Identification of a domain in apolipoprotein B-100 that inhibits the procoagulant activity of tissue factor

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The ability of low-density lipoprotein (LDL) to inhibit the procoagulant activity of tissue factor is mediated by a direct protein–protein interaction involving apolipoprotein (apo) B-100. A lysine-rich sequence within apo B-100 (residues 3121–3217), which we have termed lysine-rich apo B-100-derived (KRAD)-98 peptide, may be responsible for its activity. Within this region, residues 3147–3160 (KRAD-14) contain an exceptionally high proportion of positive amino acids. Both recombinant KRAD-98 and KRAD-14 peptides inhibited the procoagulant activity of tissue factor by preventing the activation of factor VII. KRAD-14 also inhibited the prothrombinase components, factors Xa and V. In comparison with the parent

protein (apo B-100), KRAD-14 peptide displayed a 20-fold enhancement in the rate of inhibition, whereas KRAD-98 peptide exhibited a rate closer to that of apo B-100. Mutational analysis of KRAD-14 peptide revealed three adjacent amino acids, alteration of which greatly reduced the inhibitory potential of this peptide. A peptide derived from tissue factor (residues 58–66) was found to act co-operatively with tissue factor itself, but also augmented the inhibition of tissue-factor activity by apo B-100. In conclusion, LDL may be a physiological regulator of haemostatic mechanisms through the interactions of lysine-rich domains of apo B-100 with tissue factor.

INTRODUCTION

The exposure of tissue factor, upon injury to endothelium, initiates the extrinsic pathway of coagulation and leads to formation of a blood clot on the injured surface [1,2]. Following clot formation, the procoagulant effect of tissue factor is restrained by a number of circulating inhibitors within the serum, including tissue-factor-pathway inhibitor (TFPI) [3] and apolipoprotein (apo) A-II within high-density lipoprotein (HDL) [4]. The ability of low-density lipoprotein (LDL) to inhibit the procoagulant activity of tissue factor arises from direct interaction of the protein moieties of tissue factor and LDL (apo B-100) [5–8]. The inhibition of tissue factor by LDL occurs at a slower rate, but persists longer than that of TFPI [6,8]. Therefore, LDL may be a secondary physiological inhibitor of tissue factor, supplementing the effect of TFPI. Moreover, we previously reported the involvement of a peptide within tissue factor (residues 58–66), which closely resembled one of the repeated domains within the LDL-receptor protein (residues 283–291) [8] that are responsible for binding to apo B-100 [9]. The binding of the proteins involves the positively charged residues within apo B-100 and negatively charged amino acids in tissue factor [8]. The inhibition of tissue factor may be disrupted with poly-L-lysine and, to a lesser extent, by poly-L-arginine [10]. Furthermore, blocking of lysine residues, e.g. during oxidation of LDL, also seems to disrupt the inhibition [5]. The primary sequence of apo B-100 contains two distinct sites within the putative receptor-binding domain that may interact with the similar regions identified in the LDL receptor and tissue factor [8,10]. Identification of the domain responsible for inhibiting tissue factor would give insight into the potential role of LDL in haemostasis.

EXPERIMENTAL

Materials

Oligonucleotide synthesis was carried out on a Biosearch Cyclone oligonucleotide synthesizer (PerSeptive Biosystems, Hertford, U.K.) using reagents purchased from Cambio (Cambridge, U.K.). Pinpoint Xa protein expression kit, Chromatophor protein visualization system, streptavidin–alkaline phosphatase conjugate, restriction and modifying enzymes, polymerase and *Escherichia coli* (JM109) cells were obtained from Promega (Southampton, U.K.). Deoxynucleotides and ampicillin were from Gibco Life Sciences (Paisley, Scotland, U.K.), and enterokinase, chymotrypsin, *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide and *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, isopropyl β -thiogalactopyranoside, biotin, Terrific broth and control and factor-deficient plasma were obtained from Sigma (Poole, Dorset, U.K.). The apo B-100 15mer peptide (fragment 3358–3372) (TRLTRKRGKLALATL) and the control 9mer peptide (VQGEESNDK) were also purchased from Sigma. Recombinant tissue factor was from Gamidor (Abingdon, Oxfordshire, U.K.). A synthetic lysine-rich apo B-100-derived (KRAD)-14 peptide was purchased to order (Genosys Biotechnologies, Cambridge, U.K.). The molecular mass and the purity of all synthetic peptides were confirmed by MS and HPLC.

