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FcεRI-α siRNA Inhibits the Antigen-Induced Activation of Mast Cells

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ABSTRACT

FcεRI, The high affinity receptor for IgE plays a critical role in triggering the allergic reactions. It is responsible for inducing mast cell degranulation and deliberation of allergy mediators when it is aggregated by allergen and IgE complexes. FcεRI on the mast cells consists of three subunits; α chain directly binds IgE, β chain and dimmer of γ chains together mediate intracellular signaling. Cross-linking of IgE-bound FcεRI on the surface of mast cells and basophils by the multivalent antigen induces release of chemical mediators. The present in vitro study was designed to investigate the effect of synthetic FcεRI-α siRNA on the antigen-induced activation of MC/9 cells.

MC/9 cells which are murine mast cells were transfected by FcεRI-α siRNA and negative control siRNA. After 6 h, anti-DNP (Dinitrophenyl) IgE was used for the cells sensitization. Then the cells were challenged with Dinitrophenyl-Human Serum Albumin (DNP-HSA) for mast cell degranulation induction before collection of supernatants. The amount of mRNA and protein expression was measured by Real Time PCR and western blot analysis, respectively. Determination of the expression rate of FcεRI-α on cell surface was achieved by flow cytometry. ELISA and spectrophotometry methods were used subsequently for measuring the effects of FcεRI-α siRNA on antigen-induced histamine and β-hexosaminidase release. FcεRI-α siRNA treated cells showed significant decrease in FcεRI-α mRNA and protein expression in comparison to control cells. FcεRI-mediated mast cell release of β-hexosaminidase and histamine were also inhibited.

In this study it was shown that FcεRI-α siRNA could suppress FcεRI-α expression and inhibited degranulation and histamine release in antigen-stimulated MC/9 cells. In conclusion, knock-down of FcεRI-α by siRNA could be a promising method for inhibition of the mast cell-mediated allergic reactions.

Keyword: FcεRI-α; Histamine release and degranulation; Mast cell line; siRNA;

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INTRODUCTION

Allergy is an undesirable immune response against non-infectious substances such as pollens, drugs and chemical components.[1,2] In allergic disorders, immune responses progress through T helper2 (Th2) cells and production of Th2 cytokines. The allergic reaction is primarily initiated by activation of high-affinity IgE receptors (FcεRI). It is characterized by cross-linking the cell surface-bound IgE with multivalent allergens and subsequently activation of mast cells.[3] This process triggers a tyrosine-phosphorylation cascade that results in degranulation of mast cells and production and deliberation of several mediators, such as histamine, cytokines, leuckotrins and prostaglandins. The mediators ultimately cause the allergic inflammation and anaphylactic reactions.[4,5]

Murine FcεRI is only expressed in mast cells and basophiles and forms a tetrameric structure, α, β and two disulphide-linked γ chains. FcεRI-α constitutes the extra-cellular component which directly binds to region of the Cε2 and/or Cε3 domains of IgE, and has a key role in triggering allergic reaction, while β and γ chains mediate intra-cellular signals.[6,7]

The common strategy for control of the allergic disorders is using drugs such as corticosteroids and anti histamines, but the main problem is the rapid recurrence of symptoms and inflammations when the treatment is discontinued. To overcome these problems, recent studies have been focused on the development of novel therapeutics approaches.[8,9]

RNA interference (RNAi), as an effective therapeutic strategy, has been developed in recent years. It includes a double-stranded RNA (dsRNA) which induces the sequence-specific degradation of cognate RNA and serves as a cellular defense mechanism against viral dsRNA and transposable genetic elements in plants and animals.[10,11] Small interfering RNA (siRNA) is used as a potent, selective, and easily inducible gene silencing molecule for blocking expression of the desired genes.[12,13] Several genes and molecules inducing the allergic diseases such as transcription factors, signaling molecules, cytokines, chemokines, surface receptors and adhesion molecules can potentially be targeted by RNAi.[9]

The aims of this study were to knockdown the expression of FcεRI-α subunit by siRNA and to evaluate the rate of degranulation and release of histamine from murine mast cell line, MC/9, as an allergic response index.

MATERIAL AND METHODS

Cell Culture and Media

Murine mast cell line, MC/9 (CRL-8306), was provided from ATCC (USA). The cells were cultured in RPMI 1640 medium (Gibco, USA) containing 10% heat inactivated fetal bovine serum (Gibco, USA), 3 ng/ml IL-3 (Pepro tech, USA), 100U/ml penicillin, 100µg/ml streptomycin, 100µM 2-mercaptoethanol, 10 µM minimum essential medium nonessential amino acids solution (Sigma-Aldrich, USA) and incubated in a humidified atmosphere at 5% CO2 at 37°C.

siRNA

FcεRI-α siRNA was provided from Qiagen (USA). The sequences included sense and anti-sense as follows: sense/5′-CAAGAAAGUUGAAACCGAA dTdT-3′ and antisense/5′-UUCGGUUUCAACUUUC UUGdTdT-3′ and negative siRNA (Qiagen) was also used as a control.

