Concise report

Correlations between changes in cytokines and clinical outcomes for early phase (proof of concept) trials in active diffuse systemic sclerosis using data from an imatinib study

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Abstract

Objective. Data from a small study testing imatinib to treat SSc were used to determine if cytokine changes were related to differences in clinical parameters to model future early phase trials pairing cytokine changes and clinical parameters.

Methods. Plasma and punch skin biopsy specimens collected at baseline and 6 months were analysed for levels of 26 fibrotic and inflammatory cytokines using multiplexed immunoassays and ELISA. Seven of nine patients on active treatment had paired data. Biopsies were biopulverized and standardized to protein levels in the tissue homogenate. Plasma was frozen at −80°C and analysed using multiplexed immunoassays or ELISAs standardized to CRP. Correlations between fold changes in cytokines and differences in clinical parameters (skin score, physician and patient global assessments and HAQ) were performed. \( P < 0.01 \) was considered significant.

Results. After 6 months of imatinib treatment, plasma levels of soluble vascular cell adhesion molecule 1 decreased significantly \( (P < 0.001) \), while tissue levels of soluble intercellular adhesion molecule 1 increased \( (P < 0.01) \). Some significant correlations between fold changes in certain plasma fibrotic and inflammatory cytokines and changes in clinical parameters after 6 months of treatment were found: patient global scores and IL-13 \( (r = 0.964, P < 0.0001) \); ESR and IL-12p70 \( (r = 0.903, P < 0.01) \); in tissue samples, patient global score and soluble E-selectin \( (r = 0.913, P < 0.01) \); and physician global score with sCD40L \( (r = 0.883, P < 0.01) \).

Conclusion. Some serum and tissue cytokines may have a role in early phase clinical trials of SSc, correlating with changes in clinical parameters. Serum and tissue samples could be analysed in early phase trials to determine whether they support the clinical observations.

Trial registration: http://clinicaltrials.gov/show/NCT01545427
Key words: SSc, diffuse SSs, imatinib, cytokines, proof of concept, early phase trial.

Introduction

SSc is a CTD with a variety of manifestations, including excessive fibrosis, vascular abnormalities and some inflammation, especially early in the disease [1]. Some organ involvement has been targeted with variable success, such as in pulmonary arterial hypertension [2, 3]. However, treatment for overall disease is disappointing [3–5].

Data suggest that both PDGF and TGF-\( \beta \) are mediators of the excessive persistent fibrosis characteristic of...
scleroderma [6]. There are several distinct tyrosine kinase inhibitors with distinct biological effects. PDGF and TGF-β production may require tyrosine kinase signalling. This indicates that inhibition of tyrosine kinase might be a key signal modifier for treating scleroderma [7]. In a previous report we examined a very small proof-of-concept study using imatinib, an oral tyrosine kinase inhibitor, in the treatment of active dcSSc [8]. This was a negative study that was stopped early due to poor drug tolerability, with no overall mean difference in outcome and only one placebo patient (as randomization was 4:1). We found that during 6 months of imatinib treatment, one-third improved, another third was stable and the remainder worsened with respect to clinical parameters (which could be the natural history of these trial patients or due to a drug effect). We wanted to determine whether changes in cytokines correlate with clinical outcome changes in order to determine whether some cytokines could be used as potential markers in other early phase trials. The purpose was not to determine whether a subset of patients within the small underpowered study improved with treatment, but instead to statistically determine whether cytokine changes were related to changes in clinical parameters to model future early phase trials pairing cytokine changes and clinical parameters. Changes in the expression of 26 fibrotic and inflammatory cytokines were measured in skin biopsies and in plasma (before and 6 months after starting treatment).

**Patients and methods**

**Subjects**

Ten patients with active diffuse SSc, according to worsening skin score, tendon friction rubs and/or increased ESR, were randomized into two groups with a 4:1 ratio of imatinib (200 mg twice a day) or to placebo over a 6-month period [8]. The modified Rodnan skin score (mRSS), physician and patient global assessments, HAQ disability index and cytokines were determined every 3 months and paired biopsies on the abdomen were performed at 0 and 6 months. The study was approved by the University of Western Ontario (UWO) Health Sciences Research Ethics Board (approval 12968). All subjects signed informed consent. Only 7 of 10 subjects were analysed for this post hoc study, as the placebo patient was removed and two patients on active treatment who dropped out do not have data for a full 6 months.

