Possible modifications of type I interferon signaling pathway mediated by tyrosine kinase 2 polymorphism

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Abstract

Recent studies have discovered two single nucleotide polymorphisms (SNPs) in the tyrosine kinase 2 (Tyk2) gene that are associated with the autoimmune disease systemic lupus erythematosus (SLE). In this study cell lines transfected with either wildtype Tyk2 (wt) or Tyk2 containing one of the SLE associated SNPs (rs2304256) (g) were compared in regard to phosphorylation of Tyk2 and the downstream transcription factors STAT1 and STAT2 by Western blot. Total expression of type I interferon receptor (IFNAR) was analyzed by western blot and proliferation in presence of different concentrations of interferons were compared between the wildtype and mutant cell clones. Clones with comparable Tyk2 expression have been selected and the results show no significant difference in phosphorylation of either Tyk2, STAT1 or STAT2. The proliferation assays did not show any differences even though it was indicated that the Tyk2 wt clones were more sensitive to interferon than the g clones. The amount of total IFNAR was higher in the wt clones than in the g clones. This fact and its consequences is yet to be elucidated.

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that displays a number of symptoms. The prevalence for SLE in Sweden is 70-80 cases per 100 000 persons and about 90 % of the affected patients are women. Numerous organs can be affected such as skin, joints, kidneys, brain, blood vessels, lungs and heart and the pathogenesis in many cases is caused by the formation of immune complexes. The most common immune complexes in SLE patients consists of autoantibodies and nucleic acids and/or nucleic acid binding proteins. Twin studies have shown that SLE has an inheritable component and several mechanisms that cause the formation of autoantibodies have been suggested. Genetic studies have associated 19 independent genetic effects with SLE but the functions of these are yet to be discovered (Harley et al. 2006). Although the tendency to develop SLE is inherited, environmental factors are needed for the symptoms to occur. It is also believed that since almost nine of ten affected patients are women, estrogen might be important for the development of the disease.

Type I and type II interferons (IFNs) were discovered in the middle of the 20th century as a substance produced by virus infected cells to enable surrounding cells to resist infection. The IFNs inhibit intracellular viral replication and stimulate immune response. All cells are able to produce some type I IFN but there are cells that are specialized producers; they are called natural interferon producing cells (NIPC) or plasmacytoid dendritic cells (PDC) (Siegal et al. 1999). The type I IFN include 13 IFN-α genes and one IFN-β and expression of these are primarily triggered by viruses, but also other factors such as some bacteria or nucleic acids from dying cells can trigger IFN expression. The induction of type I IFN by RNA or DNA is triggered via receptors at the cell membrane or within the cell. Typical receptors for RNA and DNA are the Toll like receptors TLR7, TLR8 and TLR9. TLR7 recognizes single stranded RNA and TLR8 and TLR9 bind CpG rich or unmethylated DNA and induce type I IFN production. This induction is mediated via myeloid differentiation factor 88 (MyD88), that by a number of tumor necrosis factor (TNF) associated factors activate the TANK-binding kinase that phosphorylates IFN regulatory factors 3, 5 and 7 (IRF3, 5 and 7). Those IRFs form an enhanceosome and bind to the IFN gene promoters, leading to transcription of type I IFN genes.

The cells respond to IFNs trough the type I IFN receptor (IFNAR1), which consists of one long and one short chain. Binding of IFN to the receptor results in activation of Tyk2 at the
long chain and Janus kinase 1 (Jak1) at the short chain (Colamonici et al. 1994). Tyk 2 is a member of the Janus kinase protein family, a protein family of five kinases, Jak1-4 and Tyk2. The Tyk2 and Jak1 phosphorylates the transcription factors, signal transducer and activator of transcription (STAT)-1 and -2, that will go into the cell nucleus and start transcription of hundreds of IFN associated genes (Fig 1) (Murray 2007). Another very important function of Tyk2 is to stabilize expression of IFNAR1 at the cell surface (Ragimbeau et al. 2003). In the mutant cell line U1A, also called 1.11, that lacks endogenous expression of Tyk2, the surface expression of IFNAR1 is almost absent and studies have shown that IFNAR1 at the cell surface increase linear with the amount of expressed Tyk2 (Ragimbeau et al. 2003).