The siRNA Transfection

Cells were resuspended in fresh medium and transfected with FcεRI-α-specific silencing siRNA or nonsilencing siRNA (Negative control) using X-tremeGENE as a transfection reagent, according to the manufacturer's protocol (Roche, Germany). Briefly, 2 $\times 10^5$ cells were cultured in six-well culture plates containing 1.8 ml of RPMI per well without any additives. For each well 70 nM siRNA was diluted in Opti-MEM to give a final volume 200 µl and incubated in the presence of 10 µl X-tremGENE for 20 min at room temperature and then the mixture was added dropwise to the culture medium. After 6 h of incubation, Fetal Bovine Serum (FBS), antibiotics and 3 ng/ml IL-3 were added. Transfected cells and Control cells were allowed to recover for 24 h and 48 h in the medium. For sensitization, 200ng/ml anti-DNP IgE (Sigma, USA) added and the plates was kept overnight and then challenged with 20ng/ml DNP/HSA (Sigma, USA) at 37°C for 30 min before proceeding with the following experiments.

Real Time PCR

Total RNA was extracted with Tripure isolation reagent (Roche, Germany), and then cDNA was

prepared with Revertaid first strand cDNA synthesis kit, according to the manufacturer's protocol (Fermentas, USA). The FcεRI-α cDNA was amplified by the FcεRI-α primer (QuantiTect Primer Assay, Qiagen, USA) and Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) by the forward primer: 5′-ACTCACGGCAAATTCAACGGC-3′, and reverse primer: 5′- ATCACAAACATGGGGGCATCG-3′.

Analysis of FcεRI-α gene expression was performed by Real Time PCR method using SYBR Green PCR Master mix (Roche, Germany) and Roche Light Cycler apparatus. Each Roche capillary contained a volume of 20 µl of solution consisting of 4 µl master mix, 2 µl primers, 12 µl water (PCR grade), and 2 µl cDNA template. GAPDH was used as an internal control for normalizing the expression values. PCR conditions were 95°C for 5 min, 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s (55 cycles). Standard curves were plotted for both internal control and our interest gene. Relative expression of FcεRI-α mRNA in MC/9 cells was quantified by the BIO-RAD software.

Western Blot Analysis

Transfected Cells (2×10^5) by siRNA and control siRNA were washed and pelleted by centrifugation. Cells were then lysed in 100 µl lysis buffer (150 mM Nacl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 1 mM PMSF, 5µ/ml leupeptin, 2 µ/ml aprotinin) on ice for 30 min. Lysates were centrifuged at 10,000 g at 4°C for 10 min and was boiled for 5 min. 20µl of the supernatant were used for western blot analysis of the FcεRI-α. For western blot analysis, proteins were separated on a 13% sodium dodecyl sulphate polyacrilamide gel. After transfer to polyvinylidene difluoride membrane and blocking of the membrane with 3% skimed milk, blots were probed with anti-FcεRI-α (eBioscience, USA) and anti β-Actin antibodies (Sigma, USA), were allowed to react for 2 h at room temperature or overnight at 4°C. Subsequently, membrane was probed with horseradish peroxidase–conjugated anti-mouse IgG antibody as the second antibody. Antibody binding was visualized by enhanced chemiluminescence (ECL, Amarsham, USA) western blotting detection reagents.

Evaluation of Surface FcεRI-α by Flow Cytometry

The 2×10^5 cells (treated cells by siRNA, control siRNA, and untreated cells) were taken, centrifuged at 200 g for 10 min and were washed by washing buffer (PBS supplemented with 1% FBS). The supernatant fluid was removed and cells were incubated with 0.06 µg/100µl of FITC conjugated anti mouse FcεRI-α and isotype control antibody (eBioscience, USA) for 30 min at 4°C and dark condition. The cells were washed 3 times and centrifuged at 400 g for 5 min and resuspended in 500 µl of ice cold PBS (with 1% FBS). Cell staining was analyzed with a FACStar Plus (Becton Dickinson, USA).