**Plasma preparation**

Blood was drawn into EDTA tubes at 0, 3 and 6 months, spun immediately and the plasma frozen at −80°C for later analysis of the 26 fibrotic and inflammatory cytokines: PDGF-AA, PDGF-AB/BB, IL-13, IL-17, VEGF, TGF-β1, soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble intercellular adhesion molecule 1 (sICAM-1), soluble E-selectin (sE-selectin), MMP-9, tissue plasminogen activator inhibitor 1, IL-1α, IL-1β, IL-4, IL-6, IL-12p70, IL-13, TNF-α, soluble cluster of differentiation 40 ligand (sCD40L), IFN-γ, monocyte chemoattractant protein 1 (MCP-1), MCP-3, macrophage inflammatory protein 1α (MIP-1α), MIP-1β and chemokine ligand 5 (CCL5; also known as RANTES (regulated upon activation normal T cell expressed and secreted)). Cytokines were measured using multiplexed immunoassays (Millipore, Billerica, MA, USA) and ELISA for TGF-β1 (BD Biosciences, Franklin Lakes, NJ, USA). Levels of cytokines in plasma were standardized to the subjects’ CRP level to normalize to an individual’s underlying inflammatory state and were used as an endogenous control protein in the cytokine assays.

**Tissue preparation**

Paired 3 mm punch skin biopsy specimens were taken in the same quadrant on the abdomen prior to first dose and again at 6 months, snap frozen immediately and stored frozen at −80°C for later analysis. At study completion, frozen tissue was pulverized using a chilled biopulverizer, resuspended in cold extraction buffer, spun and the homogenate analysed for the 26 fibrotic and inflammatory cytokines (as stated above). Levels of cytokines in the tissue were standardized to total protein levels in the tissue homogenate, as determined by a Pierce bicinchoninic acid (BCA) protein assay. There was insufficient material from the skin biopsies to measure IFN-γ, IL-4, IL-17 and TGF-β1.

**Choice of cytokines**

SSc pathogenesis is very complex and usually involves fibrotic, inflammatory and vascular abnormalities. It was not thought that there would be important alterations in the vascular abnormalities in SSc in a short trial using a drug that may decrease fibrosis. Thus potentially inflammatory and fibrotic cytokines were chosen that have been demonstrated in early SSc as being important or as potentially changed by imatinib in other studies. The list was not exhaustive but was representative of some key pathways.

**Additional laboratory tests**

Complete blood count, ESR, CRP, transaminases, urea and creatinine were measured monthly for each subject by the hospital laboratory.

**Statistics**

Modelling for changes in cytokines compared with differences in clinical parameters over 6 months of the trial were performed. Log changes and fold changes (doubling or a reduction by half) of the cytokines were explored. The most normal fit of the data for changes in cytokines were fold changes, so significant changes between baseline and 6 months were analysed on fold changes in levels of cytokines. Fold changes were used to define cytokine changes within an individual, as they normalized the data better than log transformations. Changes in clinical outcome measures were determined by the mean difference (6 months minus baseline scores). Pearson correlations were performed between fold changes in levels of cytokines in plasma or skin and clinical outcome.
measures: mRSS, patient and physician global scores and HAQ score. Data at 3 months were not used for several reasons: clinical parameters are not expected to vary in SSc trials until at least 6 months for most drugs previously studied (so we thought it would be too soon to look at correlations between differences in clinical parameters) and we did not do skin biopsies at 3 months. Thus the data at 0 and 6 months were used in the analysis. Cytokine fold changes were compared to differences from entry to the end of the trial for CRP and ESR because these are inflammatory mediators that are readily available and if the correlations were strong between the cytokine fold changes and differences in CRP and ESR, then using other cytokines could be considered less helpful. A Bonferroni correction was performed, so the data are interpreted where a $P$-value $<$ 0.01 may be less likely to be due to chance [since four outcomes (changes in patient and physician global assessments, HAQ and mRSS) were of interest, the $P$-value of 0.05 was divided by 4 and rounded down from $P = 0.0125$ to $P < 0.01$]. It is important to note that these comparisons were hypothesis generating, not testing, as the purpose of this substudy was to determine whether changes in cytokines could correlate with changes in clinical parameters even with a small sample size, in order to make decisions about moving forward with future drugs in early phase SSc studies or to understand mechanistically what is occurring when a trial patient improves (or doesn’t).

