IPTG. The expression protein, checked by SDS-PAGE first, will be purified by nickel column. Immunization will be first performed in rabbit. Around 500µg of protein with complete Freund adjuvant will be used to immunize rabbit by subcutaneous injection

over several sites on the back. Boostering will be done every 4 weeks with 500µg of protein and incomplete Freund adjuvant. Monoclonal antibody

Immunization will be performed with 200µg of protein and complete Freund adjuvant intraperitoneally in BALB/c mice, 6 w/o, female. 50µg of protein with incomplete Freund adjuvant will be used for booster at 3th week and every 2 week thereafter. Mice tail blood will be checked for the presence of antibody by ELISA. Spleen cells will be fused with myeloma cells. Myeloma cell will be split in 10% serum at a concentration of 5×10^5 cells/ml. 10 ml of the overnight culture will be diluted with 10 ml of medium supplemented with 20% FBS and 2x OPI. After sacrifice the mouse, tease apart the spleen and collect spleen cell suspension. Before fusion, wash the splenocytes in medium without serum. To the cell pellet, add 1ml 50% PEG solution with continuous stirring, then dilute the mixture with 10 ml of serum-free medium. The cells will be plated into 96-well plate, and positive clones will be selected by ELISA.

Western blot analysis

Cells will be extracted by RIPA lysis buffer. In the vial that contains cell pellet from 10 cm dish, add 100 - 300 µl of RIPA buffer, mix gently till cells were lysed completely. Put on ice for 30 mins, then centrifugate at 12,000 rpm for 30 mins at 4 °C. Carefully transfer the cell lysate to a new vial, determinate the proteins concentration by a Bio-Rad kit.

Samples were run on a 10 % SDS polyacryamide gel. Transfer proteins onto a NC membrane by a semi-dry transfer unit (Hofer) in Towbin buffer. Development will be performed using an ECL system.

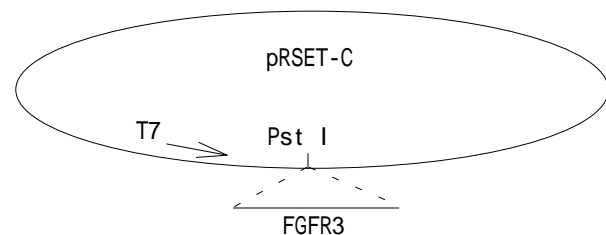
Immunoprecipitation:

Cells will be rinsed with cold PBS and lysed in lysis buffer (50mM HEPES, pH7.5, containing 1% Triton X-100, 150mM NaCl, 100µM sodium orthovanadate (NaVO_4), 1mM phenyl-methyl-sulfonyl fluoride (PMSF), 10µM/ml leupeptin, and 4µg/ml aprotinin). Lysates will be clarified by centrifugation for 10min at 13,000 x g and incubated with antibodies for 4 hr. Complexes of antigens and antibodies will be precipitated with protein A-Sepharose beads (Pharmacia). To analyze antigens in immunoprecipitates, samples will be resolved by SDS-PAGE and subsequently transferred to a nitrocellulose membrane. Antigens are then probed with anti-receptor or anti-phosphotyrosine antibody followed by second antibodies conjugated with Horseradish peroxidases and visualized by enhanced chemiluminescence (Amersham) (Zhan 1994).

Results and Discussion

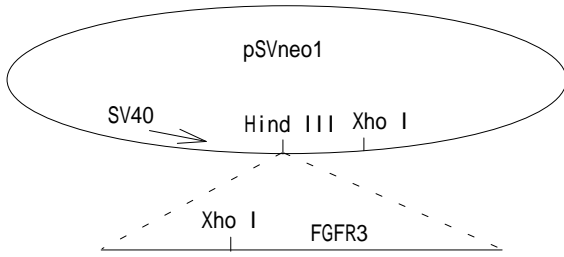
Cloning of pRSET expression vector

Map of pRSETC-FGFR3



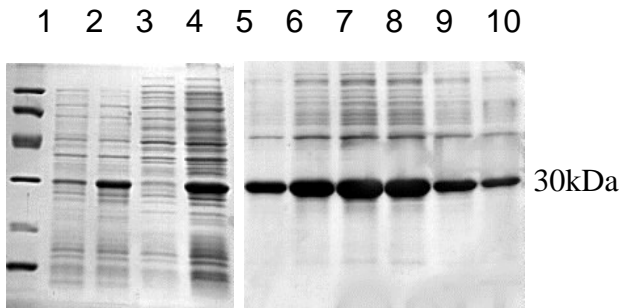
Cloning of eukaryotic expression vector

Map of pSV-FGFR3

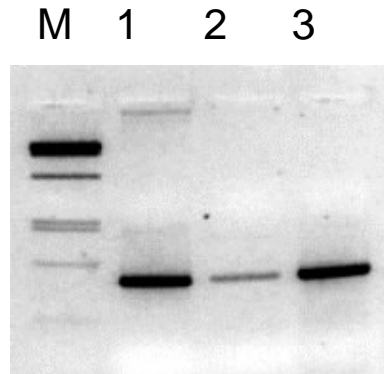


Western blot analysis of FGFR3 protein. Twenty μ g of protein was loaded on each lane. Western blot was performed with FGFR3 antibody. Lane 1: BHK cell protein. Lanes 2 to 5: 3T3 cell protein. Lanes 2 and 3: non-transfected. Lanes 4 and 5: transfected with pSV-FGFR3. lane 6: humanskin fibroblast protein. Expression in BHK cells

The purified recombinant protein

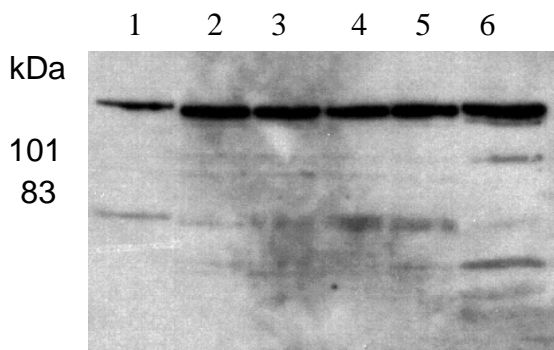


Expression of recombinant FGFR3 in E. Coli
 1: uninduced 2: induced
 3: supernatant (soluble)
 4: pellet (inclusion body)
 5-10: Fractions from nickel column



Expression of pSV-FGFR3 in BHK cells: results of RT-PCR. RNA was extracted from permanent transfectants selected by G418. PCR was performed by human FGFR3 primers FGF#5 (5'-GATGCTGTGGAGCTGAGCTGT) and FGF#6 (5'-CACAGTACGCGCTGCGTGA). Lane 1: PCR product using cDNA as template. Lane 2 & 3: two clones of transfectant.

Western blot analysis



Immunoprecipitation

This experiment failed after a lot of effort, so this project could not be continued.

計畫成果自評

本實驗的困難應該是因為 FGFR3 是一個細胞膜蛋白質，而且有大量糖醣化

(glycosylation)的現象。這樣的蛋白質由細菌表現較難，所製造出之抗體也未必能有效偵測細胞膜蛋白質，尤其是做免疫沉澱實驗時。用 FGFR3 細胞內的部分做抗體就簡單得多，可是 tyrosine kinase domain 相似性太高，抗體的特異性就不夠了。然而經由這個計畫的經驗，我們對於蛋白質分析的知識和能力都大大的增強。由於下一個世紀將是蛋白質的世紀，這個計畫的執行將有助於我們日後的研究工作。

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