NFAT and AP1 are essential for the expression of a glioblastoma multiforme related IL-13Ra2 transcript

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Abstract. Background: IL-13Ra2 is overexpressed by gliomas but not by normal tissue. However, the molecular basis for IL-13Ra2 overexpression in gliomas is unknown.

Methods: In the present study we have investigated the regulatory mechanisms that are responsible for the expression of IL-13Ra2 with mutation analysis, quantitative RT-PCR, Flow cytometry analysis, transcription factor binding assay and Elisa.

Results: Our results reveal a complex mechanism for regulating IL-13Ra2 expression that involves at least 2 promoters and 4 transcripts of human IL-13Ra2. Transcription factors NFAT and AP1 are necessary and essential for the expression of this GBM related transcript, and are responsible for the high level of expression of IL-13Ra2 in GBM. Most interestingly, we found that expression of this transcript results in the production of a secreted form of IL-13Ra2 and thus may have the potential to be used as a diagnostic biomarker for GBM patients and other cancer patients that express the soluble form of this receptor.

Conclusion: This study is the first to characterize the role of NFAT and AP1 in the regulation of IL-13Ra2 expression, and provides insight into understanding the high levels of IL-13Ra2 expressed by GBM cells.

Keywords: IL-13Ra2, promoter, NFAT, AP1, glioblasma multiforme

1. Introduction

Interleukin 13 (IL-13) is a cytokine with diverse functions on a wide variety of cells. These functions include the activation, recruitment and survival of eosinophils [19,34,41], the proliferation of B cells [17], and the activation of mast cells of the immune system [25]. In non-hematopoietic cells, IL-13 can induce the expression of vascular cell adhesion molecule 1 by endothelial cells [4], enhance the proliferation of smooth muscle cells [15], induce type 1 collagen synthesis in fibroblasts [23], and is an inducer of chemokine expression in epithelial cells [21].

IL-13 typically signals through a type I cytokine receptor. This type I receptor is a heterodimer that is formed from the IL-4 alpha receptor (IL-4Ra) as one component of this complex and the IL-13 receptor alpha 1 chain (IL-13Ra1) as the second component [18]. Another receptor to which IL-13 binds is the interleukin 13 receptor alpha 2 chain (IL-13Ra2). This receptor is a protein of 380 amino acids which was cloned from a human renal cell carcinoma cell line [7]. This receptor chain binds IL-13 with high affinity and was demonstrated to be a specific inhibitor of IL-13 signaling by functioning as a decoy receptor [2]. However, a recent report demonstrated that IL-13 can sig-
nal through the IL-13Ra2 in a Stat6 independent and AP1 dependent manner to induce activation of the TGF beta-1 promoter [16].

Human IL-13Ra2 is expressed at abnormally high levels in glioblastoma multiforme (GBM), the most common primary brain tumor, and also in several other human cancers such as AIDS kaposi’s sarcoma, head and neck cancer [26,29] and ovarian cancer [32], but is only expressed at very low levels in normal tissue except in the testis [11,38]. This finding had elicited an intense interest in targeting IL-13Ra2 with immunotoxins, active and adoptive immunotherapy, and clinical trials are ongoing [13,28,37]. The mechanism by which GBM constitutively express high levels of human IL-13Ra2 is poorly understood. David et al. [9] cloned the promoter of human IL-13Ra2 from a keratinocyte cell line HaCaT. This promoter is located 25 kb from the 5′ end of human IL-13Ra2 gene coding start site (Fig. 1A). Alternative splicing in the 5′ UTR gives rise to 3 different transcripts. To simplify this system, we designated this promoter as promoter N (Fig. 1A), and the 3 transcripts as transcript N1, N2 and N3 (Fig. 1B). The letter ‘N’ was based on the normal cell line from which the promoter was cloned. David et al. [9] demonstrated that Stat6 plays an important role in the transcriptional regulation of the IL-13Ra2 promoter N. Their result was supported by the finding that IL-13 and IL-4 can upregulate IL-13Ra2 expression through Stat6 activation in some tissues [10,50]. However, it was shown that IL-13Ra2 functioned as an inhibitor of an IL-4 dependent pathway in human GBMs [42], and that Stat6 was not activated or activated at low levels in GBM cell lines [42]. This suggests that the high expression of human IL-13Ra2 in GBMs is unlikely to be dependent on Stat6 activation, and that different regulatory mechanism for human IL-13Ra2 expression in GBMs may exist. Consistent with this hypothesis we previously cloned and characterized an alternative human IL-13Ra2 promoter from GBM cell line U118 [45], which is located 1.6 Kb before the 5′ end of the human IL-13Ra2 gene coding start site. By sequencing the different 5′ RACE clones, one transcript was found under the control of this promoter. We designate this promoter as promoter G, and the transcript controlled by this promoter as transcript G (Fig. 1B). Our choice of the letter ‘G’ was based on the GBM cell line from which the promoter was cloned.