Cloning and expression of peptides and protein isolation

In order to minimize the possibility of peptide aggregation due to presence of hydrophobic amino acids, the peptide sequence KASKKKHKHRHSTT, given the name KRAD-14, was prepared instead of the parent (KAYKKKHKHRHSIT). The secondary structure of the peptide was not compromised by these

Abbreviations used: apo, apolipoprotein; HDL, high-density lipoprotein; KRAD, lysine-rich apo B-100-derived; LDL, low-density lipoprotein; TFPI, tissue-factor-pathway inhibitor.

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substitutions, as determined by prediction methods [11,12]. Both DNA strands were synthesized on the oligonucleotide synthesizer. Either a chymotrypsin or an enterokinase cleavage site was defined between the biotinylation tag and the peptide. The oligonucleotides were prepared with unique restriction ends and were ligated into plasmid Pinpoint Xa(3) predigested with appropriate enzymes. In addition, four mutant forms of KRAD-14 peptide and three control peptides were prepared. The mutant peptides were KAQSAKNKHHSTT, KAQSKANKHHSTT, KAQSKKAKHHSTT and KAQSKKNAHHSTT, and the control peptides were reverse KRAD-14 (TTSHRHKNKSAQK), proline-rich KRAD-14 (KAQPKKPKPRHSTT) and scrambled KRAD-14 (KAHSKTRSKTHNQK). Similarly, the DNA sequence coding for tissue-factor residues 58–66 (DLTDEIVKD) was prepared and cloned as described above. A synthetic 9mer peptide (VQGEESNDK) was used as a control for this peptide. KRAD-98 peptide (98 amino acids) corresponded to residues 3121–3217 of apo B-100 sequence. Overlapping strands of DNA (each 200 bp long) with non-identical termini were engineered using the oligonucleotide synthesizer. The strands were annealed and completed by addition of Taq polymerase. The product was examined by agarose-gel electrophoresis. The KRAD-98 DNA was digested and ligated into the vector as above.

Competent *E. coli* (JM109) cells were subsequently transfected with either the plasmid containing the KRAD-14 insert, KRAD-14 mutant inserts, KRAD-98 insert, tissue-factor fragment (58–66) insert, plasmid without any insert, or with Pinpoint containing each of the control peptides, and selected by growth in Terrific broth, containing ampicillin (100 µg/ml). The cells were then grown in the presence of 100 µg/ml isopropyl β-thiogalactopyranoside/5 µg/ml biotin, and proteins were isolated using the Softlink matrix according to the manufacturer's instructions. The peptide samples were then dialysed and freeze-dried. SDS/12% (w/v) polyacrylamide electrophoresis was carried out on the samples using the Chromatophor protein visualization system and blotted and probed using streptavidin-alkaline phosphatase conjugate. In order to release the peptides from the biotinylated fusion protein, aliquots (1 mg/ml) of the isolated fusion proteins were incubated with bovine pancreatic chymotrypsin (0.1 mg/ml) or enterokinase (0.1 mg/ml). At the concentrations used, the enzymes did not have any effect on the assay.

Preparation of LDL and reconstituted apo B-100

LDL was prepared by a modification [13] of a discontinuous-gradient ultracentrifugation procedure [14]. Apo B-100 was isolated and reconstituted (1 mg/ml) in phosphatidylcholine (1 mg/ml) as described previously [5].

Modelling of the interaction of KRAD-14 peptide with tissue factor (residues 58–66)

A computer-based search of the entire primary sequence of apo B-100 was carried out to determine the concentration of positive and lysine residues within the protein sequence [10] and give clues about the span and structure of these domains. The structure of KRAD-98 was predicted using the Threader program [15] and, as was the case with KRAD-14, modelled using Alchemy III program (Tripos Associates, St. Louis, MO, USA).

Measurement of tissue-factor activity

The one-stage prothrombin time assay was carried out at 37 °C using 50 nM recombinant tissue factor in 12.5 mM CaCl₂ to

which standard plasma was added (2:1, v/v), and the clotting time was measured using a coagulometer. The two-stage amidolytic assay was carried out according to published procedures [16]. To test the effects of the various peptides, they were pre-incubated, over a range of concentrations (0–500 nM) with tissue factor for 0–120 min before application of either method. The tissue-factor activity was then calculated from a standard curve prepared previously, and the percentage of inhibition due to the peptide being tested was calculated as $100 \times [(\text{initial activity}) - (\text{residual activity})]/(\text{initial activity})$.

