

Antitumor Effect of Granulocyte Expression and Protein Tyrosine Phosphatase standing on Gene - Antitumor Effect induced by the Expression of Granulocyte Macrophage-colony stimulating Factor(II) and Rat Protein Tyrosine Phosphatase Gene expressed in embryonal Brain -

by

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Introduction

The biochemical and biotechnical study related to gene is important and fundamental for solute various biological phenomena. The former part¹⁾ of this study is the successive part which was carried on partially at Chiba Cancer Center Research Institute and Chiba University School of Medicine as the study of Tokyo Kasei University Research Institute (Quality of Life II). And the latter of that is carried on partially at Chiba University School of Medicine (sometime, Chiba Cancer Center Research Institute).

Now, the possibility of cancer gene therapy is widely studying in various systems²⁾. Some methods, the introduction of tumor suppressor gene in tumor cells³⁾, the prodrug administration with expression of virus encoded enzymes⁴⁾, and the activation of tumor-bearing host immune system⁵⁾, were published as examples. These studies tried by the author are related to actions of several organs that some genes exist to express or suppress biological biochemical reactions in tumor bearing host or undifferentiated cells. It is thought that an enforcement of host immune surveillance by cytotoxicity against tumor cells holds an advantages from eradication of tumor cells and inhibition of recurrence.

The cytokines are important key molecules for immune and inflammatory reactions. Many cytokines have been studied for induction of tumor responses⁵⁾ in animal models. The expression of cytokine gene in tumor cells enables the host ani-

mals to increase their immune responses to tumors. The application of vaccines using tumor cells that have cytokines, interleukin-4(IL-4)⁶⁻¹⁰⁾, interleukin-6(IL-6)¹¹⁻¹⁴⁾, granulocyte macrophage-colony stimulating factor(GM-CSF)^{15,16)}, and interleukin-2 are suggested by various investigations.

The effect of these cytokine genes expressed in colon tumor cells, and the reduced tumorigenicity of GM-CSF-producing tumor cells were published by the author on the previous paper¹⁾.

This experiment of the latter part was carried out as a fundamental determined part related to carcinogenesis which expressed in one organ, brain, of them. And the phosphorylation and dephosphorylation are very important in relation to the expression and suppression in carcinogenesis.

Protein tyrosine phosphatase(EC 3. 1. 3. 48) mediates the dephosphorylation of phosphotyrosine residues and is involved in many aspects of intracellular signal transduction pathways²⁵⁾. The tyrosine phosphatase is not clear against tyrosine kinase, but the phosphatase acts in cell differentiation and transformation^{26,27)}.

Also, the protein phosphatase is classified to two distinctive families depending upon their molecular structures, intracytoplasmic phosphatase and receptor-type transmembrane phosphatase. By the precise molecular structural analysis, the phosphatase²⁷⁾ catalytic domain exists in both families. About 300 amino acids constitute the conserved domain, and the cysteine residues in this region are necessary to the catalytic reaction²⁸⁾.

Many cellular processes are regulated with the pro-

tein phosphorylation in neural tissues. The tyrosine phosphorylation initiated by the receptor tyrosine kinase was implied in nervous system²⁹. The function of the receptor tyrosine kinase has been examined in *Drosophila* with several mutants³⁰. But the tyrosine phosphatase is not sufficiently investigated in a neural system. And a few protein tyrosine phosphatases are expressed in a developing brain, so it is thought that more tyrosine phosphatase is involved in the neurogenesis. To examine the expression of tyrosine phosphatase gene in an embryonal brain, a reverse transcription-based-polymerase chain reaction(PCR) method was applied. And a novel tyrosine phosphatase gene with a brain specific expression was found by the author. The deduced amino acid sequence shows that this is a homologous gene to *Drosophila* protein tyrosine phosphatase gene(DPTP10D), which expressed in an embryonal central nervous system^{31,32}.