Assays for β-hexosaminidase Release

25 µl of the culture supernatants and triton X-100-lysed cell pellets were removed from cells (2×10^5) that had been challenged by antigen for 30 min and then mixed with 100 µl of substrate solution (p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma, USA), 1 mM in 0.1 M citrate. pH 4.5) and incubated for 1 h at 37°C. The reaction was then terminated by adding 200 µl of stopping buffer (0.05M Na2CO3/NaHCO3, pH 10.0) and the absorbance was measured with a microplate reader at 405 nm (Awareness, USA).

The net percentage of β-hexosaminidase release was calculated as follows: β-hexosaminidase (OD) in supernatant / (β-hexosaminidase (OD) in supernatant + β-hexosaminidase (OD) in pellet) ×100

Histamine Release Assay

Histamine Bioassay ELISA kit was used for measurement of histamine, according to the manufacturer's protocol (USBiological, USA). 2×10^5 cells (treated cells, negative control cells, and untreated cells) were separated from the released histamine by centrifugation at 400 g for 5 min at 4°C. Residual histamine in the cells was released by disrupting the cells with 1% triton X-100 and centrifugation at 400 g for 5min at 4°C. The absorbance was read with a microplate reader at 450 nm (Awareness, USA). Histamine release (%) was calculated according to the following formula: histamine (ng/ml) in medium/ (histamine (ng/ml) in cells + histamine (ng/ml) in medium) × 100.

Statistical Analysis

The expression analysis including flow cytometry, Real Time PCR assay and histamine and β-hexosaminidase release assays were carried out in triplicates and the generated data were presented as the means ± SD. The Mann-Whitney U test was used to

evaluate differences among control, siRNA treated and untreated cells. A P value of less than 0.05 was considered as significant difference. All statistical analyses were carried out with SPSS 11 (SPSS Inc.).

RESULTS

The efficacy of FcεRI-α siRNA on MC/9 cells was assayed by Real Time PCR and western blot. Flow cytometery was used as a method for analyzing the expression FcεRI-α on cell surface.

Inhibition of FcεRI-α mRNA and Protein Expression by siRNA

Results of Real Time PCR indicated that the amounts of FcεRI-α transcripts in FcεRI-α siRNA–treated MC/9 cells were decreased to 0.24±0.09 and 0.32±0.06 fold after 24 h and 48 h, respectively (Figure 1).

Western blot analysis showed a considerable reduction of FcεRI-α protein in siRNA–treated MC/9 cells during incubation for 24 h and 48 h when compared with controls (Figure 2).

Flow Cytometry Analysis

Flow cytometry was used to determine FcεRI-α protein on the surface of mast cells (Figure 3). The surface expression of FcεRI-α was confirmed to be down- regulated by siRNA. The siRNA treated cells exhibited a reduction from 61 to 70.5% in FcεRI-α expression on mast cells surface as compared with control and untreated cultures (Figure 4).

β-hexosaminidase and Histamine Release Assay

siRNA significantly inhibited DNP-HSA induced β-hexosaminidase release from MC/9 cells. β-hexosaminidase was reduced to 52.8% and 61.2% in incubated cells for 24 h and 48 h respectively as compared with negative control cells. In addition, FcεRI-α siRNA considerably reduced DNP-HSA induced histamine release from MC/9 cells by 12 ng/ml (19%) and 10 ng/ml (18.5%) compared to control cells [31 ng/ml (60%) and 35 ng/ml (64%)] that were incubated 24 h and 48 h respectively (Figure 5).

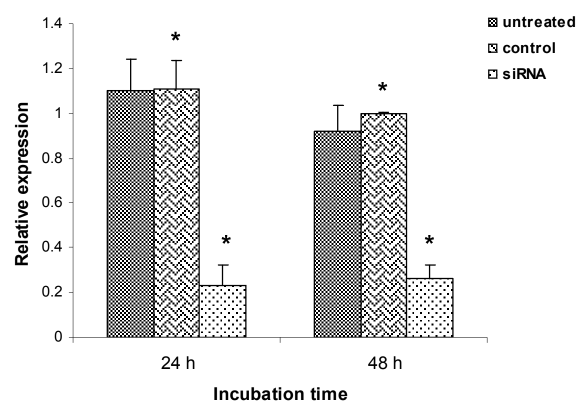

Figure 1. The amount of FcεRI-α mRNA in siRNA-treated, control and untreated MC/9 cells were analyzed by Real Time PCR (*p<0.05). Results indicated that the amounts of FcεRI-α transcripts in FcεRI-α siRNA–treated MC/9 cells were decreased to 0.24±0.09 and 0.32±0.06 fold after 24 h and 48 h, respectively.

