A combined immunostimulatory and immunoinhibitory short interference RNA reduces hypercoagulability in a rat model of acute promyelocytic leukaemia

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Summary

Acute promyelocytic leukaemia (APL) confers an increased risk of thrombosis and bleeding. Current treatments are insufficient to inhibit these complications. We recently showed that a combined immunostimulatory and immunoinhibitory short interference (si) RNA effectively inhibited leukaemic growth and metastasis in rats with APL. We now assessed if the reported anti-leukaemic effects of siRNA treatment could be explained by inhibition of hypercoagulability. We measured markers of coagulation and fibrinolysis in plasma collected from APL rats with overt leukaemia using conventional assays. Coagulopathy developed in untreated leukaemic rats evidenced by increase in several haemostatic markers. Treatment of leukaemic rats with the siRNA reduced (p < 0.05) the concentration of thrombin-anti-thrombin complex (a marker of coagulation) by 40% compared with rats treated with an inactive, control siRNA. Substantial reductions (p < 0.05) were also obtained for two markers of fibrinolysis: D-dimer (72%) and plasminogen activator inhibitor type 1 (51%). The activity of tissue factor, the main initiator of coagulation, was not increased (p > 0.05) in untreated leukaemic rats compared with healthy rats, and did not change (p > 0.05) upon treatment with the siRNA. The bifunctional siRNA reduces the hypercoagulable state in APL in addition to its direct anti-leukaemic properties, supporting testing of this small molecule in human APL.

Keywords

Acute myeloid leukaemia, coagulopathy, rat, short interference RNA

Introduction

Coagulopathy significantly adds to both morbidity and mortality in haematological malignancies. Reportedly as many as 1 in 10 patients with acute promyelocytic leukaemia (APL) may be at risk of both major thrombotic complications such as venous thromboembolism, and severe bleeding, often due to disseminated intravascular coagulation (1–3). The precise mechanism(s) underlying this neoplastic-associated coagulopathy has not been identified. The laboratory features of this abnormal haemostasis suggest that several coagulation and fibrinolytic factors are dysregulated in APL (4). Tissue factor (TF) initiates blood coagulation and has been implicated in the pathogenesis of coagulopathy in APL. Increased expression of TF has been detected in primary leukaemic cells from both blood and bone marrow of APL patients, as well as in APL cell lines (2, 5, 6).

Only intensive chemotherapy combined with all-trans retinoic acid and allogeneic stem cell transplantation can cure APL. Although the prognosis of APL generally is more favourable than the other subtypes of acute myeloid leukaemia, 30–40% have died within five years of the time of diagnosis (2). Moreover, the intensive treatment modalities are often not applicable to patients > 65 years due to the severe treatment-related morbidity. Various vaccine-like strategies have been developed, but often with dismal results, in particular for myeloid leukaemias (7). This may in part be due to limitations in breaking tolerance to leukaemic cells and/or the use of single peptides only as leukaemic antigens for T-lymphocyte stimulation (8).

The concept of RNA interference for gene silencing has emerged as a promising tool for combating neoplastic diseases (9). The small interfering RNA (siRNA) molecules can degrade mRNA with homologous sequences and thereby silence a target gene. We have refined this method by designing a bifunctional siRNA molecule that partly cleaves the mRNA of the anti-inflammatory cytokine interleukin (IL)-10 and partly increases the production of proinflammatory cytokines by stimulation of intracellular toll-like receptors (TLR) (10). Specifically, this siRNA efficiently inhibited IL-10, both at the mRNA- and protein levels when delivered to cultured dendritic cells. Recently this active siRNA molecule was transfected into rat dendritic cells and subsequently injected into Brown Norway rats with myeloid leukaemia (BNML) (11). In this model of human APL we demonstrated that injection of the siRNA reduced the death rates about four-fold, reduced the bone marrow infiltration of leukaemic cells with 65%, and nearly halved the extramedullar
dissemination of the leukaemic cells (11). We now asked if these beneficial effects could be explained by siRNA-induced alterations of the coagulopathy in the leukaemic rats. To this end we studied markers of haemostatic factors in overt BNML disease.

