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Growth Factor Receptor Expression on Brain Tumor Cell Lines : Preliminary Study for *in vitro* and *in vivo* Experiments of Immunotoxin Therapy*

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= Abstract =

뇌종양세포주에서의 성장인자수용체의 발현 : 면역독소 치료의 연구를 위한 예비실험

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Objective : Growth factor receptors on the tumor cells are known to be expressed highly allowing the tumor cells to bind growth factors to stimulate cellular division. Immunotoxin therapy is one of the novel approaches to the primary malignant brain tumor, and expression of cell-surface receptor is essential for the immunotoxin to have specific anti-tumor activity. Despite promising cytotoxic activity of immunotoxin, tumor responses are not curative on clinical trials, and additional studies are needed regarding various factors influencing the efficacy of the immunotoxin. The purpose of this study is to detect the expression of various growth factor receptors on brain tumor cell lines which are going to be used in these studies.

Materials and Methods : The authors detected transferrin receptor(TR), insulin-like growth factor-1 receptor(IGF-1R), and interleukin-4 receptor(IL-4R) on medulloblastoma cell line(Daoy) and glioblastoma cell lines(U373 MG and T98 G) by flow cytometric analysis.

Results : TR was expressed on Daoy, U373 MG, and T98 G. IGF-1R was expressed on Daoy and U373 MG, but not on T98 G. IL-4R was expressed on all cell lines tested.

Conclusion : The transferrin and interleukin-4 receptors might be good targets for immunotoxin therapy. The results should be considered in additional *in vitro* and *in vivo* studies regarding immunotoxin and in establishing the proper treatment model of the immunotoxin therapy including selection of the adequate immunotoxin.

KEY WORDS : Immunotoxin · Transferrin receptor · Insulin-like growth factor-1 receptor · Interleukin-4 receptor.

Introduction

The prognosis of primary malignant brain tumor is poor despite aggressive treatment with surgery, radiation therapy, and chemotherapy. Targeted fusion toxins, combining the cytotoxic domain of the natural toxin to carrier ligand, are being developed for the treatment of cancers including malignant central nervous system tumors[6)7)23)]. The toxins bind to receptors on the surface of target cell via the ligand specific to it and are internalized by endocytosis[14)]. It is essential for these receptors on the tumor cell to be highly ex-

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pressed in order for the immunotoxin to have anti-tumor activity.

Compared with normal cellular counterparts, several growth factor receptors on the tumor cells are known to be expressed highly and some of the growth factors for these receptors are useful in the development of immunotoxin as a carrier ligand[16)18)]. Transferrin and interleukin-4 receptors are frequently used as cell-surface targets in immunotoxin therapy[8)9)10)17)]. Insulin-like growth factor-1 and -2 are associated with glioblastoma multiforme[2)4)]. Expression of TR, IGF-1R, and IL-4R was not evaluated completely in all cell lines. Alteration of receptor expression can be influenced by environmental conditions[28)] and, and it is needed to detect factors affecting receptor expression and to develop the methods to improve the efficacy of immunotoxin. We detected expression of TR, IGF-1R, and IL-4R on Daoy, U373 MG, and T98 G by flow cytometric analysis.

Materials and Methods

1. Cell lines

One medulloblastoma cell line(Daoy) and two glioblastoma cell lines(U373 MG and T98 G) were obtained from ATCC(Rockville, Maryland). The cells were cultured in minimum essential medium(GibcoBRL, Grand Island, NY) which was supplemented with 10% fetal bovine serum (vol/vol), 1% L-glutamine, and 1% penicillin/streptomycin. The media was changed every three days and the cells were split 1 : 10 every 5 to 7 days.