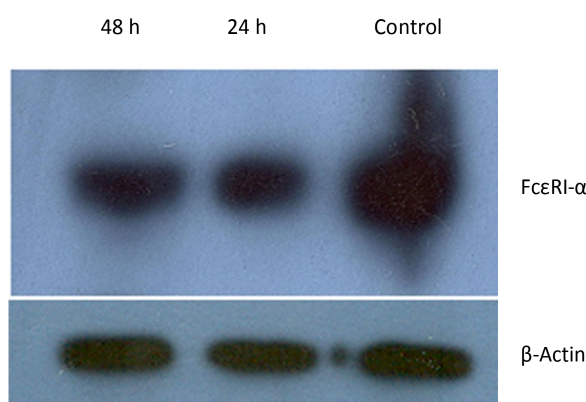

Figure 2. Effect of FcεRI-α siRNA on protein expression of FcεRI-α. MC/9 cells were transfected with either negative control or FcεRI-α siRNA. Total protein was extracted and analyzed by western blot. Western blot analysis showed a considerable reduction of FcεRI-α protein in siRNA–treated MC/9 cells during incubation for 24 h and 48 h when compared with controls.

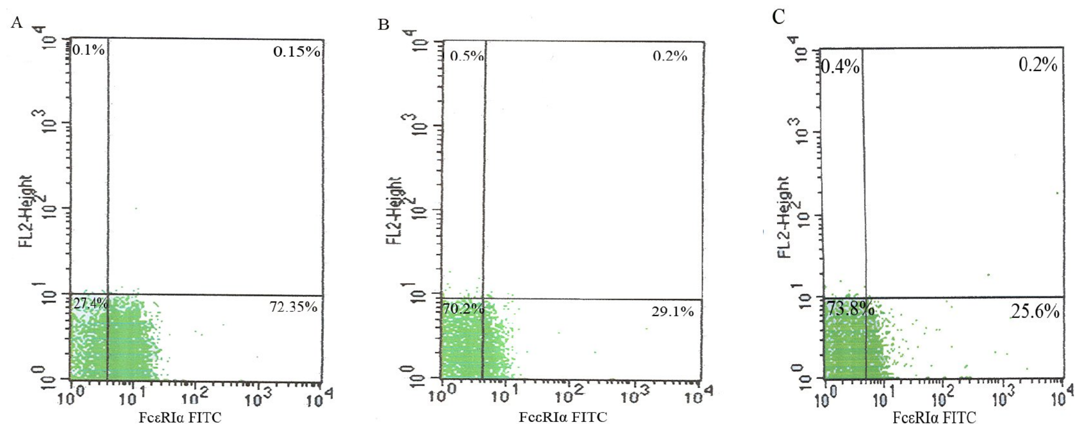

Figure 3. Surface FcεRI-α was assayed by flow cytometry as described in Materials and Methods. (A) Control cells. (B) FcεRI-α cognate siRNA treated cultures, 24 h. (C) The cells incubated by siRNA for 48 h. The lower right rectangular indicates the cells that expressed surface FcεRI-α.

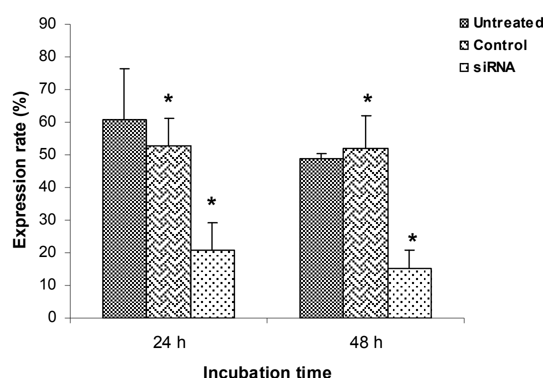

Figure 4. Effect of FcεRI-α siRNA on surface FcεRI-α expression. MC/9 cells were transfected with either control or FcεRI-α siRNA, and incubated for 24 h and 48 h (* p<0.05). The surface expression of FcεRI-α was confirmed to be down- regulated by siRNA.