In the present study, we demonstrate that the IL-13Ra2 transcript G is GBM related, and that the transcriptional factors NFAT and AP1 are essential for the expression of this transcript and are responsible for the high level of expression of IL-13Ra2 by GBM cells.

2. Material and methods

2.1. Prediction of promoters for human IL-13Ra2 gene

Putative promoters of human IL-13Ra2 gene (accession number: NM_000640) were predicted by using online software Gene2promoter (Genomatix software GmbH, Munchen, Germany). The predicted promoters were blasted with promoter N or promoter G by using Contig Express (Invitrogen, Carlsbad, CA, USA). The putative transcription factor (TF) binding sites for promoter G was predicted by using online software MatInspector (Genomatix).

2.2. Tissue culture and cell lines

GBM cells lines (U87, U118, U138, U373, CRL1620, CRL2365, SF295, GBM1, GBM3, GBM4) were cultured in DMEM supplemented with 10% FBS, 100 µg/ml of penicillin and 100 µg/ml of streptomycin. Cell lines GBM1, GBM3 and GBM4 were established from clinical samples as described previously [47]. Cell lines K562, jurkat, HL60 and colo205 were cultured in RPMI containing 10% heat inactivated FCS, 2 mM glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin. All cells were cultured in a humidified 5% CO2 incubator at 37°C. To induce the expression of IL-13Ra2, PMA (10 ng/ml) and Ionomycin (500 ng/ml) were added to K562 cells.

2.3. Mutation analysis

In vitro mutation was accomplished in the vector pSEAP-IL-13Ra2S by using a transformer site-directed mutagenesis kit (Clontech, Mountain View, CA, USA) according to manufacturer’s instructions. The mutation primer was: 5′-GGGGTTCCAGcttAAAAGCGTTCATAAT (lower case letters indicate the mutation). The selection primer was: 5′-CCATCCAGTCTAcTAgTTGTTGGCGGAAAG-3′ (lower case letters indicate the mutation that leads to the loss of the hind III site). The sequence of the mutation was verified by automated sequencing. For transfection, U118 cells were seeded into 24 well plates, 24 h later 1 µg of each pSEAP reporter plasmid and 1 µg pKT2/clp-luc [46] were transfected into U118 cells by using TransIt-LT1 reagent (Mirus, Madison, WI, USA) according to the manufacturer’s instructions. SEAP activity was quantified using a phosphatase substrate kit (Pierce, Rockford, IL, USA) according to the manufacturer’s proto-
col. SEAP activities were read at 405 nm with a microplate reader (Bio-Tek, Winooski, VT, USA). Luciferase activities were examined by using luciferase assay kit (Promega, Madison, WI, USA), and the values were used to normalize the SEAP activities. All measurements were performed in triplicate, and the measurements of SEAP were normalized to Luc activities and divided by the values from samples transfected with a promoterless pSEAP basic vector.

2.4. Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from cell lines with the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to manufacturer’s protocol. Purified RNA was treated with DNase (Ambion Inc., Austin, TX, USA). mRNA from normal human tissues (lung, spleen, liver, brain) was ordered from Origene (Rockville, MD, USA). The expression of human IL-13Ra2 mRNA was investigated with the Superscript III Platinum One Step RT-PCR kit (Invitrogen). The reactions were carried out with a reverse step of 3 min at 50°C, a polymerase activating step of 5 min at 95°C, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s and 68°C for 30 s. The primers used are shown in Table 1. Ten μl of product was analyzed on a 1.2% agarose gel containing 1/10000 Gelstar (Biowhittaker Molecular Applications, Rockland, ME, USA) and photographed.

For quantitative RT-PCR, the Superscript III Platinum SYBR Green One Step qRT-PCR kit (Invitrogen) was used to investigate the expression of IL-13Ra2 transcript G. In normal tissues, the Tissue Scan Real-Time Gene Expression panel (Human-48, Origene, Rockville, MD, USA) was used, and PCR was performed according to the manufacturer’s instructions. 48 normal human tissues were studied (http://www.origene.com/qPCR/getTissueScan.aspx?id=40). The reactions were carried out with a reverse step of 50°C for 3 min, a polymerase activating step of 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s and 68°C for 30 s. Melting curves and gel analysis were performed to confirm the presence of predicted products. The levels of gene expression were normalized to the GAPDH levels, and are presented as relative expression. Samples were analyzed in triplicate, and the results are shown as the mean value ± standard error.

