行政院國家科學委員會補助專題研究計畫成果報告

計畫名稱: TEL-JAK2 **致癌基因在血癌生成機轉的訊息傳遞路**

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中文摘要

JAK/STAT 在血液細胞的訊息傳遞路徑中扮演著非常重要的角色。藉由 JAK/STAT 使 cytokine 的訊息能藉由刺激細胞膜上的 receptor 一步步地傳達到細胞核內以啟動基因的表達。此路徑傳遞血液細胞絕大部分的生長和分化訊息。目前文獻顯示所有須與 cytokine receptor superfamily 結合的 cytokine 都能活化 JAK tyrosine kinase family。

因為 JAK/STAT 路徑在血液細胞訊息傳遞路徑中扮演著非常重要的角色，所以推論 JAK/STAT 路徑亦應在血癌的生成機轉中佔重要的角色。愈來愈多的證據支持此學說，最近 TEL-JAK2 致癌基因在三個血癌病人細胞中被發現。此致癌基因能使細胞變性轉型的能力是來自於藉由 TEL 的 PNT domain 使 JAK2 的 kinase domains 彼此結合(oligomerization)而持續保有 JAK2 kinase 的活性。

TEL-JAK2 的致癌生成機轉，主要來自於其 kinase domain 上的酪氨酸基被持續磷酸化。尋找 TEL-JAK2 的下游受脢質蛋白，對了解癌症的生成機轉之謎，是很重要的．我們已 clone hTEL-mJAK2，且證明 TEL-JAK2 的持續保有酪氨酸激脢的特性，能使 Ba/F3 細胞株由 cytokine-dependent 轉型成 cytokine-independent．而且我們利用 TEL-JAK2 的持續保有酪氨酸激脢的特性， 發現一個持續被酪氨酸磷酸化的受脢質蛋白，只存在於已轉型的 Ba/F3/ TEL-JAK2 細胞，但不出現在未轉型的 Ba/F3/ TEL-JAK2 細胞．目前正進而研究它的生物特性，以了解血癌的生成機轉．

Abstract

The JAK/STAT (Janus protein tyrosine kinase/Signal Transducers and Activators of Transcription) pathway has emerged as a major signal transduction mechanism in hematopoietic system, linking cell surface receptors on the membrane to transcriptional events in the nucleus. This pathway plays critical roles in transducing growth and differentiation signals emanating from ligand-activated cytokine receptor complexes. It was demonstrated that all the cytokines that utilize receptors of the cytokine receptor superfamily were capable of activating members of the JAK tyrosine kinase family.

The importance of the JAK/STAT pathway in cytokine signaling for the hematopoietic cells suggests a potential role in the pathogenesis of hematological malignancies. Some evidences have supported this hypothesis. The TEL-Jak2 fusion oncogene has recently been found in 3 leukemia patients. The oligomerization by the PNT domain of TEL leads to constitutive activation (phosphorylation) of the Jak2 tyrosine kinase domain, which is necessary for cellular transformation.

The constitutive tyrosine phosphorylation of the tyrosine kinase domain of TEL-Jak2 plays the critical role for the leukemogenesis. To try to find the down-stream substrate of the TEL-Jak2 is very important for the understanding the signal transduction pathway for the leukemogenesis. We have constructed the hTEL-mJAK2 oncogene including the human TEL-specific oligomerization domain and the catalytic domain of murine JAK2. And our data also showed the TEL-induced oligomerization of TEL-JAK2 resulted in the constitutive activation of its tyrosine kinase activity and conferred cytokine (IL-3)-independent proliferation to the IL-3-dependent Ba/F3 hematopoietic cell line. We have also identified one strongly constitutively tyrosine-phosphorylated protein in the Ba/F3/TEL-JAK2 cells which is not found in the Ba/F3 parent cells by using the character of the constitutively activated catalytic activity of TEL-JAK2. We are studying the biological character of this down-stream substrate of TEL-JAK2 oncogene to elucidate the signal transduction pathway for leukemogenesis in the hematopoietic system.

Background and Purposes

The JAK/STAT signal pathway is the main and the most important pathway among the hematopoietic system for signaling almost all cytokines' messages into the nucleus. This pathway was still unknown until early '90, but the whole frame has been established since '96[1-3].. This pathway links cell surface cytokine receptors to transcriptional events in nucleus. The JAK kinase family (JAK1, 2, 3 and Tyk2) is cytoplasmic kinases that bind to cytokine receptors that have no kinase domain. The STAT protein family (STAT1-4, 5a, 5b and 6) is latent cytoplasmic transcription factors. Once cytokines bind to specific receptors, they will induce receptors' dimerization. The associated JAK kinases will respond by phosphorylating each other and also tryosine residues on the receptor cytoplasmic domains, creating phosphotyrosine docking sites that allow SH2-containing signal proteins, including STATs or other down-stream substrate to bind. The recruited STATs will be activated by phosphorylation on conserved tyrosine residues, then dissociate from the receptor and dimerize, forming homo- or heterodimers, and translocation to the nucleus, where the complexes bind to specific DNA response elements and activate transcription of genes.