Materials and methods

Generation of siRNA and preparation of dendritic cells

The design of the siRNA and its transfection into dendritic cells have been detailed previously (12, 13). Briefly, the designed siRNA targeting rat IL-10 was chemically made by Eurogentec (Seraing, Belgium). A 2’-O-methyl uridine modified siRNA targeting mouse basigin was used as an inactive control siRNA (11). The target site sequences of the siRNAs were:

- active siRNA, 5’-ACGCUGUCAUCGAUUCUC-3’
- inactive (control) siRNA, 5’-GAGGCAAUCACCAAUAGCA-3’

Blood mononuclear cells were purified with density gradient centrifugation followed by adherence to plastic before culturing in six-well plates in complete RPMI medium supplemented with rat GM-CSF (50 ng/ml) and IL-4 (100 ng/ml) to generate immature dendritic cells. On day 5, half of the cells were transfected with an immunostimulatory siRNA targeting rat IL-10 (20 μg/2x10⁶ cells) using cationic liposomes (20 μg/ml), whereas the other half was transfected with the control siRNA. In both cases 50 μg of leukaemic cell protein extracts from the BNML rats and prepared by freeze thawing were added to the transfection mixture as an adjuvant to enhance the immune response of T-lymphocytes. Subsequent to an overnight incubation, the dendritic cells were harvested, washed with phosphate-buffered saline (PBS), and then immediately used for injection.

Analyses of haemostatic markers

Blood was sampled from the inferior caval vein after induction of anaesthesia with barbiturate (50 mg/kg, i.p.) and shortly before the lethal dose was given. The blood was collected in plastic tubes with sodium citrate (0.11 mM), and then centrifuged (2,500 x g, 10 minutes [min], 4°C) before the plasma was obtained and stored at −80°C until further analyses.

Fibrinogen was measured using an ACL Futura coagulometer and reagents from Instrumentation Laboratories (Milan, Italy) (16). In short, a thrombin agent was added to the sample in order to convert fibrinogen to fibrin and the ensuing clot formation was then determined. Plasminogen activator inhibitor type-1 (PAI-1) activity was measured with Spectrolyse (Biopool, Trinity Biotech, Co. Wicklow, Ireland). The thrombin-anti-thrombin complex (TAT) and D-dimer were assayed using ELISA-kits from Dade Behring (TATc, Marburg, Germany) and Diagnostica Stago (Roche, Almere, the Netherlands), respectively (17). To measure TF, we determined the conversion of coagulation factor X (FX) to its activated form Xa in the presence of exogeneous coagulation factor VIIa. This process is dependent on the prevailing TF concentrations and can be studied in a chromogenic assay (18).

The mRNA levels of TF in leukaemic cells of rats were measured with an RNase protection assay after extraction of total RNA, as described (19). We used mRNA for glyceraldehyde phosphate dehydrogenase (GAPDH) as an internal standard.

Transplantation and treatment design

The protocol conformed to the national legislation and institutional guidelines (permission no. S-2006/30555). Pathological microbes were not detected when tested according to the FELASA recommendations (14). Brown Norway rats (260–280 g; 10 per group) were kept in cages with an ambient temperature of 20°C and with a 12 hour (h) day/night cycle. We only included male rats since oestrogen levels and the ovarian cycle may significantly influence rat haemostatic processes (15). They were randomly assigned to be injected with dendritic cells (2 x 10⁶) transfected with either the active or the control siRNA and loaded with the leukaemic cell lysate. One week later (day 0) this procedure was repeated before we transplanted the rats with leukaemic cells, as previously detailed (11). Ten healthy Brown Norway rats were used as controls. The rats were killed with an intraperitoneal (i.p.) overdose of barbiturate when they developed obvious signs of disease (e.g. paralysis of extremities, failure to thrive, bleeding from mouth or nose). BNML rats treated with the inactive siRNA lived for (range) 20–25 days after transplantation whereas those given active siRNA lived for 23–32 days (11). All blood samples were collected after the rats had developed overt disease, and all analyses were therefore performed post mortem.