2.5. Flow cytometry

For flow cytometric analysis of IL-13Ra2 expression, cells were incubated with PE conjugated anti human IL-13Ra2 mAb (Cell Sciences, Canton, MA, USA) for 30 min at 4°C. After washing, cells were analyzed on BD FACstation (BD Biosciences, San Jose, CA, USA). PE labeled mouse isotype IgG1 (BD Biosciences) was used as control.

2.6. Oligodeoxynucleotides (ODNs) and transfection

NFAT decoy ODN, AP1 decoy ODN, control ODNs and biotin end labeled probes were ordered from Genedetect (Bradenton, FL, USA). The sequence of the ODNs and probes are showed in Table 2. The lyophilized ODNs and probes were dissolved in DNase and RNase free TE buffer at a concentration of 20 μM, and stored at −20°C, the transfections were performed by using Oligofectamine transfection kit (Invitrogen) for U87 cells, and superfect transfection reagent (Qiagen) for K562 cells, according to the manufacturers’ instruction. One μM final concentration of ODNs were used for the transfections.

2.7. Transcription factor binding assay

To investigate the binding of transcription factors NFAT and AP1 to the consensus sequence at promoter G, cells were transfected with decoy ODNs as described above, or treated with NFAT or AP1 inhibitors, and 2 h later PMA and Iono were added. After 2 h incubation in PMA/Iono, cells were washed twice with cold PBS, and nuclear proteins were isolated with a nuclear protein isolation kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer’s protocol. Protein concentration was determined using a Bradford Protein Assay Kit (Pierce, Pittsburgh, PA, USA). DNA
protein binding assays were performed as described in Fig. 3C. Briefly, biotin labeled double stranded ODNs (Table 2) containing the consensus sites for NFAT or AP1 were added to streptavidin coated 96 well plates. Ten µg of nuclear extracts were then added and incubated for 2 h. Unbound proteins were washed by using transcriptional factor assay buffer, and bound proteins were incubated with rabbit anti-NFAT (Abcam, Cambridge, MA, USA), rabbit anti C-fos (Abcam) or rabbit anti C-jun antibody (Abcam) for 1 h. HRP labeled anti-rabbit secondary antibody (Chemicon International, Temecula, CA, USA) was then added and incubated for another 30 min. After washing, TMB substrate was added for color development, and plates were read at 450 nm with EL311 plate reader (Biotek, Winooski, VT, USA). For cold competition, a 10-fold excess of the AP1 or NFAT double stranded ODN was added, respectively, to the binding reaction. Untreated cells were used as controls. A series of additional control experiments with an excess of unrelated ODNs were performed to assess the specificity of binding. All measurements were performed in triplicate.

2.9. Statistical analysis

Data are presented as mean ± standard error of the mean. Statistical significance was determined by paired t-tests and differences were considered significant at \( p \leq 0.05 \).

3. Results

3.1. Predicted promoters of the human IL-13Ra2 gene match identified promoters

With the completion of the human genome project, biological software has been developed to rapidly screen and analyze large quantities of nucleotide sequences. Based on the main structural features of promoters and the potential binding sites for transcription factors, several promoter prediction algorithms have been established. In this study, the online software Gene2Promoter, which showed high sensitivity and specificity in predicting promoters, was used to identify the promoter regions of human IL-13Ra2. As shown in Fig. 1A, 4 promoters were predicted. Predicted promoter 1 is located at 25 kb from the 5' end of human IL-13Ra2 gene coding start site, and was matched with promoter N. Predicted promoter 2 is located at 4.1 kb from the coding start site. Predicted promoter 3 is located at 1.6 kb from the coding start site, which was matched with promoter G. Predicted promoter 4 is located at 0.27 kb from the coding start site. These results are intriguing because the two promoters characterized for IL-13Ra2 (promoter N and promoter G) were among the predicted promoters. This reflects the accuracy of the software prediction program, and raises the question of whether predicted promoters 2 and 4 are alternative un-characterized promoters of human IL-13Ra2 gene. This issue is worth further investigation, since the identification and characteriza-
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3.2. Expression of IL-13Ra2 transcripts in tumor cell lines and normal tissues