![Figure 1](image.png)

**Figure 1.** The signaling pathway for Tyk2 and the STAT proteins.

The type I IFN system is of great importance for the development of SLE and most SLE patients have increased IFN-α serum levels at some point that correlates with the severity of the disease. Those patients and also the patients that lack increased serum levels of INF-α show a specific IFN signature which means that expression of several IFN induced proteins are elevated (Bauer et al. 2006). More than 30 cytokines and other blood proteins that are induced by type I IFN were expressed at very high levels in serum from affected patients and these proteins can in many different ways induce immune response and enhance autoimmunity.

A recent study has associated two SNPs in the Tyk2 gene with SLE (Sigurdsson et al. 2005). The *TYK2* rs2304256 (V362F) SNP promotes a change of a valine residue to a phenylalanine in exon 8. Since one hydrophobic aminoacid is changed to another hydrophobic aminoacid conformational changes of the protein may not be severe, but the site is located in a large domain of the protein that is important for the interaction between Tyk2 and the IFNAR1. The other SNP *TYK2* rs12720356 I684S causes a substitution of an isoleucine to a serine in exon 15 in the pseudo kinase region of the protein. Although this mutation changes a nonpolar residue to a hydrophilic polar aminoacid and is predicted to be disruptive by PolyPhen and SIFT tools (Sigurdsson et al. 2005) it is not thought to affect the interactions of Tyk2 to its targets.

To further investigate effects of the Tyk2 polymorphism the normal Tyk2 and Tyk2 carrying the rs2304256 SNP were cloned into expression vectors and transfected into the cell line U1A.
that lacks endogenous expression of Tyk2. The established stable clones were treated with interferon for 15 minutes and the levels of expressed Tyk2 in the different clones were analyzed with western blot. The level of expressed Tyk2 varied in all clones and no significant differences in expressed Tyk2 levels were found between the wild type clones and the mutant clones (unpublished results). These clones have also been analyzed in flow cytometry for their IFNAR1 expression at the cell surface.

The aim of this study was to investigate if the Tyk2 polymorphism causes differences in the phosphorylation of Tyk2 and if it has an effect on the phosphorylation of the Tyk2 target proteins STAT1 and STAT2. The amount of total IFNAR1 in the cells was analyzed and possible differences in cell proliferation and their ability to react to IFN was investigated. All tests were performed on clones with comparable Tyk2 expression.

Materials and methods

Cell clones

Three cell clones with the wild type Tyk2 gene (wt) and three clones with the SLE associated SNP in exon 8 (rs2304256) of Tyk2, here designated as Tyk2 (g) were chosen due to their comparable Tyk2 expression. Lysates from the same clones were used for all experiments. The parental cell line 2fTGH were used as a positive control for Tyk2 expression and a cell clone of the U1A cell line transfected with an empty vector was used as a negative control for Tyk2. All cells had been treated with interferon (100U/ml), or medium only for 15 minutes prior to preparation of the cell lysates.

Table 1. The clones used in this study.

<table>
<thead>
<tr>
<th>wt clones</th>
<th>g clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 μg wt</td>
<td>3bF1 g</td>
</tr>
<tr>
<td>3c2b wt</td>
<td>2AF6, g</td>
</tr>
<tr>
<td>2AB11 wt</td>
<td>3bE10 g</td>
</tr>
</tbody>
</table>