2. Transferrin and insulin-like growth factor-1 receptors

Expression of transferrin and insulin-like growth factor-1 receptors were detected by flow cytometric analysis. Two-color immunofluorescence staining was done for the detection of TR and IGF-1R. The cells were harvested from the flask with 0.5mM ethylenediaminetetraacetic acid(EDTA) and resuspended in 200 μl of cold phosphate buffered saline(PBS). They were divided into two 5ml polystyrene tubes in equal volume, one for control and the other for TR and IGF-1R staining. Fluorescein isothio-cyanate(FITC)-labeled anti-transferrin receptor antibody, CD71(Pharmingen, San Diego, CA), and phycoerythrin(PE)-labeled anti-IGF-1 receptor antibody, 1H7(Pharmingen, San Diego, CA), were added to the tube for the detection of TR and IGF-1R, and fluorescein-conjugated isotype antibodies were used for control staining. The reaction mixtures were incubated on ice for 20 minutes with protection from light. After incubation, the cells were washed with 3ml of cold buffer, made up of Hank's balanced salt solution with 2% fetal bovine serum and 0.02% sodium azide. The samples were centrifuged at 1000G for 8 minutes and the supernatant was removed. The cell sediment was resuspended in 1% formaldehyde in PBS and the fixed cells were placed at 4℃ with light protection until flow cytometric analysis.

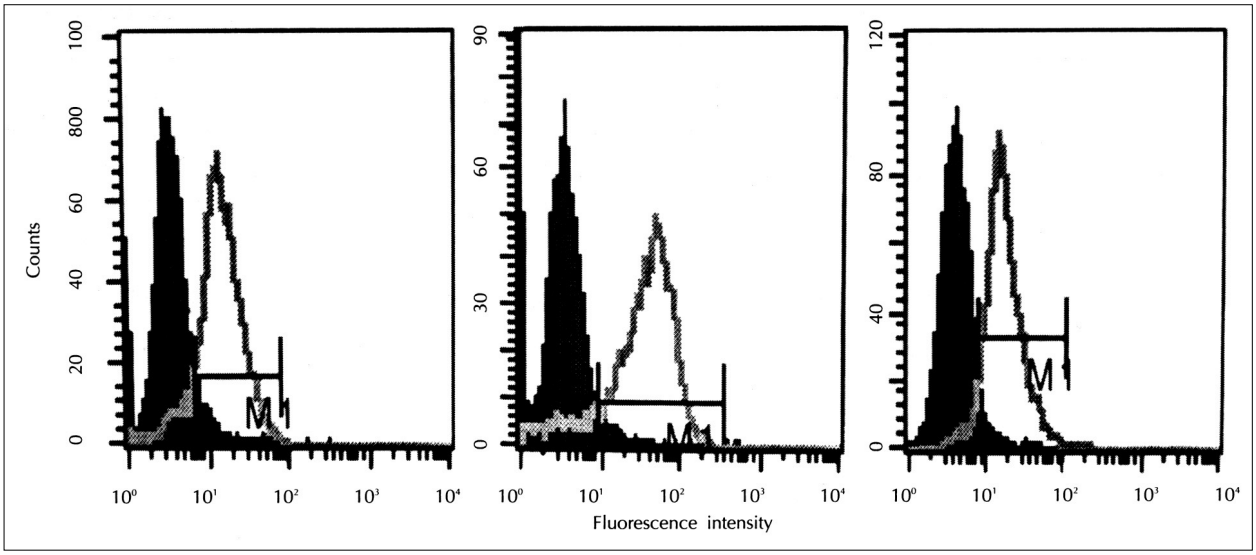

Fig. 1. Expression of transferrin receptor was detected by flow cytometric analysis. Fluorescein isothiocyanate(FITC)-labeled anti-transferrin receptor antibody, CD71, was used for receptor staining and fluorescein-conjugated isotype antibody was used for control staining. Solid curve is isotype control for background staining and blank curve is receptor staining. Medulloblastoma cell line(Daoy, Left) and glioblastoma cell lines, U373 MG(Middle) and T98 G(Right).

FACScan(Becton Dickinson, San Jose, CA)was used and 10,000 events were collected for each tube. Data were analyzed using "CELLQuest" software and histograms were obtained. The cells were gated and M1 marker was applied (Fig. 1).

