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ORIGINAL ARTICLE

Investigation of the mechanisms of tissue factor-mediated evasion of tumour cells from cellular cytotoxicity

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Abstract
Aims We previously reported that overexpression of tissue factor (TF) protected HT29 tumour cells from cellular cytotoxicity through a mechanism requiring the presence of the cytoplasmic domain of TF. In this investigation the mechanism of TF-mediated immune evasion has been examined.
Methods The influence of alanine-substitution at Ser253 and Ser258 of TF (TF_{Ala253} and TF_{Ala258}) on the induction of cytotoxic evasion, as well as expression of vascular cell adhesion molecule-1 and intra-cellular adhesion molecule-1 (VCAM-1 and ICAM-1) was investigated. Moreover, we examined the effect of transfection of four 20-mer peptides, corresponding to the C-terminal residues of TF, with different phosphorylation states, on promotion of evasion from cell cytotoxicity.
Results Cells overexpressing TF_{Ala258} and to a lesser extent overexpressing TF_{Ala253}, exhibited a reduced ability to evade cellular cytotoxicity compared to cells overexpressing the wild-type TF. Furthermore, the increase in protection acquired was greatest on transfection of Ser258-phosphsorylated form of the cytoplasmic peptide, lower in double-phosphorylated and Ser253-phosphorylated peptides respectively, and lowest in the unphosphorylated form. Finally, the expression of VCAM-1 mRNA as well as surface antigen was reduced on overexpression of TF_{wt} but was partially reverted in the cells transfected to overexpress TF_{Ala253} or TF_{Ala258}.
Conclusions These data show that the phosphorylation of TF at Ser258 and to a lesser extent Ser253, plays an essential role in the protective influence of TF on immune evasion by tumour cells, and that the mechanism could involve the downregulation of key surface antigens, such as adhesion proteins, involved in cell:cell interaction.

Keywords Tissue factor · Metastasis · Cytotoxicity · Phosphorylation · VCAM-1

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Introduction

While the association between haemostatic mechanisms and malignancy has been known for a long time [1], the mechanisms by which tissue factor (TF) can induce tumour metastasis has become a topic of great interest more recently [2, 3]. The role of TF in tumour growth has, at least in part, been attributed to the ability of TF in promoting angiogenesis [4–6]. The ability of TF to promote metastasis of TF-transfected melanoma cells in severe combined immunodeficient (SCID) mice has been previously reported [7]. Furthermore, Abe et al showed that the cytoplasmic domain of TF is responsible for the upregulation of vascular endothelial growth factor (VEGF) in melanoma cells, in a mechanism that is independent of FVIIa [8]. It is known that the prometastatic properties of TF-transfected melanoma cells are reduced by the deletion of the cytoplasmic domain of TF [7, 9]. Analysis of the human TF protein sequence has revealed a consensus sequence for protein kinase C phosphorylation in the cytoplasmic domain which is shown to be phosphorylated

in response to phorbol esters [10, 11]. Moreover, the function of the cytoplasmic domain in metastasis has been more specifically attributed to the requirement of two intracellular serine residues (Ser253 and Ser258) in this domain [12, 13].

Previously, we reported that the overexpression of TF, using a mammalian cell expression vector, conferred protection in HT29 and K562 cells against recognition and lyses by peripheral blood mononuclear cells (PBMC) [14]. Furthermore, by expressing a truncated form of TF, we demonstrated that the majority of this protective effect was dependent on the presence of the cytoplasmic domain of TF and not mediated by coagulation-dependent mechanisms. This change in the percentage of surviving cells would be sufficient to account for the increased rate of metastasis in TF-expressing tumours as suggested by others [15]. In the present study, by alanine substitution of the two serine residues (Ser253 and Ser258) in turn, and in combination, we investigated the role of phosphorylation of the cytoplasmic domain of TF in the induction of the mechanism of cellular evasion. Moreover, since the recognition of the cells by the natural killer (NK) cells requires cellular interaction, mediated through adhesion molecules [16, 17], using the mutant forms of TF, we examined the hypothesis that the influence of TF on immune evasion might be mediated through alterations in the expression of these adhesion receptors.

Materials and methods

Cell culture

Colorectal adenocarcinoma cell line (HT29) were obtained from the ATCC (Teddington, UK) and maintained in RPMI medium (90% (v/v)), foetal calf serum (10% (v/v)), containing 1% (v/v) antibiotic/antimycotic solution (Sigma Chemical Company Ltd., Poole, UK).

Plasmid preparation and site-directed mutagenesis

Alanine-substitutions were carried out at Ser253 and Ser258 in turn, to produce mutated forms of TF (TF_{Ala253} and TF_{Ala258}). Mutagenesis was carried out on previously cloned pEGFP-TF_{wt} plasmids using the QuickChange® site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) as described before [14]. Briefly, the amplification of the pEGFP-TF construct was carried out by polymerase chain reaction at an annealing temperature of 55°C and an extension time of 16 min, using the following primers (the specific mutations are underlined).