The identification of two IL-13Ra2 promoters (promoter N and promoter G) (Fig. 1A) and related transcripts (N1, N2, N3 and G) (Fig. 1B) provide possible clues regarding these mechanisms. Our hypothesis is that different IL-13Ra2 promoters are activated under different circumstances. To test this hypothesis, we designed transcript specific primers (Table 1), and performed RT-PCR to investigate the expression of different IL-13Ra2 transcripts in normal human tissues, human GBM cell lines and in other tumor cell lines (Fig. 1C). As we predicted, transcripts N1, N2 and N3 were only expressed in normal tissues such as liver, lung, and spleen, but not expressed in any GBM cells.
Fig. 1C. RT-PCR analysis the expression of human IL-13Ra2 transcripts (N1, N2, N3, G) in tumor cell lines and some normal tissues. Primers for human GAPDH were used as positive control, for every reaction, and water without template was used as a negative control. (a) The expression of IL-13Ra2 transcripts in tumor cell lines (1. U87, 2. U118, 3. U138, 4. U373, 5. CRL1620, 6. K562, 7. Jurkat, 8. CRL2365, 9. HL60, 10. GBM3, 11. GBM4, 12. SF295, 13. GBM1, 14. colo205). (b) The expression of human IL-13Ra2 transcripts in some normal human tissues (lung, liver, spleen, brain, PBMC). RT-PCR products were electrophoresed and stained with Gelstar (Biowhittaker Molecular Applications, Rockland). The gel electrophoresis from one of three representative sets of PCR reactions is shown.

or other tumor cell lines. In contrast, transcript G was expressed in all of the GBM cell lines we investigated except GBM1, whereas normal tissues and other cell lines did not show any expression of this transcript (Fig. 1C). This result suggests that transcript G is a GBM related IL-13Ra2 transcript. Since it would be important to know whether or not this GBM related transcript is expressed in any normal tissues, we investigated its expression in 48 normal human tissues using the Tissue Scan Real Time PCR kit (Origene, Rockville, MD, USA). The results of this scan indicate that most normal human tissues do not express this IL-13Ra2 transcript, except kidney and adrenal glands.

3.3. NFAT binding site in promoter G is necessary for transcriptional regulation

The specific expression of transcript G by GBM cells suggests that the activation of promoter G may be responsible for the high expression of human IL-13Ra2 by GBM cells. Using the online software MatInspector, the potential transcript binding sites NFAT and AP1 were predicted for promoter G (Fig. 2A). In a previous study we demonstrated that the AP1 binding site plays an important role in regulating the activity of the human IL-13Ra2 promoter G [45]. AP1 is a very prevalent transcription factor, and usually cooperates with other transcriptional factors such as NFAT to modulate gene expression [35]. The prediction of an NFAT binding site near the AP1 binding site (Fig. 2A) may suggest such a cooperative action. To assess the importance of the NFAT binding site in regulating the expression of human IL-13Ra2, a mutation analysis using a SEAP reporter plasmid was performed. The expression of reporter protein SEAP in this plasmid was controlled by the human IL-13Ra2 promoter G (Fig. 2B) that we previously cloned [45]. In transient transfection experiments using U118 GBM-derived cells, mu-
Fig. 2A. The nucleotide sequence of the promoter G region. The promoter G sequence (Genbank Accession No: AF473808) is indicated by a single underline. The transcriptional initiation site is in bold and assigned by +1. Putative binding sites for transcriptional factors were predicted by using the Matinspector program (Genomatix, Germany) and are indicated by labels above the sites.

Fig. 2B. In vitro mutagenesis analysis. (a) Scheme of plasmids used. (b) Human glioma cell line U118 was transfected with various reporter gene constructs and assayed for SEAP activity. NFAT mutation is indicated. SEAP activity of every transfection was normalized with the luciferase activity by cotransfection of pKT2/CLP-luc plasmid, and relative to the basal SEAP activity obtained with the pSEAP basic plasmid. The column and bars represent the mean and standard deviation, respectively. All the values were obtained from 3 independent experiments.
3.4. Induction of human IL-13Ra2 transcript G expression in K562 cells

Based on the NFAT mutation analysis and our previous studies [45], we identified that the cis-acting elements (NFAT and AP1 binding sites) are required for the expression of IL-13Ra2 transcript G. We next sought to determine if the NFAT and AP1 transcription factors were both necessary and required for IL-13Ra2 expression in GBM cells. This analysis was performed using an erythroleukemia cell line K562. K562 has been used as a model cell line for the investigation of AP1 regulated gene expression [6,14]. A K562 NFAT reporter cell line has been developed by Panomics (Fremont, CA, USA) as a product. There is evidence that 12-myristate 13-acetate phorbol (PMA) and ionomycin can activate NFAT and AP1 in K562 cells [3,6,14], RT-PCR analysis revealed that K562 cells do not express IL-13Ra2 transcripts (Fig. 1C). We postulated therefore that treatment with PMA and ionomycin might induce the expression of IL-13Ra2 transcript G in K562 cells via NFAT and AP1 activation. To test this, we cultured K562 cells in the presence of PMA and ionomycin for 24 h, and monitored the induction of IL-13Ra2 transcripts by RT-PCR. As expected, treatment with PMA and ionomycin resulted in the induction of IL-13Ra2 transcript G expression in K562 cells, and the induction was confirmed at both mRNA and protein levels (Fig. 2C, D). In contrast, no induction of transcripts N1, N2 or N3 was detected (Fig. 2C).