3. Interleukin-4 receptor

For the detection of IL-4R, 'FluorokineTM Biotinylated Human IL-4 'kit(R & D Systems, Minneapolis, MN) was used. The cells in the flask were harvested with 0.5mM EDTA. After centrifugation, the cells were resuspended in 10mM PBS to a final concentration of 4×10^6cells/mL. Twenty-five microliter of the washed cell suspension was transferred to 5ml polystyrene tubes and 10 μl of biotinylated cytokine reagent was added. Ten microliter of biotinylated negative control reagent was added to 25 μl of cell suspension in control tube. The mixtures were incubated on ice for 60 minutes. After that 10 μl of avidin-FITC reagent was added to each tube for the fluorescein staining and incubated on ice for 30 minutes in the dark. The cells were washed twice with 2ml of 'RDF 1 cell wash buffer 'and resuspended in 0.2ml of 1% formaldehyde in 'RDF 1 buffer'. The fixed cells were stored at 4℃ with light protection until flow cytometric analysis. Flow cytometric analysis and data analysis were done as in detection of TR and IGF-1R.

Results

Data obtained from FACScan were analyzed using CELLQuest software. Percent-gated fluorescence intensity of transferrin receptor were 200.7 for background staining and 1389.1 for receptor staining on Daoy, 58.5 for background staining and 2931.1 for receptor staining on U373 MG, and 42.1 for background staining and 1617 for receptor staining on T98 G(Table 1). Percent gated fluorescence intensity of IGF-1R were 193.2 for background staining and 1378.9 for receptor staining on Daoy, 266.4 for background staining and 2375.1 for receptor staining on U373 MG, and 52.5 for background staining and 123.1 for receptor staining on T98 G(Table 2).

Percent-gated fluorescence intensity of IL-4R were 270 for background staining and 1953.8 for receptor staining on Daoy, 93.8 for background staining and 1356.3 for receptor staining on U373 MG, and 61.9 for background staining and 948.9 for receptor staining on T98 G(Table 3).

Histograms were obtained and solid curve represent isotype control for background staining and blank curve represent receptor statining. Transferrin receptor was highly expressed on Daoy, U373 MG, and T98 G(Fig. 1). Insulinlike growth factor-1 receptor was detected on Daoy and U373 MG, but not on T98 G(Fig. 2). Interleukin-4 rece-

Table 1. Mean fluorescence intensity and '% gated' of the background staining and receptor staining of the transferrin receptor on medulloblastoma cell line(Daoy) and glioblastoma cell lines(U373 MG and T98 G)

	Daoy		U373 MG		T98 G	
	% gated	Mean	% gated	Mean	% gated	Mean
Background staining	28.84	6.96	2.67	21.91	4.09	10.30
Receptor staining	97.48	14.25	97.9	29.94	95.12	17.00

Table 2. Mean fluorescence intensity and ''% gated' of the background staining and receptor staining of the insulin-like growth factor-1 receptor on medulloblastoma cell line(Daoy)and glioblastoma cell lines(U373 MG and T98 G)

	Daoy		U373 MG		T98 G	
	% gated	Mean	% gated	Mean	% gated	Mean
Background staining	12.94	14.93	9.41	28.31	6.93	7.58
Receptor staining	98.21	14.04	98.92	24.01	17.77	6.93

Table 3. Mean fluorescence intensity and '% gated' within M1 marker of the background staining and receptor staining of the interleukin-4 receptor on medulloblastoma cell line(Daoy)and glioblastoma cell lines(U373 MG and T98 G)

	Daoy		U373 MG		T98 G	
	% gated	Mean	% gated	Mean	% gated	Mean
Background staining	21.50	12.56	12.71	7.38	11.54	5.37
Receptor staining	94.25	20.73	95.31	14.23	99.26	9.56