Experimental and Results

Cells and animals: BALB/c mice (6-to 8-week old females) were purchased from Shizuoka Laboratory Animal Center(Japan). Colon 26, a carcinogen induced undifferentiated adenocarcinoma cell line^{17,18} and the packing cells used for retrovirus vectors, ecotropic ψ 2 and amphotropic PA 317 cells, which were cultured in Dulbecco's modified medium supplemented with 10% heat-inactivated fetal calf serum were obtained from Ajinomoto Co. Ltd. (Japan) and American Tissue Culture Collection(USA) respectively.

Also, cytokine-producing colon 26 cells(1×10^6) or wide-type colon 26 cells(1×10^6) were injected subcutaneously into syngeneic BALB/c mice(5 mice each group). The mice were assessed for survival.

Construction of retrovirus: Retrovirus vector, LXS_N¹⁹ and human IL-6 cDNA²⁰ were supplied from Fed Hutchinson Center and Osaka University respectively. IL-4 cDNA²¹ was obtained from Tokyo University. The subcloning of each cDNA into LXS_N vector were undergone. Each plasmid DNA with a cytokine cDNA was transfected into PA317 cells using lipofectin reagent which was obtained from Life Technologies Inc. (USA). After drug-selection with G418 obtained from Life

Technologies Inc. (USA), cell-free culture supernatants of P317 cells were used for infecting ecotropic ψ 2 cells in the presence of 8μ g/ml polybrene which was purchased from Aldrich Chemical Company Inc. (USA). After the infection, G418-resistant ψ 2 cells were examined for corresponding cytokine mRNA production.

Detection of cytokine-producing cells: Infection process of colon 26 cells with each retrovirus were same as the above description. G418-resistant cells which produced each cytokine were obtained and cultured at 3×10^5 cells per 6 cm diameter dish for 72 hr. The amount of cytokines in the culture supernatants were determined with enzyme-linked immunosorbent assay kits purchased from Emdogen Inc. (USA).

Data treatment: Survival analysis were undergone with Kaplan-Meier test. Statistical analysis was performed with the generalized Wilcon test.

Morphological microscopic observation: Tumor samples obtained were fixed with 10% formalin and embeded in paraffin. There were no distinct morphological differences among the transduced and untransduced colon 26 cells. All transduced cells were not immunogenically different from wide-type cells. Histological analysis on tumors with cytokine production was performed. The mild migration of macrophages and neurophilis was detected in the vicinity of IL-6 producers and there was no major cellular infiltration in the case of IL-4 producers.

Detection of transduced cells: The transduced colon 26 cells which produce each cytokine were obtained with G418 drug selection and the amount of cytokine secreted was determined with the enzyme-linked immunosorbent assay. And the amount of IL-4 or IL-6 was 555 pg/ml or 448 ng/ml per 1×10^5 cells/ml/72 hr respectively.

Survivals of the mice inoculated with cytokine-producing cells: These transduced cells were subcutaneously inoculated into BALB/c syngeneic mice to study the effect of cytokines which were secreted from tumor cells.

The two cases with IL-4 producer cells and the one case with IL-6 cells showed similar regression of tumor masses, therefore the mice with IL-4 producing cells were alive longer than those with wild-type

cells. In the case of the mice with IL-6 producers, the survival was relatively inconsistent and was not prolonged, compared with that of untransduced cells. The results were shown in Table 1.

Table 1

Antitumor effect by the expression of cytokine genes in murine colon carcinoma cells

Cytokine gene	Number
-	5
IL-4	5
IL-6	5
Survival days	Tumorigenicity
29,38,41,55,55**	100%
53,57,82,180>,180>*	60%
19,44,51,76,180>	80%

BALB/c mice(5 mice per a group) were subcutaneously inoculated with corresponding cytokine producer cells.

* : $p < 0.01$, # : < 0.05

Chain reaction with reverse transcription polymerase: The double stranded cDNAs were synthesized with the cDNA synthesis kit purchased from Amersham Plc. (UK), with poly(A)⁺ RNA from a rat brain of day 15 p.c. (post coitum) embryo.