In some experiments, recombinant tissue factor (1 mg/ml) was delipidated as described previously [5,6], to remove phosphatidylserine and other lipids, and reconstituted with phosphatidylcholine (1 mg/ml).

Measurement of thrombin, factor Xa and V activity

KRAD-14 peptide (0–500 nM) or control peptides were incubated with thrombin (100 nM) at 37 °C, and the thrombin activity was monitored for 60 min. The proteolytic activity of thrombin was measured using the substrate *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide according to published procedures [16]. The procoagulant activity of thrombin was measured using control plasma and measuring the clotting time. Similar assays were carried out on factor Xa using another substrate, *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide, and factor Xa-deficient plasma. The procoagulant activity of factor V was assayed using factor V-deficient plasma, pre-activated by incubation (2 min) with tissue factor (200 nM) in the presence of CaCl₂ (12.5 mM), and measurement of the clotting time. The percentage of inhibition was calculated against a control.

Measurement of factor VII activation and activity

Tissue factor (200 nM) and factor VII (200 nM) were incubated for 10 min with 25 mM CaCl₂, in the presence or absence of factor Xa (100 nM) before addition of KRAD-14 peptide (500 nM) and a further 10 min incubation. The procoagulant activity was then measured in factor VII-deficient plasma.

RESULTS

Modelling of the interaction of KRAD-14 peptide and tissue factor (residues 58–66)

The computer-based analysis of the entire apo B-100 sequence revealed two distinct regions with a large proportion of positively charged amino acids. The sequences, residues 3121–3217 (KRAD-98) and residues 3300–3400, are both within the receptor-binding domain of apo B-100 [17–20]. The former domain is also especially rich in lysine residues. KRAD-98 was predicted to be a distinct four-helix-bundle domain within apo B-100 (Figure 1). In this model the domain is surrounded by positively charged amino acids, particularly lysines, which make the domain hydrophilic and exposed. The highest concentration of lysine residues was within the amino acid regions 3147–3160 (KRAD-14). In order to explain a possible mode of interaction between KRAD-14 peptide and the complementary peptide within tissue-factor peptide (residues 58–66) the KRAD-14 peptide, essentially an α-helix, was positioned at an approximate angle of 20° [21] to tissue factor (residue 58–66), hence indicating the interacting amino acids. Approximate positioning of tissue-factor peptide (residues 58–66) and KRAD-14 sequence (Figure 2) showed that the lysine and histidine residues within KRAD-14 peptide form pockets of positive charge around the negatively charged aspartate and glutamate amino acids within tissue-factor peptide. Of special significance was the interaction of

Figure 1 Top view of the four-helix-bundle model of KRAD-98 peptide

KRAD-98 sequence (apo B-100 residues 3121–3217) was predicted to be a four-helix-bundle structure using the Threader program and was constructed using the Alchemy III program. The amino acids involved in the turns were omitted for clarity. The positively charged amino acids, lysine (K), arginine (R) and histidine (H) residues are highlighted to demonstrate the positive envelope surrounding the domain. The KRAD-14 sequence has been indicated.

Figure 2 Space-filling model of KRAD-14 peptide and tissue-factor fragment (56–66) indicating the interacting amino acids

The KRAD-14 sequence was constructed using Alchemy III, and energy minimization carried out, until the energy gradient was below 0.01. In order to explain a possible mode of interaction between KRAD-14 peptide and the complementary domain within tissue factor (residues 56–66), the KRAD-14 peptide, essentially an α -helix, was positioned at an approximate angle of 20° to tissue factor (residue 56–66), hence indicating the interacting amino acids. The interacting positive and negative residues are marked, and the two sequences are turned by 90° in opposite directions to reveal the interacting charged pockets. In the model, Asp-56 (tissue factor) interacts with Lys-3147 (apo B-100), Asp-58 (tissue factor) interacts with Lys-3151 and -3152 (apo B-100), Asp-61 (tissue factor) interacts with Arg-3156 and His-3155 (apo B-100), and Glu-62 (tissue factor) interacts with His-3157 (apo B-100).