The importance of the JAK-STAT pathway in cytokine signaling suggests a potential role in the leukemogenesis of hematological malignancies. Some evidences support this hypothesis: A single amino-

acid mutation in the Drosophila melanogaster JAK causes leukemia-like hematopoietic defects [4; 5]. The constitutive activation of the JAK-STAT pathway has been shown in malignant cutaneous T-cell tumor [6; 7]. And, the constitutively activated STATs have been shown in cells from patients with chronic myelomonocytic leukemia, acute lymphoblastic leukemia, acute myeloid leukemia and chronic lymphocytic leukemia [8-10]. The leukemic cells from patients with relapsed ALL have constitutively activated JAK2, and inhibition of JAK2 activity by a specific blocker (AG-490) selectively blocks leukemic cell growth in vitro and in vivo [11]. And, the most direct evidence for involvement of JAK family in leukemogenesis is the chromosomal translocations from leukemic cells involving the JAK2 kinase gene on 9p24 and the TEL gene on 12p13(TEL-Jak2 oncogene) [12].

The TEL is a member of the ETS family of transcription factors. It contains a 60-amino acid oligomerization domain(PNT) at its N-terminus. Three TEL-tyrosine kinase fusion proteins (TEL-PDGFR (platelet-derived growth factor receptor), TEL-ABL and TEL-JAK2) have been found in human leukemia [12-14]. The TEL-JAK2 oncogene was characterized to fuse the C-portion (catalytic kinase domain) of JAK2 to the N-portion (oligomerization domain) of TEL. The oligomerization by the PNT domain of TEL leads to constitutive activation of the PDGFR, ABL and JAK2 tyrosine kinase domains (JH1) activity (Fig.1.), which is critical and necessary for transformation of hematopoietic cells. There are several expected purposes in this study:

1. To clone the TEL-JAK2 oncogene into retroviral vector.
2. To do site-directed mutagenesis on the supposed critical tyrosine residues of the TEL-JAK2.
3. To sequence the cloned genes.
4. To transfect the TEL-JAK2 and mutant oncogene into various cells.
5. To check the protein expression of TEL-JAK2 by GFP marker.
6. To check the TEL-JAK2 protein activity by :

   In vitro: immunoprecipitation and western blotting

   In vivo: the transformation ability of TEL-JAK2 in cells
7. To try to identify the substrate(s) of TEL-JAK2.
8. To try to purify the substrate(s) of TEL-JAK2.

Results and Discussion

Clone TEL-JAK2 oncogene into retroviral vector:

The JAK kinase family share structural domains designated as JH segments 1-7. The critical domain which is involved in the TEL-JAK2 oncogene is the JH1, the kinase domain located at the C-terminus. The JH2 to JH7 domains appear to be involved in the protein-protein interactions. The sequence of the human JAK2 was 90% identical to that of murine JAK2 at the nucleotide level and 96% identical at the protein level over the JH1 domain. We prepared a chimeric cDNA construct containing the sequence encoding for the human TEL PNT domain (nucleotide 1-1032) fused to the murine JAK2 kinase domain (JH1, nucleotide 2931-3893) by RT-PCR strategy and cloned into PMSCV retrovirus vector with GFP(Green Fluorescence Protein) selective marker. All of the cDNA fragments are checked by sequencing (data not shown).

Produce retrovirus and transfer into murine hematopoietic cell line (Ba/F3):

We prepared the retroviral stocks by using the 293-T cells as the first virus packaging cell line for transient transfection of the PMSCV retrovirus vector with encoding the TEL-JAK2 construct and the GP+E86 cells as the second virus producer cell line for harvesting the retrovirus. Virus titer was checked by using the 3T3 cells. The virus titer was 10×10^7 /ml. We used this harvested supernatant which contained the cDNAs encoding TEL-JAK2 retrovirus to transfect the murine Ba/F3 hematopoietic cell line, which is strictly dependent on IL-3 for survival and proliferation. Mock-transfected (with retrovirus vector only) cells were used as control. The calcium phosphate method is used for this transfection.

Check the protein expression of TEL-JAK2 by GFP marker:

After 48-72 hours of transfection in the presence of IL-3, the infected Ba/F3 cells were sorted by checking the selective marker GFP in the vector by flow cytometry. The sorted GFP positive cells were also frequently checked directly under fluorescent microscopy to assure the stable expression of exogenous TEL-JAK2 protein.