3.5. Inhibition of NFAT or AP1 can prevent the induction of IL-13Ra2 transcript G in K562 cells

We next asked whether the induction of IL-13Ra2 transcript G expression can be suppressed by the inhibition of NFAT or AP1. To address this question, we selected SP600125 as an AP1 inhibitor. It is well known that SP600125 can inhibit the ability of JNK mediated C-Jun phosphorylation and AP1 transcriptional activity [5]. We previously demonstrated that the expression of human IL-13Ra2 gene in GBM-derived U87 cells can be partly inhibited by SP600125 [45]. NFAT activity is regulated by Calcineurin, and the specific NFAT inhibitor (INCA-6) we used in this experiment is a cell permeable compound that acts as a potent and selective inhibitor of Calcineurin–NFAT association [24]. Unlike other NFAT inhibitors such as CSA and FK506, INCA6 is much more specific and does not affect other signaling pathways that are also regulated by calcineurin [24].

K562 cells were pretreated with JNK inhibitor SP600125 or NFAT inhibitor INCA6 for 2 h followed by stimulation with PMA and Ionomycin. Eighteen hours later the expression of the human IL-13Ra2 transcript G was evaluated by realtime-RT-PCR. As we predicted, the induction of the human IL-13Ra2 transcript G in K562 cells was suppressed by these two inhibitors, and the inhibition was dose dependent (Fig. 3A, B). At a relatively high dose (35 µM), the NFAT inhibitor INCA-6 completely abolished the expression of IL-13Ra2 transcript G, and SP600125 at 30 µM inhibited expression to a very low level. These results indicate that both the JNK pathway and NFAT pathway were essential in the regulation of IL-13Ra2 expression.

3.6. NFAT ODN or AP1 ODNs can inhibit the induction of IL-13Ra2 transcript G in K562 cells

In order to directly confirm the involvement of NFAT and AP1 transcript factors in the regulation of the GBM related IL-13Ra2 transcript, a sequence specific inhibitor was used. Double stranded decoy ODNs have been widely used as sequence specific inhibitors to block the function of TFs [43]. Transfection of double stranded decoy ODNs results in the attenuation
Fig. 2D. (a) Flow cytometric analysis of human IL-13Ra2 expression on K562 cells after the stimulation of PMA/iono. (b) The induction of human IL-13Ra2 expression on the surface of K562 cells was effectively inhibited by using NFAT or AP1 specific ODNs. Each histogram is representative of three independent evaluations. The flow cytometric analysis was performed using an anti-human IL-13Ra2 mAb and an isotype IgG as a control.

of cis–trans interactions, leading to the suppression of genes in a sequence specific manner. Therefore, we used double stranded DNA ODNs which are specific for AP1 or NFAT in this experiment. We transfected decoy AP1, NFAT ODNs or control ODNs (mutant ODNs) into K562 cells, followed by PMA/Ionomycin stimulation. The expression of human IL-13Ra2 was measured by both realtime RT-PCR and flow cytometry. As expected, by silencing NFAT and AP1 using sequence specific ODNs, the induction of human IL-13Ra2 mRNA expression in K562 cells was significantly attenuated (Fig. 3A, B). In contrast, the mutant ODNs (that do not interact with AP1 or NFAT binding sites) do not show any inhibitory effects on the induction of IL-13Ra2 expression (Fig. 3A, B). These results demonstrate that the inhibition is sequence specific and provide direct evidence that transcription factors NFAT and AP1 are both necessary for the expression of GBM related IL-13Ra2 transcript.

3.7. Transcription factors NFAT and AP1 (C-fos and C-jun) can bind consensus sequences in the GBM related IL-13Ra2 promoter

To initiate gene expression, transcription factors need to be activated, translocated to the nucleus, and bound to cis-elements. We therefore sought to investigate whether or not NFAT and AP1 can bind to relative consensus sites in the promoter region of IL-13Ra2 transcript G. To determine the binding of AP1 and NFAT to the promoter sequence, a simple, quantitative, non-radioactive strategy was used as illustrated in Fig. 3C. Our result showed that after incubation with nuclear extracts of PMA/Iono stimulated K562 cells,
Fig. 3A. (a) The induction of human IL-13Ra2 transcript G in K562 cells by PMA/Iono was inhibited by AP1 inhibitor Sp600125 (in a dose dependent manner) and AP1 decoy ODN, but not mutant AP1 ODN. The expression was evaluated by real-time RT-PCR. Values were normalized to GAPDH and indicated as relative expression. Data are shown as means ± SD of three separate experiments. (b) Induction of human IL-13Ra2 transcript G in K562 cells by PMA/Iono is inhibited by NFAT inhibitor (in a dose dependent manner) and NFAT decoy ODN, but not mutant NFAT ODN. The expression was evaluated by real time RT-PCR. Values were normalized to GAPDH and indicated as relative expression. Data are shown as means ± SD of three separate experiments.