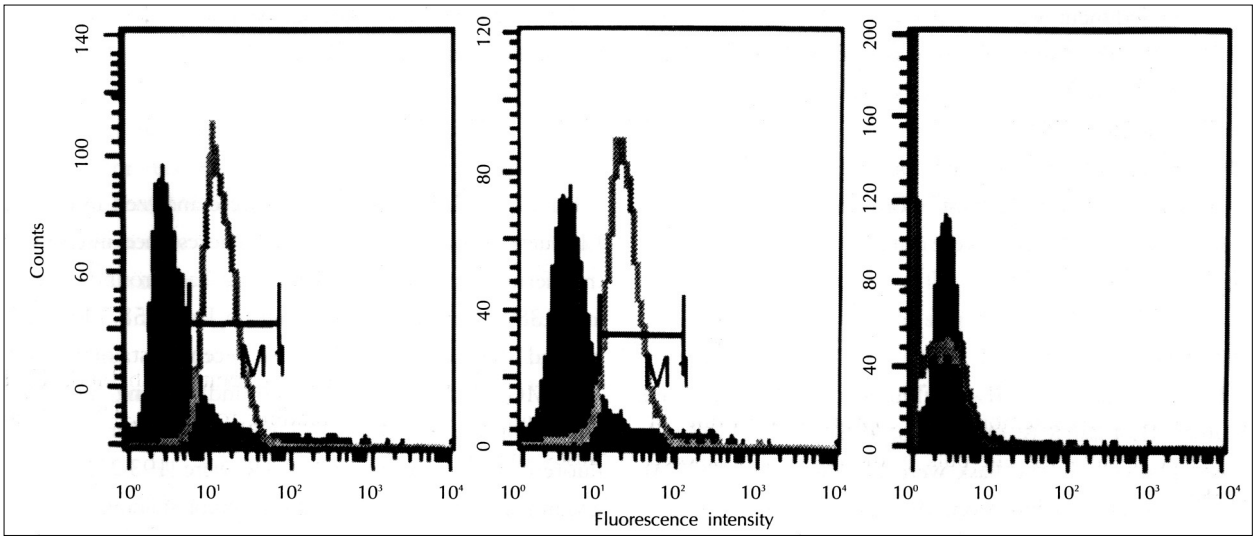

Fig. 2. Expression of insulin-like growth factor-1 receptor was detected by flow cytometric analysis which was done at the same procedure detecting transferrin receptor by two-color immunofluorescence staining technique. Phycoerythrin(PE)-labeled anti-IGF-1 receptor antibody, 1H7, was used for receptor staining and fluorescein-conjugated isotype antibody was used for control staining. Solid curve is isotype control for background staining and blank curve is receptor staining. Daoy(Left), U373 MG(Middle) and T98 G(Right).

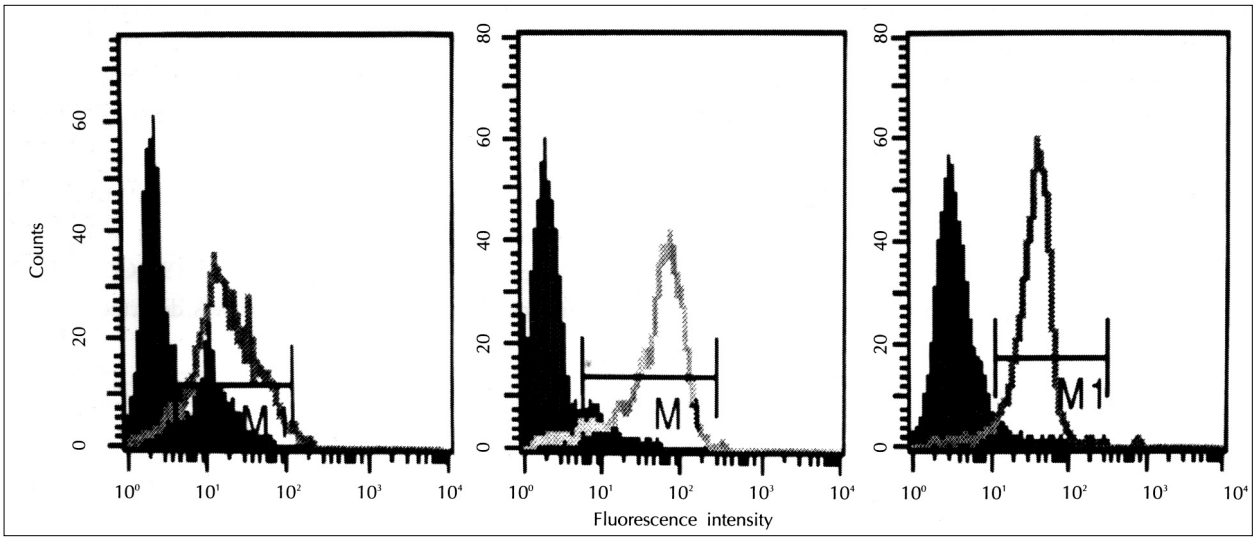

Fig. 3. Expression of interleukin-4 receptor was detected by flow cytometric analysis using 'FluorokineTM Biotinylated Human IL-4' kit. Solid curve is isotype control for background staining and blank curve is receptor staining. Daoy(Left), U373 MG(Middle) and T98 G(Right).

ptor expression was noted on Daoy, U373 MG, and T98 G (Fig. 3).