Statistics

Data are reported as medians and interquartile ranges. Differences were evaluated with the Mann-Whitney and Kruskal Wallis tests followed by the Bonferroni correction, as appropriate, and assumed significant for p < 0.05.

Results

Coagulation is activated in BNML and can be inhibited by the siRNA

APL is characterised by coagulopathy, we therefore first examined the plasma concentrations of TAT, which serves as a marker of activated coagulation. Whereas BNML rats given the inactive siRNA
exhibited a profound increase in the plasma concentration of TAT, it was substantially reduced in BNML rats treated with the active siRNA, and to levels similarly to those of healthy rats ($p < 0.001$; Table 1).

**Attenuated hyperfibrinolysis in BNML rats treated with the siRNA**

To examine fibrinolysis we measured the plasma concentration of D-dimer, a marker of fibrin degradation. Table 1 shows that the plasma concentration of D-dimer was markedly increased in BNML rats given the inactive siRNA, while those treated with the active siRNA had D-dimer levels comparable to those of healthy rats ($p < 0.001$). In support of these data were similar findings from measurements of plasminogen activator inhibitor (PAI) type 1, a main regulator of fibrinolysis (Table 1).

The plasma fibrinogen concentrations are given in Table 1. As expected BNML rats given the inactive siRNA had low levels of fibrinogen, indicating a consumptive coagulopathy, whereas those of BNML rats treated with the active siRNA and the healthy rats had higher plasma concentrations of fibrinogen ($p < 0.001$).

**No changes in TF levels upon treatment with siRNA in BNML**

Physiologically TF is the main initiating factor of the coagulation process and it as been postulated also as the main triggering factor of cancer-associated hypercoagulopathy. We therefore measured the rate of conversion of FX to its active form, a process that requires the presence of TF and hence serves as marker of TF activity. It is evident from Table 1 that the TF activity did not differ ($p = 0.68$) between the BNML rats given either the active or the inactive siRNA. Moreover, we could not detect any difference ($p = 0.73$) in the TF transcript levels between leukaemic cells samples from rats treated with either inactive ($n = 10$) or active siRNA ($n = 10$). The ratios of mRNA-TF to mRNA-GAPDH were 2.7 (2.2–3.0) and 2.7 (2.1–2.8), respectively.

**Discussion**

In this study we show that a bifunctional siRNA molecule could efficiently reduce the hypercoagulable state in this rat model of APL. Specifically, the siRNA markedly lowered the plasma concentrations of the thrombin-anti-thrombin complex, a marker of activated coagulation. Similarly, the markers of fibrinolysis, plasma D-dimer and PAI-1, were also substantially reduced. Interestingly, the siRNA had no apparent effect on either the plasma concentration or the mRNA level in leukaemic cells of TF, the main initiator of blood coagulation. Collectively, these findings are in agreement with the inhibitory effects on leukaemic growth, viability and dissemination (11).

The BNML rat is an established model of APL and shares important features with human APL with regard to development, progression and dissemination patterns of the leukaemic process as well as the response to chemotherapy (20, 21). In addition, BNML rats with overt disease frequently develop disseminated intravascular coagulation, hence mimicking the coagulopathy in human APL. In line with this all our BNML rats showed signs of pulmonary bleeding upon autopsy (data not shown).

A possible limitation of our study is that the siRNA was given prior to the injection of leukaemic cells. This design should be complemented with studies in rats with established disease. Moreover, our experimental set-up does not allow a firm conclusion as to whether the beneficial effects of the active siRNA are primarily due to direct anti-leukaemic properties or via a reduced hypercoagulability or a combination of these. However, the inhibition of extramedullary dissemination observed in BNML rats treated with the active siRNA, may suggest that the anti-leukemic effects are of prime importance.