Western blot

Electrophoresis was run on a 26 wells, 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (BioRad, Hercules, CA, USA) according to the manufacturer's protocol. Equal amounts of protein was loaded on each lane and electrophoresis was run at 150V for 1.5 hours. Blotting to a PVDF membrane (Amersham Biosciences, Uppsala, Sweden) was performed at 0.68 mA for 1 hour and the membrane was blocked with 5% nonfat milk in TBS-T (20 mM Tris, 140 mM NaCl pH 7.6; 0.1 % Tween-20). To detect the phosphorylated forms of Tyk2, STAT1 and STAT2 polyclonal rabbit antibodies that only recognize the protein in their phosphorylated form was used. For Tyk2 we used anti-phospho-Tyk2 (Tyr 1054/1055) (Santa Cruz, Bergheimer, Germany) diluted 1:200, for STAT1 we used anti-phospho-STAT1 (Tyr701) (Chemicon International, Hampshire, United Kingdom) diluted 1:1000 and for STAT2 we used anti-phospho-STAT2 (Tyr 689) (Upstate, Temecula, USA) diluted 1:1000. Antibodies that detect all forms of Tyk2, STAT1, STAT2 and IFNAR1 in the cells were; anti-Tyk2 (Upstate, Temecula) diluted to 0.75 μg/ml, anti-STAT2 (Santa Cruz) diluted to 0.75 μg/ml and for STAT1 we used a monoclonal mouse antibody (Anti-STAT1, clone 79) from Upstate diluted 1:1000. The IFNAR1 was detected by using a mouse-anti-human IFNAR (AA3) diluted to 10 μg/ml that was a kind gift from Dr. Zafari, Biogen Idec, Cambridge, UK. The membranes were incubated with the primary antibody at +4°C over night. The membranes were then washed 1x 15 minutes plus 3x 5minutes with TBS-T before incubateing with the secondary antibody. For the polyclonal antibodies a goat-anti-rabbit HRP
antibody (Upstate) was used as secondary antibody. For anti-STAT1 and anti-IFNAR an anti-mouse HRP (Santa Cruz) 1:3000) was used. The membranes were incubated for 1,5 hours at room temperature with the secondary antibody and washed as described above. Finally, the membrane was incubated with ECLplus reagent (GE Healthcare) exposed to film and developed.

**Proliferation assay**

The same clones, with comparable Tyk2 levels as used in western blot were used in the proliferation assay. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing L-glutamine and penicillin and streptomycin with 10% heat inactivated fetal calf serum (FCS) and 0,25 mg/ml hygromycin B. The cells were counted and diluted in culture medium to a concentration of 150000 cells/ml. A titration of Introna (IFNalpha2b: Schering-Plough Europe) was performed in the wells; the final concentrations of IFN were 2000, 1000, 500, 250, 125, 62.5, 31.2 and 0 U/ml. Each clone was run in triplicate for each interferon concentration. The total culture volume in each well was 200 μl with a total of 15000 cells at incubation start. Proliferation was tested with the EZ4U proliferation assay at 24, 48 and 72 hours according to manufacturers instructions. The EZ4U reagent converts a yellow tetrazolium compound to a red formazan derivative in living cells. The intensity of the color can be measured with a spectrophotometer at 450 nm and can thereby give a value of the cells metabolic ability and their ability to proliferate.

The cell line U1A carries a plasmid containing the upstream region of the 6-16 gene that is induced by type I IFN, and this promoter region has in the plasmid been linked with the Escherichia coli guanine phosphoribosyltransferase gene (gpt). Cells that are treated with interferon will express this gene and in presence of 6-thioguanine (6TG) cells will die (Pellegrini et al. 1989). The goal with this assay is to see if there is a difference in the sensitivity to interferon between the Tyk2 wt and the Tyk2 g clones. Cells that can respond to the interferon will die and clones with less ability to react to IFN will have a higher degree of survival.

Cells were grown in DMEM cell culture medium containing L-glutamine and PEST with 10% FCS and 0,25 mg/ml hygromycin B. The cells were counted and diluted in culture medium and in culture medium containing 60 μM 6TG to a concentration of 75000 cells/ml. A titration of Introna was performed as described above. Each clone was run in triplicate for each interferon concentration with and without 6TG. The total culture volume in each well was 200 μl with a total of 7500 cells at incubation start, the final 6TG concentration in the wells was 30μM. Viability of the cells was tested with the EZ4U proliferation assay at 24 hours, 48 hours and 72 hours according to manufacturers protocol. The absorbance was measured at 450 nm.