The oligo(dt) was used for a primer, 20 ng of double-strand cDNAs were used as templates and three degenerate oligonucleotides as PCR primers. For sense primers, 5'AA(A/G)TG(T/C)GC(T/C)CA(A/G)TA(T/C)TGGCC-3' (corresponding to consensus sequence, TCAQYWP) and 5' AA(A/G)AG(T/C)GA(T/C)CA(A/G)TA(T/C)TGGCC-3'(corresponding to KCDQYWP), for an antisense primer,5'(G/C)CC(A/G)ATGCCTGCACT(A/G)CAGTG-3' (corresponding to HCSAGIG) were used. Those base sequences correspond to the nucleotide sequence 448-467 and 730-750 of rat PTP1B³³ correspondingly. The applied reaction was performed with a standard procedure³⁴. As main process, 30 cycles of PCR reaction consisted of denaturation at 94°C for 1 min, annealing at 50°C for 2 min and extension at 72°C for 2 min. The final PCR products were subjected to agarose gel electrophoresis and the

expected-sized bands were isolated. The determination of sequence was carried out with dideoxy chain termination method. The homology of the amino acid sequence was searched with the catalase of National Biomedical Research Foundation(NBRF, Rel. 38).

A new gene (D30) which was novel based on the homology search with NBRF protein database [Rel. 38] was discovered in PCR products from embryonal rat brain mRNAs. The D30 amino acid sequence with those of the phosphatase showed that the interval between the consensus region of the catalytic domain(used for primers) and the amino acids intervening between the primers were highly conserved. This D30 may be a tyrosine phosphatase gene from the structural similarities to tyrosine phosphatase. These certainty and expression of gene were examining subject to publication on the next papaer under the high probability.

Southern blot analysis: To Southern blot, 20 μ g of genomic DNA from rat fibroblasts were digested with several restriction enzymes. The samples were subjected to agarose gel electrophoresis and transferred to nylon filter. The 288-bp D30 DNA labeled ³²Pd CTP was used for a probe. The hybridization and the washing condition were same as the above description. And 10 μ g of genomic DNAs from several species were used after the digestion with *Bam*HI for the cross-hybridization. D30 probe detected several distinctive bands in Southern blot analysis with rat genomic DNA. 20 μ g of this genomic rat DNA digested with *Eco*RI, *Bam*HI, *Hind*III, and *Xba*I were hybridized with D30 DNA. This result showed that the gene existed as a single copy. The used probe which corresponds to the catalytic domain did not cross-hybridize with other phosphatase genes. The presence of homologous genes in other species by Southern blot analysis was detected. The distinct bands in rodents, human and chicken DNAs were detected under a high stringent condition. This gene was well conserved among these species. This gene did not cross-hybridize with other phosphatase gene. Those results may be published on the following paper.

Northern blot determination: For the analysis of the developmental expression, 1 μ g of poly(A)⁺ RNAs

from an embryonal were subjected to agarose gel electrophoresis and transferred to a nylon filter. 288-bp D30 DNA and ribosomal DNA were labeled³²Pd CTP and were used for probes. The hybridizations were carried out in the solution of 50% formamide/5X SSC/50 mM NaH₂PO₄/2X Denharts' solution/0.1% sodium dodecyl sulfate

(SDS)/0.1 mg/ml salmon sperm DNA at 42°C for 12 hr. The filters were washed with the solution of 0.2X SSPE/0.1% SDS several times at 50°C. They were exposed to Fuji X-ray film RX with an intensifier at -80°C. The radioactivities of each band were calculated with Molecular Imager GS-250(Bio-Rad Laboratories, USA). The results were shown in Fig.1.

Fig. 1. The alignment of amino acid sequence of rat D30 gene and other protein tyrosine phosphatase genes.