but not with extracts from unstimulated cells, ODN probes designed to exhibit NFAT or AP1 binding sites identical to that of the IL-13Ra2 promoter were capable of binding NFAT or AP1 (C-fos and C-Jun), respectively (Fig. 3D). This provides evidence that NFAT and AP1 can bind to the putative/predicted NFAT and AP1 binding sites in the IL-13Ra2 promoter. Additionally, it suggests that PMA/Iono treatment can activate NFAT and AP1 in the K562 cells, as shown in T cells [33]. Most importantly, the binding of TFs to the relative promoter sites can be blocked by excess of competitor probes, but not by mutant probes, which demonstrates the specificity of this binding (Fig. 3D). When the cells were treated with an AP1 inhibitor (SP600125) or an NFAT inhibitor (INCA-6), followed by PMA/Iono stimulation, we only detected a weak binding of tran-
Fig. 3B. Scheme of transcriptional-DNA binding assay. Biotin labeled double stranded NFAT or AP1 decoy ODN were added to streptavidin-coated 96-well plates. 10 µg of nuclear extracts were added. After incubation for 2 h unbound proteins were washed away, bound proteins were identified with rabbit anti NFAT, rabbit anti C-fos or rabbit anti C-jun antibody. HRP labeled anti-rabbit secondary antibody was then added and incubated for 30 min. After washing, TMB substrate was added for color development, and plates were read at 450 nm with EL311 plate reader.

transcription factors with the associated DNA binding sites (Fig. 3D), which indicates that the AP1 inhibitor and the NFAT inhibitor can specifically inhibit the activation of the respective transcription factors, and decrease their DNA binding. This is consistent with the finding that SP600125 and INCA-6 decrease IL-13Ra2 expression in a dose dependent manner. These data reinforce the notion that AP1 and NFAT are necessary and sufficient for the transcriptional regulation of IL-13Ra2 transcript G. In addition, the DNA binding assays indicated that C-Jun and C-fos are components of the AP1 transcription factor that bind to the IL-13Ra2 promoter.

3.8. NFAT ODN or AP1 ODN can inhibit the expression of human IL-13Ra2 in GBM cell line U87

Our results indicated that NFAT and AP1 may be responsible for the high level of expression of IL-13Ra2 in human GBM. To support this notion further, we transfected NFAT or AP1 decoy ODN into U87 GBM cells. The results of this experiment showed that both NFAT and AP1 decoy ODNs can significantly suppress the expression of IL-13Ra2 in U87 cells compared with mutant ODNs (Fig. 4A). These results support our hypothesis that NFAT and AP1 are essential for the expression of IL-13Ra2 in GBM cells. Consistent with these results is the evidence showing that transcription factors AP1 and NFAT are activated in GBMs [1,39].

3.9. Human IL-13Ra2 transcript G can be induced in PBMCs

Our results thus far suggest that IL-13Ra2 transcript G is inducible. To provide further support for this concept, we incubated human PBMC with PMA/Iono, and 24 h later we were able to detect IL-13Ra2 transcript G in PBMC cells using real time RT-PCR. Most interestingly, soluble IL-2 receptor had a significant inhibitory effect on this IL-13Ra2 induction in PBMC cells (Fig. 4B). This indicates that the IL-2 signaling pathway and other transcription factors in addition to NFAT and AP1 may be involved in the regulation of transcript G expression. Using the decoy ODN approach, we found that the expression of IL-13Ra2 in U87 cells can be inhibited by the ODN of Stat5 (Fig. 4C). This result suggests that Stat5, a transcription factor in IL-2 signal pathway may be involved in the regulation of IL-13Ra2. However, the exact mechanism for Stat5 regulation needs to be further investigated, and is not the focus of the present study.
Fig. 3C. Transcriptional factor-DNA binding assay. (a) The binding of transcript factors NFAT with the probes containing NFAT binding sites at promoter G. (b) The binding of transcript factor AP1 (C-fos and C-jun) with the probe containing AP1 binding site at promoter G. For both (a) and (b), nuclear lysate was prepared from PMA/Iono stimulated K562 cells as indicated in material and methods. The binding assay was performed as illustrated in Fig. 3C, samples without primary antibody were used as control. (c) The binding of NFAT to its consensus sequence can be significantly inhibited by NAFT inhibitor or NFAT decoy ODN. Two hours before the PMA/Iono stimulation, cells were treated with NFAT inhibitor or transfected with NFAT decoy ODN. Two hours after the PMA/Iono stimulation, the nuclear lysate from the cells were prepared, and the DNA binding assay was performed as indicated above. (d) The binding of C-jun (component of AP1) to AP1 consensus sequence in the promoter G can be significantly inhibited by AP1 inhibitor or AP1 decoy ODN. Two hours before the PMA/Iono stimulation, cells were treated with AP1 inhibitor or transfected with AP1 decoy ODN. Two hours after the PMA/Iono stimulation, the nuclear lysate from the cells were prepared, and the DNA binding assay was performed as indicated above. Columns and bars represent the mean and standard deviation, respectively. All the values were obtained from 3 independent experiments.