Figure 3 Time-course assay of the inhibition of tissue factor by KRAD-14 and KRAD-98 peptides and reconstituted apo B-100

Three 1 ml samples containing recombinant tissue factor (100 nM) were mixed with 500 nM KRAD-14 peptide (■), 500 nM KRAD-98 peptide (□), 500 nM reconstituted apo B-100 (▲), 500 nM control 15mer apo B-100 (fragment 3358–3372) peptide (◆) or 500 nM recombinant control protein (◇) (All three recombinant control peptides had similar activities, although, for clarity, only one, that of the reverse control peptide, is shown here). The concentrations used are equivalent to that of apo B-100 in normal plasma. A portion (100 μ l) of each sample was removed immediately and assayed for tissue-factor activity, by means of the one-stage prothrombin time assay. The rest of the samples were incubated at 37°C , and further 100 μ l samples were removed at intervals up to 120 min and measured. The tissue-factor activity was calculated from a standard curve prepared previously, and the percentage of inhibition was calculated as $100 \times [(\text{initial activity}) - (\text{residual activity})]/(\text{initial activity})$. The data were obtained from four independent experiments, each performed in duplicate.

lysine-3151 and lysine-3152 of apo B-100 with aspartate-58 of tissue factor. The involvement of the tissue-factor sequence (58–66) [22], and particularly aspartate-58, in binding and activation of factor VII has been demonstrated previously [23].

Preparation of recombinant peptides

Western blotting of the peptides revealed bands of biotinylated protein with molecular masses of 15 kDa (tissue-factor fragment 58–66), 18 kDa (KRAD-14, mutants of KRAD-14 and control peptides) and 27 kDa (KRAD-98), which agree with theoretical molecular masses calculated (results not shown).

Effect of KRAD-14 and KRAD-98 peptides on tissue-factor activity

The results from the one-stage prothrombin time assay and the two-stage amidolytic assay were similar. The percentages of inhibition were calculated from the data generated using both methods, but only those from the one-stage assay are shown. Samples of recombinant tissue factor were incubated with KRAD-14 peptide, KRAD-98 peptide, reconstituted apo B-100 or each of the control peptides at 37°C , and tissue-factor activity monitored up to 120 min before measurement of tissue-factor activity. Both recombinant and synthetic KRAD-14 peptides inhibited the procoagulant activity of recombinant tissue factor by $> 90\%$ following a 2 min incubation at 37°C (Figure 3). KRAD-98 peptide inhibited tissue-factor activity by $> 90\%$, after a 40 min incubation; reconstituted apo B-100 inhibited tissue-factor activity following a 90 min incubation. Neither the control 15mer apo B-100 (fragment 3358–3372) peptide (TRLTRKRLKLATAL) nor any of the three recombinant

Figure 4 Effect of the KRAD-14 peptide concentration on inhibition of tissue factor

Aliquots (1 ml each) of recombinant tissue factor (100 nM) were incubated at 37 °C with a KRAD-14 peptide at concentrations 50 (◆), 100 (▲), 200 (■), 300 (◇) and 500 (□) nM (final concentration). A portion (100 µl) of each sample was removed immediately and assayed for tissue-factor activity, by means of the one-stage prothrombin time assay. The rest of the samples were incubated at 37 °C, and further 100 µl samples were removed at intervals up to 40 min and measured. The percentage of inhibition was calculated as $100 \times [(initial\ activity) - (residual\ activity)] / (initial\ activity)$. The data were obtained from three independent experiments.

Table 1 Influence of alanine mutations of KRAD-14 peptide

Samples of KRAD-14 peptide, four mutant peptides and the three controls (500 nM) were incubated with tissue factor (100 nM) at 37 °C for 10 min before assaying using the one-stage prothrombin time assay. The activity was calculated from a tissue-factor standard curve. The data are expressed as means $\pm$ S.E.M., obtained from three independent experiments. * $P > 0.01$ against control (no peptide); † $P > 0.01$ and ‡ $P > 0.05$ against KRAD-14. —, not applicable.