D30	KCAQYWP-FT	EEPIAYGD--	ITVEMVSEEE	QEDWASRHRFR
DPTP10D	KCDQYWP-ND	TVPVFGYGD--	IKVQILNDSH	YADWVMTEFM
Rat PTB1B	KCAQYWPQKE	EKEMVFDDTN	LKLTLISEDV	KSYYTVRQLE
Consensus	KCAQYWP-.D	...YGD--	.V..LSE.V	...WT.R.F.
D30	I--NYA-DEA	QDVMHFNYTA	WPDHGVPPAN	AAESILQFVY
DPTP10D	LCRGSE---Q	RILRHFHFTT	WPDFGVP--N	PPQTLVRFVR
Rat PTP1B	L-ENLATQEA	REILHFHYTT	WPDFGVP--E	SPASFLNFLF
Consensus	L-.N...E.	R.I.HFHYTT	WPDFGVP--E	.P.S.L.FV.
D30	TVRQQA--TK	SKGPMIIHCS	AGIG	
DPTP10D	AFRDRI--GA	EQRPIVVHCS	AGVG	
Rat PTP1B	KVRESGSLSP	EHGPIVVHCS	AGIG	
Consensus	.VR.....P	..GP.VVHCS	AGIG	

Discussion

The antitumor responses which were induced by the local production of cytokines from mouse colon carcinoma cells were investigated in this experiment. As the previous publication¹⁾, in the cytokines examined, the integration of GM-CSF gene into tumor cells induced antitumor effect in vivo. The effect was shown by prolongation of the survival of inoculated animals and the spontaneous regression of the tumor masses. Since GM-CSF is thought to be involved in antigen presentation²⁾, the local production of this cytokine may enforce the host immunity by providing immunogenic epitopes to CD4⁺ T cells, and consequently supplying signals for generating cytotoxic T cells⁵⁾.

The infiltrated cells around the tumor in this study is different from the previous reports. According to the previous reports the filtration of CD4⁺/CD8⁺ T cells and the macrophages were observed in GM-

CSF producers^{3,23)}. The macrophages and neutrophils were detected in IL-6 producers^{5,12)} and the eosinophils and macrophages in IL-4 producers^{6-9, 24)}, respectively. It was considered that the difference might be attributable to the stages of tumor development or regression as well as the amount of cytokines produced. Since the migration might be induced by other chemotactic factors which were driven from the tumor-mediated inflammatory reactions, the cells migrated around tumor cells are not directly responsible for the tumor immunity.

On the other hand, the culture supernatants of the clone producing the highest level of corresponding cytokine mRNA were used for transducing tumor cells. It is considered that there are several directions to extend the responses examined in this study. The required amount of cytokines, the acquisition of immunological memory and the possibility of elimination of preexisting tumor cells are important issues to be pursued. The investigation of these

subjects may point out the possibility of cancer gene therapy using cytokine-modified tumor cells.

The biochemical phospho-reaction is classified to forward reaction: phosphokinase, and backward reaction: phosphorylase. This phospholation-dephospholation is related closely to the expression or suppression of oncogene. This protein phosphatase is classified to two subgroups by one categorical consideration; (1) cytosolic phosphatase of low molecular weight and (2) receptor-type transmembrane phosphatase of high molecular weight. The difference between high molecular weight enzyme and low molecular weight enzyme shows the classification of catalytic phosphatase domain soluting the dephosphorylation of tyrosine residue. The conserved domain is made from about 300 amino acids. The cysteine residue in the domain is essential for the catalytic reaction. In a neutral system, many cellular processes are regulated by protein phosphorylation. For example, tyrosine phosphorylation acted by the receptor tyrosine kinase has been suggested in the developing nervous system³⁴. The function of these receptor tyrosine kinases was determined in *Drosophila* which included several mutants³⁵. While some protein tyrosine phosphatases were expressed in developing brain. Many protein tyrosine kinases are involved in the neutral development during the neurogenesis. So, the expression of tyrosine phosphatases genes in the embryonic brain of mouse and rat was examined with the method of one reverse transcription based polymerase chain reaction(PCR). Two putative new tyrosine phosphatase genes were obtained from mouse and rat respectively. Then, the sequence alignment showed that these were homologous each other. Above all, tyrosine protein kinase, protein tyrosine kinase, is related to oncogene, *src*, *yes*, *fgr*, *fyn*, *lyn*, *lck*, *abl*, *fes/fps*, *kit*, *ros*, *erbB*, *fms*, etc. These oncogenes and these products of oncogenes and proto-oncogenes and these products of oncogenes and proto-oncogenes have tyrosine kinase activity. The *ros*, *erbB*, *fms*(pro-oncogene) have the acceptor of insulin, epidermal growth factor(EGF) and macrophage colony-stimulating factor(CSF). The acceptor of platelet-driven growth factor (PDGF) and insulin-like growth factor(somatomedin