Fig. 4A. The expression of human IL-13Ra2 transcript G in U87 cells can be inhibited with NFAT decoy ODN or AP1 decoy ODN. NFAT mutant ODN or AP1 mutant ODN was used as control.
Fig. 4B. The induction of human IL-13Ra2 transcript G in PBMC. Human IL-13Ra2 transcript G can be induced in PBMCs with the stimulation of PMA/Iono, and the induction can be inhibited by soluble IL-2 receptors.

Fig. 4C. The expression of human IL-13Ra2 transcript G in U87 cells can be inhibited with stat5 decoy ODN. Stat5 mutant ODN was used as control. For Fig. 4A–C, the expression of human IL-13Ra2 transcript G was evaluated with real-time RT-PCR. Normalization of the data was done against control GAPDH expression, and indicated as relative expression. Columns and bars represent mean and SD, respectively, and represent values from three independent experiments.

3.10. IL-13Ra2 transcript G can be detected in culture supernatant

Previous studies have shown that soluble IL-13Ra2 exists in mouse urine and serum [51]. We therefore wanted to investigate whether or not the human IL13Ra2 protein could also be secreted. Using methods of ELISA, the IL-13Ra2 protein was detected in the supernatant of GBM U118 and PMA/Iono treated K562 cells, but not in the supernatant of untreated K562 cells (Fig. 4D). This observation confirms the secretion of soluble human IL-13Ra2, and at the same time indicates that the secreted IL-13Ra2 may be related to transcript G, but more work needs to be done to characterize this property of transcript G.

4. Discussion

Human IL-13Ra2 is highly expressed in human GBM, but present at extremely low levels in normal human cells [11,38]. The identification of promoter G
and promoter N that drive the expression of IL-13Ra2, and the further characterization of the regulatory mechanisms for these promoters may provide an explanation for this difference in expression levels. In this study, we demonstrated that IL-13Ra2 promoter G is responsible for the high expression of human IL-13Ra2 in GBM, and suggests its possible usage in GBM-specific gene therapy. We found that transcripts NFAT and AP1 are necessary for the expression of GBM related IL-13Ra2 transcript G. Our results indicate that the high expression of IL-13Ra2 in GBMs may be a result of the combined activation of NFAT, AP1 and possibly other TFs. The expression of IL-13Ra2 may only be a phenomenon following the activation of specific transcription factors, and thus may not provide growth advantage for tumor cells. This was partly supported by Kawakami et al. [27], who demonstrated that in vivo overexpression of IL-13Ra2 chain inhibits tumorigenicity of human breast and pancreatic tumors in mice. However, further investigation to define the role of IL-13Ra2 in GBM progression is needed.

The expression of IL-13Ra2 has been found in gliomas other than GBM, such as some low grade astrocytomas [48], anaplastic astrocytomas [48], medulloblastomas [30], ependymomas [12,30], oligodendrogliaomas [12]. Debinski et al. [12] demonstrated that only a small portion of low grade gliomas express IL-13Ra2, while high grade gliomas ubiquitously demonstrated high levels of IL-13Ra2 expression. This result indicated that IL-13Ra2 is associated with the progression of glioma from low to high grade. The expression tendency of IL-13Ra2 in glioma can be explained by the activation of AP1 and NFAT, which we confirmed to be responsible for the specific expression pattern of IL-13Ra2. Assimakopoulou et al. [1] demonstrated that AP1 was activated in the majority of GBM whereas only minimal expression was noted in low grade gliomas. In one other experiment (manuscript in preparation), we found NFAT was activated in gliomas and the activation was related to the glioma progression. Pilocytic astrocytoma is a low grade glioma that expresses a high level of IL-13Ra2 [12], however this is not contradictory to our result, since Assimakopoulou et al. [1] confirmed the highly activated AP1 in pilocytic astrocytomas.