Peptide	Sequence	Procoagulant activity (units/ml)	Inhibition (%)
Control	—	46.5 $\pm$ 5	—
KRAD-14	KAQSKKNKRRHSTT	10.0 $\pm$ 3*	78.5
Mutant-1	KAQSAKNKRRHSTT	47.8 $\pm$ 4†	—2.7
Mutant-2	KAQSKANKRRHSTT	36.5 $\pm$ 4*†	21.5
Mutant-3	KAQSKAKRRHSTT	22.0 $\pm$ 3*†	52.7
Mutant-4	KAQSKKNAHRHSTT	2.1 $\pm$ 2*‡	95.5
Control-1 (reverse)	TTSHRHKNKKSQAK	47.8 $\pm$ 6†	—2.7
Control-2 (proline-rich)	KAQPKKPKPRHSTT	49.6 $\pm$ 7†	—6.7
Control-3 (scrambled)	KAHSKTRSKTHNQK	42.6 $\pm$ 5†	8

control peptides, TTSHRHKNKKSQAK, proline-rich KRAD-14 (KAQPKKPKPRHSTT) and scrambled KRAD-14 (KAHSKTRSKTHNQK), had any detectable effect on tissue factor. The pre-incubation of tissue factor with a range of KRAD-14-peptide concentrations (50–500 nM) at 37 °C, demonstrated that the inhibition is concentration dependent and exhaustible (Figure 4) ($K_{d,app} = 1.5 \times 10^{-7}$).

In order to establish whether all of the amino acids within the lysine-rich region of KRAD-14 were essential to its inhibitory activity, the properties of a number of mutants of KRAD-14 were tested, where each of these residues was substituted by

Table 2 Inhibition of tissue factor, reconstituted in phosphatidylcholine, by KRAD-14 and KRAD-98 peptides and reconstituted apo B-100

Recombinant tissue factor was delipidated to remove phosphatidylserine and other lipids and reconstituted at 1 mg/ml with phosphatidylcholine (1 mg/ml). Four 1 ml samples were incubated with 500 nM KRAD-14 peptide, 500 nM KRAD-98 peptide, 500 nM reconstituted apo B-100, each of the four control peptides (500 nM) or distilled water at 37 °C for 30 min, before assaying. The percentage of inhibition was calculated against the control. The data are expressed as means $\pm$ S.E.M., obtained from three independent experiments. * $P > 0.01$ against control (no peptide). —, not applicable.

Sample	Procoagulant activity (units/ml)	Inhibition (%)
Distilled water	4.70 $\pm$ 0.06	—
Apo B-100 control peptide (3358–3372)	4.68 $\pm$ 0.06	0.4
Reverse KRAD-14	4.68 $\pm$ 0.08	0.4
Proline-rich KRAD-14	4.71 $\pm$ 0.04	—0.2
Scrambled KRAD-14	4.61 $\pm$ 0.08	1.9
Recombinant KRAD-14 peptide	0.05 $\pm$ 0.04*	99
Recombinant KRAD-98 peptide	0.71 $\pm$ 0.04*	85
Reconstituted apo B-100	1.79 $\pm$ 0.05*	62

Figure 5 Time-course assay of the inhibition of the procoagulant activity of factor X by KRAD-14 peptide

Three 1 ml samples containing recombinant KRAD-14 peptide [100 (◆), 200 (▲) and 500 (■) nM] were mixed with factor Xa (100 nM). A portion (100 µl) of each sample was removed immediately and assayed for factor Xa activity by addition to factor Xa-deficient plasma in the presence of CaCl₂ (25 mM) and measuring the clotting time. The rest of the samples were incubated at 37 °C, and further 100 µl samples were removed at intervals up to 40 min and measured as before. The activity was measured against a standard curve, and the percentage of inhibition was calculated against the initial factor Xa activity. The data were obtained from three independent experiments.

alanine. The procoagulant activity of tissue factor was greatly reduced by the mutation of Lys-3151 or Lys-3152 (Table 1) and, to a lesser extent, by mutation of Asn-3153. However, an enhanced inhibitory potential was detected in the sample with a Lys-3154 mutation.

The importance of lipids to the activity of KRAD-14 was also investigated. Reconstitution of delipidated recombinant tissue factor in phosphatidylcholine reduced the activity of the sample by 200-fold. However, the presence of either KRAD-14 or KRAD-98 peptides inhibited this activity by > 99 and $> 85\%$ respectively (Table 2).