Our findings of a concomitant activation of both coagulation and fibrinolysis in untreated BNML rats fit well with similar observations in APL patients (2, 4). Several mechanisms have been proposed of how malignant cells can induce a hypercoagulable state, including alterations in haemostatic factors as well as in inflammatory- and adhesion molecules (22). Among the procoagulant molecules, TF is the main initiator of the coagulation cascade. Several studies have attributed the hypercoagulable state in APL to an up-regulation of this TF/factor VIIa complex (1, 2). Reportedly, both primary APL cells and APL cell lines express TF, and circulating microparticles harbouring membrane-bound TF have also been detected in acute myeloid leukemia (2, 22). However, in the present study neither TF activity nor TF transcripts in leukaemic cells were altered upon treatment with the siRNA. This is in agreement with the early observation that leukaemic cells from BNML rats with overt disease and disseminated intravascular coagulation do not display procoagulant activity (23). Furthermore, in our recent study of 93 unselected patients with various haematological malignancies excluding APL, we could not find any support for TF as a

Table 1: Effects of siRNA on blood haemostatic marker concentrations.

<table>
<thead>
<tr>
<th>Haemostatic marker</th>
<th>Inactive siRNA</th>
<th>Active siRNA</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAT (ng/ml)</td>
<td>9.9 (8.6–12.0)</td>
<td>6.0 (4.8–7.4)</td>
<td>6.2 (5.7–7.4)</td>
</tr>
<tr>
<td>D-dimer (μg/l)</td>
<td>310 (260–360)</td>
<td>85 (75–130)</td>
<td>85 (78–150)</td>
</tr>
<tr>
<td>PAI-1 (%)</td>
<td>46 (37–50)</td>
<td>23 (18–29)</td>
<td>20 (16–25)</td>
</tr>
<tr>
<td>Fibrinogen (μg/l)</td>
<td>1.8 (1.4–2.0)</td>
<td>2.7 (2.3–3.0)</td>
<td>2.7 (2.4–2.9)</td>
</tr>
<tr>
<td>Tissue factor (% of control)</td>
<td>102 (97–107)</td>
<td>100 (95–107)</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are expressed as medians (interquartile ranges), n = 10. * denotes p < 0.05 for inactive group vs. active group and controls.
main initiator of the hypercoagulable state among these patients (24). We have no definite explanation for why the BNML cells did not display enhanced TF-activity compared to controls. Possibly this reflects a difference between the human and rat form of APL, e.g. the BNML rats do not harbour the 15:17 chromosome translocation.

As recently reviewed, platelet abnormalities may occur in acute myeloid leukemias, and this may possibly contribute to hypercoagulability (25). Platelet function was not assessed in the present study.

Although the BNML cells apparently do not initiate hypercoagulation via TF, these cells may possibly activate haemostasis via other mechanisms, e.g. involving cytokines that can be inhibited upon treatment with the active siRNA. In our previous study we found among BNML rats given the active siRNA compared to those given the inactive siRNA, a marked increase in the serum concentrations of the proinflammatory cytokines tumour necrosis factor (TNF-α) and interferon γ, while that of IL-1β was unaltered (11). Among the anti-inflammatory cytokines the serum concentrations of IL-10 declined whereas that of IL-13 remained unchanged. Reportedly both pro- and anti-inflammatory cytokines can modify the haemostatic process, but whether these cytokine changes can directly modify haemostasis in APL remains to be verified (26).

Despite the putative potential of applying specific immunotherapy to myeloid leukemias, the clinical results have mostly been disappointing (7). A major obstacle has been self-tolerance often mediated via suppression of T cell activation (8). The main rationale of the present vaccine approach using the bifunctional siRNA transfected into dendritic cells, was to break self-tolerance by partly delivering a TLR signal to stimulate innate immunity, and partly by inhibiting the immunosuppressive IL-10. In line with this, we could demonstrate that the active siRNA efficiently stimulated TLRs, measured as an increase in TNF-α, while it concomitantly silenced the IL-10 gene (11). Collectively this combined immunostimulation and immunoinhibition led to a profound anti-leukaemic activity evidenced by increased life-span and less metastasis among the BNML rats (11). Here we extend these observations by showing that treatment with the active siRNA also profoundly reduced the hypercoagulation that frequently accompanies the development and progression of APL, and which increases morbidity and mortality among these patients. It is therefore likely that the positive effect of siRNA treatment on leukaemogenesis in BNML at least partly can be explained by its effect on the haemostatic process. The present approach may be particularly useful as therapy in patients that will not tolerate intensive chemotherapy combined with transplantation, e.g. among elderly AML patients.

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References