Discussion

In primary malignant brain tumors, a significant number of activated oncogenes have been identified. Some of these oncogenes are growth factors that result in the stimulation of cell proliferation[5]. The receptors on the cell surface bind to their specific growth factors usually with high affinity. It has also been reported that growth factor receptors are overexpressed on many malignant brain tumor cells, allowing the tumor cells to bind growth factors to stimulate cellular division[2)16)22)]. Malignant brain tumors show biological heterogeneity and it is extremely difficult to identify a tumor-specific receptor.

Growth factor receptors, such as TR, IL-4R, IL-13R, transforming growth factor-alpha receptor, are known to be

expressed highly on the brain tumor cells and some of the growth factors for these receptors are useful for the development of immunotoxin as a carrier ligand[1)16)18)26)]. In addition to the role in tumorigenesis, overexpression of growth factor receptors is an important aspect in the treatment of tumor with targeted toxins. Because these receptors have preferential expression on the tumor cells compared with normal brain tissue, they are frequently chosen as targets for immunotoxins[7)8)10)].

TR binds transferrin and mediates cellular iron uptake, and its expression is closely related to the cell proliferation and iron requirement of the cells[24)]. The transferrin receptor is a transmembrane glycoprotein consisting of two identical subunits. The bulk of the receptor is exposed on the cell surface and each subunit has a single transferrin-binding site. The expression of TR was reported to reflect the proliferative status of tumors[18)]. Transferrin receptors were not detectable on normal brain tissue but are expressed in great number by malignant brain tumors[18)30)]. In the study with glial tumors and meningioma, TR-positive cells were contained in the tumor, where the intensity and pattern of TR staining were reported to be varied with the histological type of the tumor[18)]. An immunohistochemical study of human glioma tissue using monoclonal anti-transferrin receptor antibody showed that rapidly proliferating cells expressed high levels of TR on the cell[22)], suggesting that TR may be a potential target for immunotoxins in treating high-grade gliomas.

IGF-1 and IGF-2 belong to the insulin family of peptides and act as growth promotors on certain human cells and are known to play a role in the growth and differentiation of the CNS[20)]. Two types of IGF receptors were identified[3)21)]. Type-1 IGF receptors bind IGF-1 and type-2 IGF receptors bind IGF-2 preferentially and the concentration of the type-1 receptor is more dense than that of type-2 receptor[3)]. In glioma cells, the molecular size and binding affinity of IGF receptors is reported to be quite different from those of normal brain, that is, the size of the α-subunit of the type-1 receptor is significantly larger than that of receptors in normal brain, type-1 receptors bind IGF-1 and IGF-2 with equal affinity, and type-2 receptors are expressed in higher amount than type-1 receptor[2)3)]. The type-1 IGF receptor is composed of an α-subunit which binds IGF-1 and IGF-2, and a β-subunit which has tyrosine kinase activity and demonstrates phosphorylation stimulated by IGF-1 and IGF-2[2)].

IL-4, one of the immune recognition-induced lymphokines, is a growth and differentiation factor for human B- and T-lymphocytes[15)29)]. IL-4 binds to IL-4R expressed on target cells to induce its function. Many cell types are known to express receptors for IL-4, and IL-4 responsive cells express relatively small numbers of receptors per cell[12)13)]. IL-4 receptors have been found to be highly expressed on many kinds of human cancer including primary CNS tumors[16)17)25)]. The IL-4 receptor is a transmembrane protein and an α-chain of IL-4 receptor constitutes the primary subunit which binds IL-4 with high affinity[12)]. IL-4 binding to the IL-4R α-chain causes the activation of receptor-associated kinases followed by tyrosine phosphorylation of insulin receptor substrate 1(IRS-1) located in the IL-4R α-chain, resulting in IL-4-induced growth and differentiation[19)27)].