Figure 6 Time-course assay of the inhibition of the procoagulant activity of factor V by KRAD-14 peptide

Three 1 ml samples containing recombinant KRAD-14 peptide [100 (◆), 200 (▲) and 500 (■) nM] were mixed with factor V (1 μ M). A portion (100 μ l) of each sample was removed immediately and assayed for factor Xa activity by addition to factor V-deficient plasma, pre-activated by incubation (2 min) with tissue factor (200 nM) in the presence of CaCl_2 (25 mM), and measuring the clotting time. The rest of the samples were incubated at 37 °C, and further 100 μ l samples were removed at intervals up to 40 min and measured as before. The activity was measured against a standard curve, and the percentage of inhibition was calculated against the initial factor V activity. The data were obtained from three independent experiments.

Effect of KRAD-14 on thrombin and factor Xa and factor V activity

Neither KRAD-14 peptide nor the control peptides exhibited any significant influence on either the clotting ability or the proteolytic activity of thrombin. However, a small but significant inhibition of coagulation (e.g. 40 % at 500 nM after 10 min) was observed upon pre-incubation of KRAD-14 with either factor Xa ($K_{d,\text{app}} = 1.6 \times 10^{-8}$) (Figure 5) or factor V ($K_{d,\text{app}} = 1.9 \times 10^{-8}$) (Figure 6).

Influence of KRAD-14 on factor VII activation and activity

When tissue factor (200 nM), factor VII (200 nM) and Xa (100 nM) were pre-incubated at 37 °C to activate factor VII, before addition of KRAD-14, the peptide did not inhibit the procoagulant activity significantly [12 ± 8 (S.E.M.) %] compared with any of the control peptides. Conversely, in the absence of factor Xa, KRAD-14 peptide was able to inhibit the activation of the pro-enzyme factor VII [74 ± 8 (S.E.M.) %], indicating that the action of KRAD-14 was directed towards the formation of an active factor VII/tissue factor complex rather than the proteolytic activity of the factor VIIa/tissue factor complex against factors X and IX.

Influence of tissue-factor peptide (58–66) on tissue-factor activity and inhibition

It has previously been proposed that tissue factor may dimerize and thereby increase its activity [24]. Therefore, recombinant tissue factor (100 nM) was incubated with 1 μ M of either the isolated tissue-factor peptide or control 9mer peptide for 5 min, in the presence or absence of reconstituted apo B-100 (250 nM), at 37 °C, and the procoagulant activity was monitored over

Table 3 Influence of tissue-factor fragment 58–66 on the procoagulant activity of tissue factor

Three aliquots of tissue factor (100 nM) were incubated for 5 min with the isolated tissue-factor peptide (1 μ M), control peptide (1 μ M) or distilled water at 37 °C before assaying using the one-stage prothrombin time assay. The activity was calculated from a tissue-factor standard curve. The data are expressed as means $\pm$ S.E.M., obtained from three independent experiments. * $P > 0.01$ against control (no peptide).

Sample	Procoagulant activity (units/ml)
Distilled water	6.70 ± 0.11
Control peptide	6.75 ± 0.06
Tissue-factor fragment (58–66)	$7.45 \pm 0.06^*$

Figure 7 Influence of peptide 58–66 on the interaction and inhibition of tissue factor by apo B-100

Samples of tissue factor (100 nM) were incubated with reconstituted apo B-100 (250 nM final concentration) in the presence (■) and absence (▲) of the isolated tissue-factor peptide (1 μ M), at 37 °C. Aliquots (100 μ l each) were removed at the start (time zero) and at various intervals up to 180 min and were assayed by means of the one-stage prothrombin time assay. The data were obtained from five independent experiments.

180 min. The isolated tissue-factor-derived peptide (58–66) had no endogenous procoagulant activity in the absence of tissue factor, but incubation of tissue factor with the recombinant tissue-factor peptide (58–66) enhanced its procoagulant activity to a small (15 %) but significant extent (Table 3). In contrast, the presence of the tissue-factor peptide also accelerated the rate of inhibition of tissue-factor activity by reconstituted apo B-100, so that by 15 min the inhibition was already clearly evident (Figure 7).

