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REGULAR ARTICLE

Suppression of HUVEC tissue factor synthesis by antisense oligodeoxynucleotide

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Abstract

Tissue factor (TF) is an important regulator and effector molecule of coagulation. It is primary known as a cofactor for factor VIIa-mediated triggering of blood coagulation, which proceeds in a cascade of extracellular reactions, ultimately resulting in thrombin formation. In sepsis, expression of TF by activated monocytes, macrophages and endothelial cells may lead to disseminated intravascular coagulation. Further studies have suggested that TF also plays non-haemostatic roles in blood vessel development, tumor angiogenesis, metastasis and inflammation. In the present study we examined the feasibility of inhibiting lipopolysaccharide (LPS)-induced TF expression in cultured human umbilical vein endothelial cells (HUVECs) using a modified phosphorothioate antisense oligodeoxynucleotide targeted to the TF mRNA. CD31 receptor-mediated endocytosis was used as a means of delivering TF antisense oligomer to HUVECs. This DNA carrier system consists of anti-CD31 antibody conjugated to the antisense. Co-exposure of HUVECs with TF antisense and LPS resulted in 54.6 ± 3.2% suppression of TF activity when compared with control LPS stimulated cells. The antisense also reduced the LPS-induced TF mRNA level. Control experiments with TF sense and mismatched antisense oligomers were performed to exclude non-specific inhibitory effects. The cytotoxicity of the antisense oligomer conjugate was also evaluated. Results demonstrate that this TF antisense oligomer specifically suppressed the synthesis of biologically active endothelial TF and that antisense oligomers might represent a useful tool in the investigation of endothelial TF function/biology.

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Tissue Factor is now recognized to be the initiator of blood coagulation *in vivo* [1,2] and is a principal participant in coagulation disorders associated with

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endotoxemia [3], malignancy [4], immune dysfunction [5,6] and atherosclerosis [7,8]. It is well established that upon exposure to stimulants such as bacterial LPS, cytokines, mitogens and immune complexes, peripheral blood monocytes synthesize and express functional TF [9–11]. However the *in vivo* expression of TF by endothelial cells remains uncertain [12–14]. TF normally remains encrypted within the cells of origin and in this state does not initiate coagulation activation. TF vesicle release from monocytes provides an important route for dissemination and de-encryption via platelet membrane phosphatidyl serine interaction [15].

So far production of functional and antigenic TF by endothelial cells has been demonstrated in studies using cultured human umbilical vein endothelial cells (HUVEC) [16,17]. Whether the production of TF by HUVECs *in vitro* is a true reflection of *in vivo* generation following endotoxin exposure is debated. TF expression in certain disorders, endotoxemia, malignancies and in atherosclerosis, may be an important component of the hypercoagulable nature of these conditions. Endotoxemia, the inflammatory response and the associated widespread thrombosis that may occur in septic shock all contribute to the development of multiorgan failure, the requirement for amputations and even death. The exploration of TF expression inhibitory modalities which offer the potential for therapeutic intervention in the above conditions is an important objective. The attraction of inhibiting intravascular TF sources whilst not impeding perivascular TF availability should reduce the haemorrhagic complications posed by systemic anticoagulants and inhibitors.

Of interest is the use of antisense oligodeoxynucleotides (aODN) to inhibit/suppress specific protein production [18,19]. aODN are designed to inhibit selected gene expression, thus providing the prospect of developing safe and effective therapies for a large number of diseases [20]. They are also powerful research tools for the investigation of gene function and in target validation studies. Several studies have demonstrated the efficacy of antisense ODN *in vivo* [21–24], and in recent years, the number of antisense therapies employed in clinical trials has steadily increased [25]. Even with such increasing evidence of activity *in vivo*, poor cytosolic uptake of aODN remains a central problem. Most cell types require specific delivery systems for efficient uptake of aODN in cell culture. Methods of antisense/gene delivery *in vitro* include calcium phosphate/DNA co-precipitation, DEAE-dextran, microinjection, controlled-release polymers and electroporation [26–28]. However these methods are generally not applicable for use *in vivo* due to problems associated with cytotoxicity or low efficiency. Liposomes, which have the advantage of improved efficiency [29] and lower toxicity [30,31], suffer from the lack of cellular specificity. As an alternative strategy, we have previously demonstrated the use of receptor-mediated endocytosis (RME), [32,33], to deliver antisense to human blood monocytes.

In the present study RME has been employed to deliver antisense to HUVECs by targeting the endothelial cell receptor CD31 (PE-CAM-1) [34,35].

The suppression of TF activity was assessed by measuring the activity of coagulation factor Xa generated by the action of the TF-VIIa complex on factor X. FXa activity was detected using a specific substrate for factor Xa. Here we present results obtained for the suppression of human umbilical vein endothelial cell tissue factor activity following exposure to the antisense oligodeoxynucleotide directed against TF mRNA.

Materials and methods

Preparation of anti-CD31 immunoglobulin-poly(L-lysine) carrier

The fab fragment of mouse anti-human CD31 antibody (Dako Corp.—Carpinteria, CA) was linked to poly(L-lysine) (hydrobromide, MW 30KD), (Sigma Chemical Co.—St Louis, MO) using the heterobifunctional cross-linking reagent *N*-Succinimidyl 3-(2-Pyridyldithiol) proprionate (SPDP) [36], (Pierce Chemical Co.—Rockford, IL). 2.5 μl of 20 mM SPDP in absolute ethanol was incubated with the anti-CD31 fab fragment (1 mg/ml) in phosphate buffered saline (PBS) at room temperature for 1 h. Unreacted SPDP and low molecular weight reaction products were removed by dialysis. This was done by placing the mixture in a dialysis membrane (cut off limit 13 kDa, Medicell International Ltd, London, England), and dialysing against PBS for 6 to 8 h at 4 °C. The modified fab fragment was then reduced by the addition of 25 mM dithiothreitol (DTT) (Sigma Chemical Co.), pH 4.5 for 1 h at room temperature. A 15-fold molar excess of poly(L-lysine) and SPDP relative to the modified fab fragment were added, and the reaction incubated at 4 °C for 24 h. The resulting fab polylysine conjugate was dialysed (as described above) to remove low molecular weight reaction products and then stored at 4 °C in 0.9% (*w*/*v*) NaCl solution for further use.

Synthesis of mixed-backbone antisense oligodeoxynucleotide (MB-aODN)

The published DNA sequence of TF [37,38] was used as a template for the synthesis of a end-modified MB-aODN; this consisted of 2′-*o*-methyl RNA placed at both the 3′-and 5′-ends of the phosphorothioate oligo of eighteen bases (Oswel DNA Service, Southampton, UK). The sequence that we chose was shared by tissue factors from human and non-human sources, and contained the rare tripeptide motif, Trp–Lys–Ser (WKS) which had been predicted as a functional motif involved in the interaction with serine proteases [38,39].

Two further mixed-backbone ODNs were also synthesized for the purpose of providing negative controls; (a) TF sense mixed-backbone oligodeoxynucleotide (MB-sODN)—of identical sequence to part of the human TF cDNA sequence, (b) mismatch MB-aODN—of identical bases to the TF MB-aODN but with the sequence order mismatched at four bases. Sequence identity of all three MB-ODNs with other known