Immunotoxins targeting TR were developed and exhibited potent and specific cytotoxicity. Targeted toxin Tfn-CRM107, a conjugate of human transferrin(Tfn) and a genetic mutant of diphtheria toxin(CRM107)that lacks native toxin binding, was delivered to the patients with recurrent malignant brain tumors by direct interstitial infusion. The initial results were promising, but the tumors recurred eventually. There are no clear explanations for tumor recurence. One of the reasons to be considered is heterogeneity of the malignant tumor, that is, expressibility of receptor can be different according to the status of tumor cells. Among ten human cell lines derived from glioma tissues, four cell lines did not expressed TR[22)]. Other possible reason is difference of receptor expression in vivo compare to that in vitro. The TR expression was reported to be negligible in the Daoy tissue in spite of significant expression in vitro assays[28)].

The activity of immunotoxin can be influenced by other factors, such as drugs or irradiation, and additional in vitro and in vivo studies are required. Daoy, U373 MG, and T98 G human cell lines are frequently used for experiments. Published data are not available about TR expression on T98 G, IGF-1R expression on U373 MG, and IL-4R expression on Daoy and U373 MG. Our results showed expression of TR on Daoy, U373 MG, and T98 G, IGF-1R expression on Daoy and U373 MG, but not on T98 G, and IL-4R expression of Daoy, U373 MG, and T98 G.

Conclusion

We studied the expression of transferrin receptor, insu-

lin-like growth factor-1 receptor, and interleukin-4 receptor on Daoy, U373 MG, and T98 G cell lines by flow cytometric analysis, and found that all receptors were highly expressed on all cell lines tested except insulin-like growth factor-1 receptor on T98 G. The transferrin and interleukin-4 receptors can be good targets for immunotoxin therapy. The results of study of insulin-like growth factor-1 receptor expression may reflect the heterogeneity of the malignant glioma. More studies of other growth factor receptor expression on various kinds of brain tumor cell lines and primary culture of the surgical specimens are required in the treatment of the patients with primary malignant brain tumor using immunotoxins.

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뇌종양세포주에서의 성장인자수용체의 발현 : 면역독소 치료의 연구를 위한 예비실험

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= 국 문 초 록 =

목 적 : 종양세포의 세포면에서는 성장인자 수용체들이 과발현되고 이들이 성장인자들과 결합함으로서 세포의 성장을 촉진하게 된다. 원발성 악성 뇌종양에 대한 새로운 치료법 중 하나가 면역독소를 사용하는 방법이다. 독소는 대상 세포의 세포면에 있는 수용체를 통해 세포질로 이행되며, 면역독소가 선택적인 항종양작용을 나타내기 위해서는 수용체가 과발현되어야 한다. 강력한 세포독성의 효과가 있음에도 불구하고 면역독소를 환자에 투여하였을 때 종양을 완치시키지는 못하였는데, 면역독소의 효과에 영향을 미치는 여러 인자들에 대한 더 많은 연구가 요구된다. 이러한 연구에 사용되어질 뇌종양세포주들에서 성장인자수용체들의 발현 여부를 알아보기 위해 본 연구를 시행하였다.

대상 및 방법 : 한 개의 수모세포종 세포주(Daoy)와 두 개의 교모세포종 세포주(U373 MG, T98 G)에서, transferrin 수용체, insulin-like growth factor-1 수용체, 그리고 interleukin-4 수용체들의 발현을 flow cytometric analysis를 이용하여 조사하였다.

결 과 : Transferrin 수용체와 interleukin-4 수용체는 Daoy, U373 MG, 그리고 T98 G 모두에서 발현되었다. Insulin-like growth factor-1 수용체는 Daoy와 U373 MG에서는 발현되었지만 T98 G에서는 발현되지 않았다.

결 론 : Transferrin 수용체와 interleukin-4 수용체는 면역독소 치료에 적합한 것으로 보인다. 본 실험의 결과는 상기의 세포주를 사용하여 면역독소와 관련된 실험을 하는데 참고가 되어야하며, 면역독소 치료에 있어서 적절한 면역독소를 선택하는 문제 등 치료 모델을 확립하는데 고려되어야 할 것이다.

중심 단어 : 면역독소 · Transferrin 수용체 · Insulin-like growth factor-1 수용체 · Interleukin-4 수용체.