DISCUSSION

We previously reported that the interaction of tissue factor with apo B-100 occurs in at least two sites [8]. Thus, whereas the first interaction involves the attainment of a more hydrophobic configuration occurring within 20 min, the second interaction involves negatively charged residues and positive free amino groups and occurs over a longer period of time (up to 60 min). Furthermore, a possible apo B-binding site on tissue factor, due to its similarity to apo B-100-binding domain within the LDL-receptor protein, was indicated. In addition, the interaction and

inhibition of tissue factor by apo B-100 is dependent on lysine residues within the latter [8,10]. On the other hand, the presence of cationic amino acid was not sufficient for the inhibition, which seems to require a specific sequence of basic amino acids [10]. The ability of KRAD-98 peptide to inhibit tissue-factor activity in the same manner as native apo B-100 suggests that the inhibitory potential of apo B-100 arises from this domain (residues 3121–3217), which may also be involved in the interaction of apo B-100 with the cellular receptor for LDL [9]. The interaction between apo B-100 and LDL-receptor protein may involve the sequence identified here and a negative peptide sequence [8] similar to that of tissue factor (residues 58–66) [22,24] within the LDL-cellular receptor. Therefore, we propose that the interaction of apo B-100 with tissue factor is essentially similar in nature to that with its receptor.

KRAD-14 peptide was an efficient inhibitor of tissue factor, and the rate of inhibition was much faster than that observed with either LDL or apo B-100 [5–8,10]. This may be due to the immense size of apo B-100 and LDL. Also, the inhibition was more persistent than that observed with TFPI [6]. The ability of KRAD-14 and KRAD-98 peptides to inhibit tissue-factor activity, when reconstituted in phosphatidylcholine, indicate that the activity of the peptide arises from a direct interaction with tissue factor rather than a non-specific association with the negatively charged phosphatidylserine molecules required for tissue-factor activity [2,25]. The marked reduction of the inhibitory potential of KRAD-14 peptide upon alanine substitution of Lys-3151 and -3152 and Asn-3153 indicates the importance of these residues in the interaction of the peptide with tissue factor. The finding that substitution of Lys-3154 enhanced the inhibitory action may be explained by the charge conflict with the adjacent Lys-65 of tissue factor (Figure 2). These findings, and the fact that the randomized control peptide of KRAD-14 is inactive, indicate that a highly specific structure (Lys-Lys-Asn motif) is required for this interaction.

Furthermore, for the period tested, KRAD-14 peptide did not significantly affect the procoagulant activity, once factor VII had been activated (factor VIIa) by incubation with factor X and tissue factor. However, it severely reduced the procoagulant activity when incubated with tissue factor and zymogen factor VII. This implies that the mechanism of inhibition by KRAD-14 peptide mainly involves the prevention of factor VII activation on the tissue-factor surface. This does not preclude the possibility that a complex between factor VII and tissue factor can be formed in the presence of this peptide.

It has been reported that dimerization of tissue factor can lead to an augmentation of the procoagulant activity of tissue factor [25]. The tissue factor (58–66) peptide enhanced the procoagulant activity of tissue factor modestly by acting co-operatively. The tissue-factor peptide (58–66) may by its small size allow a rapid rate of formation. This combination had a more significant effect of enhancing the inhibition of tissue factor by apo B-100. This is consistent with the hypothesis that the inhibitory action of apo B-100 on the extrinsic pathway of inhibition arises from its direct interaction involving the sequence residues 58–66 within tissue factor as proposed previously [8].

Pre-incubation of KRAD-14 peptide with factor X and factor V but not thrombin inhibited the procoagulant activity to an extent. The ability of KRAD-14 peptide to inhibit factor Xa and V may be due to a repeated sequence, with the consensus of DLSQTNLSPE, within factor V that has some similarity to that of tissue factor (58–66) and LDL-receptor protein (283–291).

The four negative amino acids are often conserved in these positions within factor V and may be the site of interaction of KRAD-14 peptide with the factor Xa–factor V complex. Nevertheless, the small magnitude of these inhibitions suggests that the principal effect of KRAD-14 is with tissue factor.

This work has identified the interacting site, between apo B-100 and tissue factor, that results in the inhibition of the procoagulant activity of the latter. Tissue factor is structurally related to class II cytokine receptors [23,24]. The peptide ligands for such receptors are normally helical [23], and a number of cytokines have been shown to be four-helix-bundle domains. Therefore, we propose that KRAD-14 peptide, KRAD-98 and apo B-100 are suitable ligands for tissue factor, and their interactions mask amino acids essential for binding and activation of factor VII, particularly Asp-58 [22,23], hence inhibiting the procoagulant activity. From this work, we conclude that the inhibitory action of the lysine-rich domain (3147–3160) within apo B-100 on the extrinsic pathway of coagulation arises from the direct interaction with tissue factor [8] and is dissimilar, but complementary, to that of TFPI, which is essentially a protease inhibitor, which inhibits the activities of factor VIIa and Xa [26]. Therefore, LDL may be a slow-acting secondary inhibitor of tissue factor subsequent to the initial inhibition by TFPI. The accelerated effect of KRAD-14 peptide, as compared with apo B-100, as well as its size and ease of preparation, makes this a potential agent in treatment of hypercoagulable conditions.