A second cytotoxicity test was performed with higher cell concentration at incubation start. Cells were grown as described above, and counted and diluted in culture medium to a concentration of 150000 cells/ml. All clones were set in two triplicates for each Introna concentration. 15000 cells were put in each well for 24 hours prior to the adding of Introna at the same titration concentrations as above. To one of the two triplicates for every clone 20 μl of 300 μM 6TG was added to a final concentration of 30 μM. The cells were grown for another 24 and 48 hours before viability was measured with the EZ4U proliferation assay.
Results

Western blot of Tyk2 and phospho-Tyk2

Western blot with an anti-Tyk2 antibody confirmed that there were no significant differences in the expression level of Tyk2 in the wt clones versus the g clones (Fig. 2). When using an anti-phospho-Tyk2 antibody that only detects the phosphorylated form of Tyk2 no significant difference in amount of activated Tyk2 could be seen between the Tyk2 wt and g clones (Fig. 3). Unspecific binding to a slightly smaller phosphoprotein was detected in all clones, as well as in the positive and negative controls (not shown), but was not detected by the anti-Tyk2 antibody on the stripped membrane.

![Figure 2. The 135 kDa Tyk2 is expressed in all clones.](image)

Western blot of STAT1 and -2 and phospho STAT1 and -2

Small levels of STAT1 were phosphorylated in cells unexposed to IFN. After IFN treatment the level of phosphorylated STAT1 increased. STAT2 was highly expressed in all clones prior to activation by IFN, and only a small part of the expressed protein was phosphorylated after IFN treatment. No significant difference of either expressed or activated STAT2 was detected between the Tyk2 g-clones and the wt-clones (Fig. 3).

Western blot of total IFNAR1

Earlier the surface expression of IFNAR1 has been investigated by flow cytometry in all clones and no significant difference could be found between the g clones and the wt clones. However this was not performed in clones with comparable Tyk2 expression (not published). As earlier described, Tyk2 is important for the stabilization of IFNAR1 at the cell surface. The present study shows that the total expression of IFNAR1 is higher in the wt clones than in g clones (Fig. 4).

![Figure 3. Western blot of transfected cell U1A cell clones expressing a wt or mutated Tyk2. The clones were treated with medium only (-) or with 100 U/ml Introna (+) for 15 minutes. The first panel shows phospho-Tyk2 expression in the Introna treated clones. The third and fourth lines show that there are some phosphorylated STAT1 (weak bands) in the non interferon treated cells but that interferon increases the phosphorylation level. There is no significant difference between the clones in either expression or phosphorylation levels. The fifth and sixth lines show that STAT2 is activated by interferon in both variants and that the expression level is the same.](image)
Figure 4. Result of western blot of total IFNAR. All the Tyk2 g-clones showed weak bands in both IFN treated and untreated cells. 2 out of 3 of the wt clones showed slightly stronger bands.

**Proliferation and cytotoxicity**

Proliferation for all the six clones was measured after one, two and three days. The clones showed high proliferation rate with all Introna concentrations. In day 3 the viability for the wild type clones was high in absence of Introna but decreases when the concentration of Introna increases. The Tyk2 g clones appear to have a higher tolerance for interferon (except for the highest concentration), and their viability drop in absence of Introna (Fig. 5).

Figure 5. Proliferation of 3 Tyk2 wt (grey lines) and 3 Tyk2 g (blue lines) The clones were treated with different concentrations of Introna or medium alone and the viability was measured after 1, 2 and 3 days.
Cytotoxicity in presence of 6TG

All clones reacted to IFN and showed sensitivity to 6TG in the presence of IFN, which indicate that their IFNAR is functional (Fig 6). There were some differences between the proliferation capacity when using 15000 or 7500 cells at incubation start. The viability of the cells treated with 6TG was very low for all clones and after 3 days the cells with high introna concentrations have all died. The cells without 6TG did not show the same pattern as in the proliferation test.