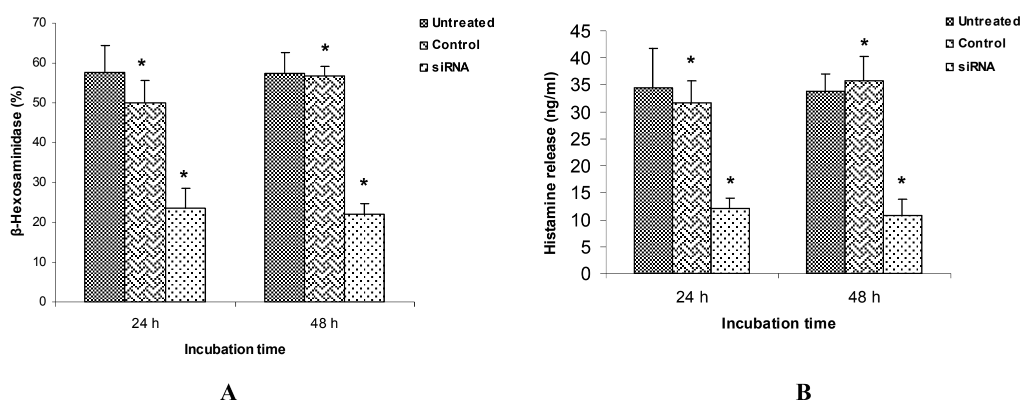

Figure 5. Effect of FcεRI-α siRNA on the FcεRI-mediated release of β-hexosaminidase and histamine from MC/9 cells. 2×105 cells were incubated for 24 h and 48 h in the medium containing 200ng/ml anti-DNP IgE. Cells were then incubated with DNP-HSA (20 ng/ml) for 30 min at 37°C. (A) β-hexosaminidase release and (B) histamine release. Results were extracted as mean ± SD, p*<0.05. siRNA significantly inhibited DNP-HSA induced β-hexosaminidase and histamine release from MC/9 cells.

DISCUSSION

Allergy is an undesirable reaction of the immune system in response to foreign substances such as an allergen.[1] In this study, effects of FcεRI-α siRNA on FcεRI-α mRNA and protein expression as well as its consequent effects in mast cell degeranulation and histamine release were evaluated. As histamine and β-hexosaminidase release are indexes of mast cell activation and initiation of allergic reaction cascade,[14,15] evaluation of these factors are a direct evaluation of these activities.

It has been reported that FcεRI deficient mice, as a result of deletion in α chain gene, were completely protected from IgE mediated anaphylaxis.[16] Therefore, the high affinity IgE receptor, FcεRI, is one of the important components in the allergic reactions.[6] Binding IgE to α subunit of its receptor is the first step in initiation of the allergic reactions. Thus the knock down of this subunit results in the arrest of the allergic cascade reactions.[3,16]

Several approaches for inhibition of the allergic reactions including anti-IgE and anti-FcεRI antibodies, antisense oligonucleotides and RNAi have been proposed.[17,18] The RNA interference-based therapeutic strategies, designed to precisely recognize and down regulate the target mRNAs, are the potential candidate in allergy treatment. In addition, siRNA technologies involve a well-argued and a possible less expensive design of new drugs for allergy.[17]

In a study, Kim et al. using antisense FcεRI-α oligodeoxynucleotides, demonstrated that the histamine release was inhibited in a concentration-dependent manner in mouse peritoneal mast cells. Maximum inhibition, about 80%, was obtained in a concentration of 10 Mm.[3] In comparison, our study showed the same results with a lower concentration of siRNA (70 nM). Moreover it has also been shown that using a higher concentration of antisense can produce both nonspecific and side effects[19] whereas, RNAi in a low level of concentration can silence the target genes and cause potential long-term therapeutic effect.[9] In agreement with this comparison, two studies were conducted on intracellular adapter Gab2, as a component in FcεRI-mediated signaling events in mast cells.[20] For inhibition of the antigen-induced activation of RBL-2H3 cells, Gab2 mRNA has been knocked down by both siRNA and antisense Gab2. Both of these treatments showed a significant decrease in the level of Gab2 expression. Although a higher concentration of antisense was used rather than siRNA, β-hexosaminidase release was reduced by two fold in RNAi approach, indicating its higher efficiency.[20,21]

Since all subunits of FcεRI complex on the mast cell surface are necessary for signal transduction of allergic reaction, knock down of only α subunit is sufficient for stopping of an allergic response.[22] Our results showed that siRNA FcεRI-α inhibited anti-DNP IgE-induced β-hexosaminidase release. This finding also confirmed by analysis of the surface and intracellular protein by western blotting and flow cytometry. The significant reduction of β-hexosaminidase release in comparison to the control cells shows that siRNA FcεRI-α results in the arrest of allergic response cascade and thereby inhibition of mast cell degranulation.

In summary, the FcεRI-α siRNA is qualified to suppress the expression of FcεRI-α and inhibits degranulation and histamine release in antigen-stimulated MC/9 cells. Our study indicated that FcεRI-α siRNA, as a potential therapeutic tool, can effectively reduce mast cell-mediated allergic reactions. Considering high efficacy of FcεRI-α siRNA in mouse cell line, further in vivo studies should be conducted to substantiate these results.

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