REFERENCES

- Nemerson, Y. and Pepke, D. (1985) *Thromb. Res.* **51**, 165–173
- Nemerson, Y. (1988) *Blood* **71**, 1–8
- Broze, Jr., G. J. (1995) *Thromb. Haemostasis* **74**, 90–93
- Carson, S. D. (1987) *J. Biol. Chem.* **262**, 718–721
- Ettelaie, C., Howell, R. M. and Bruckdorfer, K. R. (1995) *Biochim. Biophys. Acta* **1257**, 25–30
- Ettelaie, C., Wilbourn, B., Adam, J. M., James, N. J. and Bruckdorfer, K. R. (1996) *Blood Coag. Fibrinolysis* **7**, P11
- Ettelaie, C. and Howell, R. M. (1992) *Thromb. Res.* **68**, 175–184
- Ettelaie, C., James, N. J., Wilbourn, B., Adam, J. M., Naseem, K. M. and Bruckdorfer, K. R. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16**, 639–647
- Goldstein, J. L. and Brown, M. S. (1985) *Annu. Rev. Cell Biol.* **1**, 1–39
- Ettelaie, C., Haris, P. I., James, N. J., Wilbourn, B. R., Adam, J. M. and Bruckdorfer, K. R. (1997) *Biochim. Biophys. Acta* **1345**, 237–247
- Chou, P. Y. and Fasman, G. D. (1978) *Annu. Rev. Biochem.* **47**, 251–276
- Garnier, J., Osguthorpe, D. J. and Robson, B. (1978) *J. Mol. Biol.* **120**, 97–120
- Naseem, K. M., Goodall, A. H. and Bruckdorfer, K. R. (1997) *Platelets* **8**, 163–173
- Chung, B. H., Wilkinson, T., Geer, J. C. and Segrest, J. P. (1980) *J. Lipid Res.* **21**, 284–291
- Jones, D., Taylor, W. R. and Thornton, J. M. (1992) *Nature (London)* **358**, 86–89
- Sandset, P. M., Abildgaard, U. and Pettersen, M. (1987) *Thromb. Res.* **47**, 389–400
- Milne, R., Théolis, R., Maurice, R., Pease, R., Weech, P., Rassar, E., Fruchart, J.-C., Scott, J. and Marcel, Y. (1989) *J. Biol. Chem.* **264**, 19754–19760
- Lawn, A. and Scott, J. A. (1990) *J. Lipid Res.* **31**, 1109–1120
- Chan, L. (1992) *J. Biol. Chem.* **267**, 25621–25624
- Olsson, U., Camejo, G., Hurt-Camejo, E., Elfsber, K., Wiklund, O. and Bondjers, G. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17**, 149–155
- Chothia, C., Levitt, M. and Richardson, D. (1981) *J. Mol. Biol.* **145**, 215–250
- Banner, D. W., D'Arcy, A., Chene, C., Winkler, F. K., Guha, A., Konigsberg, W. H., Nemerson, Y. and Kirchhofer, D. (1996) *Nature (London)* **380**, 41–46
- Ruf, W., Schullek, J. R., Stone, M. J. and Edgington, T. S. (1994) *Biochemistry* **33**, 1565–1572
- Harlos, K., Martin, D. M. A., O'Brien, D. P., Jones, E. Y., Stuart, D. I., Polikarpov, I., Miller, A., Tuddenham, E. G. D. and Boys, C. W. G. (1994) *Nature (London)* **370**, 662–666
- Bach, R. R. and Moldow, C. F. (1997) *Blood* **89**, 3270–3276
- Girard, T. S., Warren, L. A., Novotny, W. F., Likert, K. M., Brown, S. G., Miletich, J. P. and Broze, G. J. (1989) *Nature (London)* **338**, 518–520