![Figure 6](image)

Figure 6. Proliferation of 3 Tyk2 wt (grey lines) and 3 Tyk2 g (blue lines) clones with 7500 cells per well at incubation start. The clones were treated with different concentrations of Introna or medium alone ± 6TG, and the viability was measured after 1, 2 and 3 days. The red line indicates the cell clone transfected with the empty plasmid.

In order to let the cells accommodate before addition of Introna and/or 6TG another protocol for cytotoxicity measurement was introduced. In this assay 6TG and Introna were added 24 hours after the cells were seeded into plates. The cell clones showed less sensitivity to 6TG in this assay. At day two, 24 hours after adding of 6TG and Introna most of the clones still showed some metabolic activity even if it had decreased compared to the clones grown without Introna, but there was not much difference from the results without 6TG (Fig. 7). When looking at each individual clone over the two days they all display the same proliferation pattern (Fig. 8). One of the wild type clones differed from the others and it is possible that it has lost its ability to react to IFN.
Figure 7. Results for the cytotoxicity test with 15000 cells at incubation start. 6TG and Introna was added after 24 hours. The cells do not show the same sensitivity to 6TG in this assay except for the highest concentration of Introna. The cells not grown with 6TG show the same pattern as for day one and two in the proliferation test, indicating that it is the time of treatment with Introna that is crucial for the sensitivity to 6TG.

Figure 8. Proliferation pattern of the individual clones at days 2 and 3. The blue lines are 6TG treated cells and the red lines are cells without 6TG in the media. 15000 cells were set in each well and grown for 24 hours in growth media before adding 6TG and Introna.

Discussion
A recent study showed an association with two SNPs in the Tyk2 gene and SLE (Sigurdsson et al. 2005) and the present study investigated whether there is a difference in the phosphorylation pattern between cell clones expressing a wildtype Tyk2 or a mutated form of Tyk2 (SNP rs2304256). It was confirmed that IFN could activate phosphorylation of Tyk2 in both the transfected cell clones and the parental cell line (2fTGH). This indicates that the transfected cell clones can be used as a tool to study possible differences in the phosphorylation pattern caused by mutations in the Tyk2 gene.

The phosphorylated form of STAT1 but not STAT2 could be detected prior to IFN stimulation and the former was not dependent of active Tyk2 for phosphorylation. This is not surprising since other cytokines than type I IFN signals via STAT1. The phosphorylated form of STAT1 increased in IFN treated cells, and it can be concluded that Tyk2 functions as an important kinase on STAT1.
The main question in this study was whether there are differences between the Tyk2 wt clones and the g variants. No significant difference was detected, although Tyk2 was activated in both forms. It is not clear if there is a difference in amount of phosphorylated Tyk2 in the different clones though it is an important question since a very small difference in activated protein could have a large impact in the biological system.

The proliferation assay in this study indicates some difference between the different clones at day three. The EZ4U assay measures the metabolic activity of the cells and thereby their viability. The wt clones appears less viable after three days when grown in the presence of Introna compared to the Tyk2 g clones. But since they all had grown in high cell density in the wells it is possible that the wt clones grown in Introna had a higher proliferation rate and therefore had grown to death while the g clones had grown slower and therefore were still metabolically active. The second experiment with reduced number of cells at the start did not confirm this assumption because there was no difference in the proliferation between the clones after 3 days. The in vitro assays performed in this aspect might indicate some differences between the Tyk2 wt and the g clones but the method used here is not sensitive enough to make it legible.

In conclusion, there is no significant difference in phosphorylation of Tyk2, STAT1 or STAT2 or total expression of IFNAR1 between the Tyk2 g and wt clones. Although, in a biological system a very small change in amount can have a large impact and it cannot be excluded that the methods used in the present study are not sensitive enough to detect slight differences in the function between Tyk2 wt and the mutant clones.

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References