The high expression of IL-13Ra2 on the surface of GBM cells has been used for targeted delivery of IL-13 based immunotoxin, which showed potent antitumor effects [20,28,31]. Furthermore, researchers have demonstrated that IL-13Ra2 can be used as a novel GBM related antigen in immunotherapy [13,40]. However, in a completed Phase III clinical trial, IL-13 based immunotoxin IL-13-PE38QQR was found to be no more effective than an existing therapy in prolonging survival, Jarboe et al. [22] explained this result as the low frequency of IL-13Ra2 overexpression in GBMs. However, our result indicated that the expression of soluble IL-13Ra2 may be partly responsible for the low treatment efficiency. We proposed that the soluble IL-13Ra2 secreted by the glioma cells will bind the immunotoxin and may decrease the treatment effect. Thus it will be important for us to further investigate
the secretion mechanism of IL-13Ra2, which may help us to develop a strategy to decrease the expression of soluble IL-13Ra2 and increase the expression of membrane bound IL-13Ra2 in the surface of glioma cells. On the other hand, the expression of soluble IL-13Ra2 may affect the role of IL-13, and thus may affect the local immune microenvironment at the tumor site. We may develop adjuvant immunotherapy methods by up-regulating or downregulating the expression of soluble IL-13Ra2.

In addition to serving as a target for glioma therapy, this receptor may have the potential to serve as a biomarker of malignant glioma for monitoring recurrence. This is possible since we found that the activation of the GBM related IL-13Ra2 transcript G can result in the secretion of soluble IL-13Ra2 into the supernatant. This is consistent with other researchers’ results, which have showed that IL-13Ra2 exist as a soluble receptor in the urine and serum of mice [51]. It was reported that soluble receptors such as soluble interleukin 2 receptor alpha (sIL2Ra) has been detected in the serum of patients with malignant glioma [49]. Actually, a growing body of evidence demonstrated that a wide variety of immune related cytokines and their cognate receptors are expressed to some extent by human malignant glioma cells. We think the soluble IL-13Ra2 may be also examined in the serum like the soluble IL-2Ra. However the concentration of IL-13Ra2 in serum and CSF and its relationship with the clinical pathological characteristics of glioma patients are unknown, this is an important question requiring further investigation and is out of the focus of this research.

Soluble cytokine receptors are important regulators of inflammation and immunity. It is now clear that IL-13Ra2 plays an important role in the regulation of immune response [8], and some investigators have demonstrated a critical role for IL-13Ra2 in the down regulation of the granulomatous inflammation in schistosomiasis [36]. Soluble IL-13Ra2 in the serum has been reported to act as an inhibitory protein regulating IL-13 responses [44]. In this study, we demonstrated that the tumor related IL-13Ra2 transcript G is inducible, and can be induced in PBMCs by incubation with PMA/Iono. Most interestingly, our results indicated that the IL-2 pathway may regulate IL-13Ra2 transcript G expression in PMA/Iono stimulated PBMCs, and Stat5, a TF in IL-2 pathway, may play a role in its regulation. Further investigations are needed to fully understand the regulatory mechanisms for this GBM related IL-13Ra2 transcript.

The importance of IL-13Ra2 in immune regulation emphasizes the need for detailed studies on its expression and regulation. Although two human IL-13Ra2 promoters were cloned and characterized, the software prediction program used in this study indicates that other uncharacterized IL-13Ra2 promoters and transcripts may exist. Until now, most research related to IL-13Ra2 has been performed in a mouse model whereby human tumor xenografts are employed. It will be very interesting to investigate whether similar IL-13Ra2 promoters, transcripts or similar regulatory mechanisms also exist in the mouse. This may be important to better understand human diseases based on mouse data.

In summary, our results indicate that there is a complex mechanism for regulating IL-13Ra2 expression. At least 2 promoters and 4 transcripts of human IL-13Ra2 exist. Transcript G is GBM related and inducible. Transcription factors NFAT and AP1 are necessary and essential for the expression of this GBM related transcript, and are responsible for the high level of expression of IL-13Ra2 in GBM. In addition to NFAT and AP1, other TFs such as Stat5 may also be involved in the transcriptional regulation of IL-13Ra2 transcript G. Most interestingly, although this transcript does not change the predicted protein, its expression and potential cleavage from the cell membrane may lead to a soluble form of IL-13Ra2 protein. The soluble IL-13Ra2 protein may have the potential to be used as a diagnostic biomarker for GBM patients and other cancer patients that express the soluble form of this receptor, although the mechanisms of this secretion are still unknown. This study is the first to characterize the role of NFAT and AP1 in the regulation of IL-13Ra2 expression, and our results provide important clues for understanding the high level of IL-13Ra2 expression in GBM cells.

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References


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