Check the TEL-JAK2 fusion protein activity by:

In Vitro: Immunoprecipitation and Western blotting:TEL-JAK2 oncogene results in the constitutive activation of JAK2 's tyrosine kinase activity:

To check the TEL-induced oligomerization properties and protein kinase activity of TEL-JAK2, the Ba/F3/TEL-JAK2 cells were analyzed with in vivo-translated protein. We have checked the autophosphorylation of TEL-JAK2 which is mediated by the TEL-induced oligomerization by immunoblotting with a phosphotyrosine-specific antibody (anti-PTyr). The Ba/F3/TEL-JAK2 cell lysate was firstly specifically immunoprecipitated by antibodies directed against the C-terminal domain of JAK2 (anti-JAK2), then immunoblotted by anti-Ptyr and anti-JAK2 respectively. A higher level of tyrosine phosphorylation occurred with hTEL-mJAK2 protein than that with endogenous JAK2 protein. The expression and strong tyrosine-phosphorylation of the hTEL-mJAK2 fusion protein shows hTEL-mJAK2 fusion protein is expressed and constitutively tyrosine-phosphorylated in Ba/F3/TEL-JAK2 cells.

In Vivo: To assay the transformation ability of TEL-JAK2 protein in cells: The constitutive activation of JAK2's tyrosine kinase activity transforms the IL-3-dependent Ba/F3 cells into growth factor(cytokine)-independent proliferation:
We deprived the Ba/F3/TEL-JAK2 cells and parent Ba/F3 cells of IL-3 and monitored the cell proliferation by daily cell counting. The growth curve was recorded. In contrast to control cells (Ba/F3, no IL-3), which died with decreased cell number in the absence of IL-3, the similar proliferation curves were traced between the Ba/F3/TEL-JAK2 cells in the absence of IL-3 and Ba/F3 cells in its presence. According to the above data, the TEL-JAK2 has the property of constitutive activation of its protein tyrosine kinase activity. And this constitutively activated tyrosine kinase activity conferred cytokine-independent proliferation property to the IL-3-dependent Ba/F3 hematopoietic cell line.

Comparison of Ba/F3 and Ba/F3/TEL-JAK2 cells: Identification of one constitutively tyrosine phosphorylated protein(P100) in the Ba/F3/TEL-JAK2 cells which is not phosphorylated in the Ba/F3 parent cells:
The total cell lysates of either Ba/F3 or Ba/F3/TEL-JAK2 cells were used in immunoprecipitation with anti-Ptyr antibody, resolved by SDS-PAGE and transferred to PVDF membrane. The blot was probed with the same anti-Ptyr antibody which was initially used for IP. We identify there is one strongly constitutively tyrosine-phosphorylated protein (P100) in the Ba/F3/TEL-JAK2 cells which is not phosphorylated in the Ba/F3 parent cells.

This constitutively tyrosine phosphorylated protein(P100) is not STAT5 or other known proteins:
To examine whether or not the P100 was similar to the known molecules, Ba/F3 and Ba/F3/TEL-JAK2 cell lysates were prepared and subjected to immunoprecipitation with anti-Ptyr antibody. Immune complexes were separated by 7.5% SDS-PAGE and transferred to membrane. Many identical blots were prepared and probed with anti-STAT5, anti-PLCγ1,2, anti-SHIP(SH2-containing-inositol phosphatase), anti-CAS(cellular apoptosis susceptibility), anti-HPK-1(hematopoietic progenitor kinase 1),and anti-SP1(sequence-specific transcription factor). All of the above molecules are considered as the potential down-stream substrates of TEL-JAK2 because of their published biological character and/or their similar molecular weights. But, all of these commercialized antibodies were unable to immunoblot the P100 in anti-Ptyr immunoprecipitates from lysates of Ba/F3/TEL-JAK2 cells (some of these data shown in Fig.5.). For further reassurance that the P100 is not STAT5, the Ba/F3 and Ba/F3/TEL-JAK2 cell lysates were firstly specifically immunoprecipitated by anti-STAT5a and anti-STAT5b antibodies, then these lysates were subsequently immunoprecipitated again by the anti-Ptyr antibody, then immunoblotted by anti-Ptyr, anti-STAT5a and anti-STAT5b respectively. The dada showed the anti-Ptyr antibody still can immunoprecipitate the P100 from the cell lysates which were firstly immunoprecipitated by the anti-STAT5a,b antibodies, and this P100 immune complex still could not be recognized by the anti-STAT5a,b antibodies. These immunological discrepancies suggested that the tyrosine phosphorylated P100 presented in lysates of Ba/F3/TEL-JAK2 cells was different from the above known molecules.

Self-Estimation

We have completed almost all of the expected goals except the cloning this new substrate of of the TEL-JAK2. We are studying the character of this protein. To study its biological character is very important to try to eluciate the mechanism of leukemogenesis by the oncogene.

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