Results

Subjects

After enrolling 10 subjects (9 imatinib, 1 placebo), the study was stopped due to poor tolerability of the study drug. Only four of nine patients completed the study on the original recommended dose of 200 mg imatinib twice a day [8]. Seven were analysed for this article (two dropped out entirely prior to study end and the placebo patient was not analysed for biomarkers). The characteristics of the patients (active diffuse SSc) have been previously published [8].

Cytokines and baseline parameters and differences in parameters and fold changes in cytokines

At baseline there were some significant correlations between ESR and sICAM-1 ($r = 0.957$, $P < 0.001$), as shown in Table 1. After 6 months of imatinib treatment, plasma levels of sVCAM-1 decreased significantly ($P < 0.001$), while tissue levels of soluble ICAM-1 increased ($P < 0.01$) (prior to transforming the cytokine data). At 6 months, differences between the baseline and 6-month values for patient global assessment were significantly correlated with fold changes in IL-13 and sCD40L in plasma ($r > 0.9$ for each). In tissue, there were significant correlations for sE-selectin and patient global changes, as described above ($r = 0.913$), and physician global and sCD40L ($r = -0.883$) (Table 1). Many tests were not shown due to lack of any statistical significance, and when correcting for multiple testing, several strong correlations were also not significant.

Discussion

The objective of this investigation was to determine whether changes in clinical outcomes could be supported by changes at the molecular level; more specifically, by changes in fibrotic and inflammatory molecules. This approach may support or reject a decision to continue development for SSc treatment, i.e. where clinical data suggest improvement but there is inadequate information to determine if a drug should move into later-phase testing. Small changes in clinical parameters without cytokine alterations may suggest stopping further drug development despite having small patient numbers, non-optimal drug dosing, short trial duration and only a minimal clinical difference. These data are from a trial that had only one placebo patient [8], so the response studied could be the natural history in a short study.

Changes in HAQ scores were strongly associated with fold changes in plasma IL-17 and for patient global assessment score. Plasma IL-17 and sE-selectin are pro-fibrotic proteins and, not surprisingly, each has been implicated in inflammatory skin diseases including SSc [9–11]. IL-17 has been proposed as a potential therapeutic target to treat these diseases [12, 13]. Plasma levels of sE-selectin have been shown to correlate with the extent of internal organ involvement and lung fibrosis in SSc [9, 14].

Changes in physician global assessments were associated with fold changes in plasma and tissue sCD40L. Imatinib may regulate fibrosis via sCD40L [13, 15], PDGF [16], TGF-β1 [17] and IFN-γ [18].

There were some associations between inflammatory markers and various cytokines. The positive correlation between skin score and VEGF was unexpected, as VEGF is elevated in SSc and may be responsible for increased vascular permeability and stimulating angiogenesis [19], but the result was not significant when the $P$-value was corrected for multiple comparisons. A possible explanation of the relationship between skin involvement and VEGF (if real and not spurious) may be that uncontrolled, chronic overexpression may lead to abnormal blood vessel formation. Thus decreased VEGF may reset angiogenesis [19]. Interpretation of the results should be made with caution, as this is a post hoc analysis. Other studies could show different results depending on the patient population, and this current sample was very small with only conservative (i.e. large) changes in cytokines being studied (fold changes).