C) has tyrosine activity in intra-cellular domain.

On the other hand, tyrosine protein phosphatase is considered as the enzyme of the reverse reaction to kinase.

The experiment could identify a novel cDNA encoding tyrosine phosphatase gene from a rat embryonal brain. D30 gene showed the homology to the membrane type-phosphatase gene DPTP10D^{36,37} which was cloned from an embryonal central nervous system of *Drosophila*. As the expression of DPTP10D gene is restricted to developing neurons, D30 gene may be a mammalian homologue of DPTP10D gene. It has a single phosphatase domain against other membrane type-phosphatases which have two phosphatase domains in cytoplasmic region.

Summary

The murine colon carcinoma cells which secrete the several kinds of cytokine after the retrovirus transduction with corresponding gene, were studied for their antitumor effects in syngeneic mice. The mice inoculated with granulocyte macrophage-colony stimulating factor (GM-CSF) producer cells which were published on the previous paper showed not only prolonged survival but also reduced tumorigenicity. The antitumor effect caused by the expression of interleukin-4(IL-4) was less than that of GM-CSF, and interleukin-6(IL-6) producer cells did not show any effects to the survival of the host animals. Then, the feasibility of cancer gene therapy with the expression of GM-SCF gene in tumor cells in comparison with IL-4 and IL-6 was shown.

The new protein tyrosine phosphatase gene was detected from the embryonal rat brain by reverse transcription-based polymerase chain reaction. As this transcription is expressed in embryonal, it is specific to brain and is regulated developmentally. This gene is belongs to the same family of the membrane-type tyrosine phosphatase gene of *Drosophila* (DPTP10D) according to the homology search of the deduced amino acid sequence. Its expression is specific to an central nervous system of fly embryo.

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By the limitation of page, the descriptions of Experimental and others should be cut or abbreviated. But this publication could not be out by the obligation of scientific funds.

顆粒性白血球発現の抗癌効果と遺伝子に基づくタンパクチロシンリン酸酵素—
顆粒性白血球大食細胞集落刺激因子（Ⅱ）による抗癌効果と胎生脳で発現したネ
ズミタンパクチロシンリン酸酵素遺伝子—（英文）

堀 津 圭 佑

（平成8年9月30日受理）

対応する遺伝子でレトロウイルス形質導入後、いくつかのサイトカインを分泌するネズミ結腸癌腫細胞は同遺伝子組成のネズミにおいてそれらの抗癌効果につき研究された。前報で発表した顆粒球マクロファージコロニー刺激因子（GM-CSF）産生細胞を接種したネズミは生存延長のみならず腫瘍形成性も減少した。インターロイキン4（IL-4）の発現による抗癌効果はGM-CSFのそれより少なくインターロイキン6（IL-6）産生細胞は宿主動物の生存にいかなる効果も示さなかった。そしてIL-4やIL-6との比較で腫瘍においてGM-CSF遺伝子の発現による癌遺伝子治療の可能性が示された。逆転写によるポリミラーゼが連鎖反応で胎生ネズミ脳からの新タンパクチロシンホスファターゼ遺伝子が検出された。この転写は胚で発現されるので、これは脳に特異的で発生的に制御されている。この遺伝子は推定アミノ酸配列の相同関係調査よりショウジョウバエの膜型チロシンホスファターゼ遺伝子と同じファミリーに属する。その発現はハエ胚の中枢神経系に特異的である。