Imatinib may have increased skin oedema, as we did not do a histological examination of the skin tissue before and after treatment. The data are underpowered, but our objective was to determine if there is a rationale to measure cytokines in skin and plasma in early phase active dCSSc studies to determine if they correlate with changes in clinical outcomes. Furthermore, although many variables showed strong correlations, many of the $P$-values may have been by chance. There are many alterations in SSc. The cytokines we tested were obtained from the
### Table 1 Correlations between clinical outcomes in SSc and changes in cytokines between 0 and 6 months

<table>
<thead>
<tr>
<th>Sample</th>
<th>Outcome</th>
<th>Cytokine</th>
<th>Baseline Correlation of baseline variable and baseline cytokine (P-value*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin score</td>
<td>sE-selectin</td>
<td>$r = -0.803$ (P = 0.030)</td>
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<tr>
<td>Physician global</td>
<td>IL-1α</td>
<td>$r = 0.853$ (P = 0.015)</td>
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<tr>
<td>Patient global</td>
<td>TGF-β1</td>
<td>$r = 0.843$ (P = 0.017)</td>
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<tr>
<td>HAQ</td>
<td>No significant data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>IL-12p70</td>
<td>$r = 0.763$ (P = 0.046)</td>
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<tr>
<td>ESR</td>
<td>sICAM-1</td>
<td>$r = 0.957$ (P = 0.001)</td>
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<tr>
<td>Tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin score</td>
<td>No significant data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physician global</td>
<td>PDGF-AA</td>
<td>$r = 0.774$ (P = 0.041)</td>
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</tr>
<tr>
<td>Patient global</td>
<td>IFN-γ</td>
<td>$r = -0.825$ (P = 0.022)</td>
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<tr>
<td>HAQ</td>
<td>MMP-9</td>
<td>$r = -0.772$ (P = 0.042)</td>
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<tr>
<td>CRP</td>
<td>IL-13</td>
<td>$r = 0.964$ (P &lt; 0.001)</td>
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<tr>
<td>ESR</td>
<td>IL-12p70</td>
<td>$r = -0.903$ (P = 0.005)</td>
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</tbody>
</table>

*Correlation of fold-change in cytokine and difference in clinical parameters (between 0 and 6 months)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Outcome</th>
<th>Cytokine</th>
<th>Correlation of change in outcome variable (6 months – baseline) and fold change in cytokine between 0 and 6 months (P-value*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
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</tr>
<tr>
<td>Skin score</td>
<td>No significant data</td>
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<td>Patient global</td>
<td>sE-selectin</td>
<td>$r = 0.913$ (P = 0.004)</td>
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</tbody>
</table>

sE-selectin: soluble E-selectin; MCP-1: monocyte chemotactic protein 1; MIP-1α: macrophage inflammatory protein 1α; RANTES: regulated upon activation normal T cell expressed and secreted. This table shows significant differences/correlations between skin score (modified Rodnan skin score), improved health status based on physician/patient global assessments and HAQ, CRP and ESR, and changes in cytokine levels between 0 and 6 months. *Only 7 of 10 subjects were analysed, as the placebo patient was removed and two patients on active treatment who dropped out did not have 6 months of data. **Levels of cytokines in plasma were standardized to CRP levels at each time point. Pearson correlation: correlation between the mean difference in clinical score and fold change in cytokine levels between baseline and 6 months. *P-value is significant at <0.01 using Bonferroni correction for four clinical outcomes. **Indicates significant correlations at baseline and 6-month fold change. Only data with a P-value <0.05 are provided in the table, so most of the analyses are not shown. There were multiple comparisons that are not shown for the 26 cytokines for each clinical parameter.
literature of SSc and also from the literature of what could be altered from imatinib in other conditions. They are numerous, but not totally inclusive, and some have stronger associations than others. A further lesson learned for future trials is that a 3 mm punch biopsy specimen may be too small. There may also be some question regarding the location of the biopsy, as often the forearm is used, but an area less involved may be more amenable to change.

This study may be a template to guide further early phase dcSSc trials, where large cytokine changes (related to the drug under study and/or the disease) can be compared with changes in clinical parameters. Some cytokines such as TGF-β may have differences in tissue (skin) and plasma, so collection of both blood and skin may be more informative. However, dcSSc has heterogeneous responses in trials, so early phase trials should be interpreted within this context and most will be underpowered for clinical and biological significance.

Rheumatology key messages

- Early phase randomized controlled trials in early dcSSc may benefit from analyses of serum and skin biopsies.
- Skin and serum analyses can help determine whether cytokine changes correspond to clinical outcome measurements in early dcSSc.
- Cytokines and outcome measurements may expedite later-phase trials in diffuse SSc.

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