TF_{Ala253} mutagenesis

Forward primer: 5′CAGGAGTGGGGCAG**GCC**TGGAAGGAGAAC
Reverse primer: 5′GTTCTCCTTCCA**GGC**CTGCCCCACTCCTG

TF_{Ala258} mutagenesis

Forward primer: 5′GCTGGAAGGAGAAC**GCC**CCACTGAATG
Reverse primer: 5′CATTCAGTGG**GGC**GTTCTCCTTCCAGC

Following the reaction, the wild type DNA was digested by incubation with *Dpn*I and full digestion confirmed using a non-amplified sample of the plasmid, treated similarly.

Transient transfection of HT29 cells

Cells (5×10^5/well) pre-adapted to OptiMEM-1 media in a 12-well plate were transfected with pEGFP-TF_{wt}, pEGFP-TF_{Ala253} and pEGFP-TF_{Ala258} constructs (1 μg), using Lipofectin (Invitrogen, Paisley, UK) as previously described [14[t1]]. Following transfection, cells were allowed to express the hybrid protein for at least 72 h prior to assaying. All assays were carried out against two sets of controls, one transfected with the empty pEGFP-C3 plasmid and the other non-transfected cells. The plasmids were found to be free of endotoxin when tested using the Limulus Amebocyte Lysate kit (LAL, Cambrex Bio Science, Wokingham, UK). Transfection efficiency was consistently above 40%.

One-stage prothrombin time assay

The activities of TF_{wt}, TF_{Ala253} and TF_{Ala258} overexpressed in 4×10^5 HT29 cells were measured and compared by the one-stage prothrombin time assay. The clotting times were converted to TF activity (U/ml) using a standard curve prepared using serial dilutions of recombinant human TF (Dade Behring, Milton Keynes, UK). The stock TF preparation was assumed to contain 1,000 U/ml which corresponds to 0.13 ng/ml of recombinant TF. Cells were resuspended in PBS (100 μl) prior to assaying. 25 mM $CaCl_2$ (100 μl) and the cell suspension (4×10^5 cells in 100 μl) were incubated together for 30 s before the addition of normal human plasma (Helena Laboratories, Sunderland, UK) (100 μl). The time taken for the clot to form was recorded using a Cascade-M coagulometer (Helena Laboratories, Sunderland, UK). The activity of the TF was confirmed as previously described using an excess of murine monoclonal antibody (ID:4509) (5 μl) capable of blocking human TF (Axis-Shield, Cambridge, UK) [18].

Peptide synthesis and transfection

Peptides corresponding to amino acids Lys_{244} through to Ser_{263} of TF were prepared by F-moc based solid phase synthesis. Peptide synthesis was carried out using established manual synthesis procedures as before [19, 20]. Four forms of the peptide, un-phosphorylated, Ser_{253}-phosphorylated, Ser_{258}-phosphorylated and double-phosphorylated were prepared by substituting Fmoc-phosphoserine for Fmoc-serine at the appropriate positions. The phosphate group is protected and remains so until the final deprotection step of the peptide. Moreover, a fluorescein-labelled TF peptide was used to assess the efficiency of the peptide transfection into cells. Finally, a random 20-mer mixed peptide was used as control, i.e. a mixture of all 20 amino acids was used at each step, hence 20^{20} potentially different combinations of peptides were possible.

To transfect the cells, HT29 cells (5×10^5/well) were adapted to serum-free media (SFM) and seeded out into 24 well plates. The transfection reagent was prepared by mixing 500 ng of each peptide (Endotoxin-free using the LAL assay) and 2 µl of the Chariot reagent (Active Motif, Rixensart, Belgium) in 200 µl of PBS and incubated for 1 h at room temperature. The transfection reagent was then mixed with 400 µl of SFM and overlaid onto washed cells and incubated for a further 1 h at 37°C. Finally 1 ml of SFM was added and incubated for a minimum of 2 h before assaying. The cells were washed and the presence of fluorescein assessed by flow cytometry.

Preparation of PBMC

PBMC were prepared from anticoagulated human blood as described previously [14]. The PBMC were resuspended in 10 ml of PBS and cell density was adjusted to 10^6 cells/ml in DMSO freeze medium (TCS CellWorks, Botolph Claydon, UK) and cryopreserved until use.

Qualitative analysis of VCAM-1 and ICAM-1 mRNA expression

Sets of HT29 cells were transfected and allowed to express pEGFP-TF_{wt} and pEGFP-C3 over 72 h. RNA isolation was performed using the TRI-reagent system (Sigma), according to the manufacturer's instructions. One-step RT-PCR reaction was carried out using Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech Inc, Giles, UK) and 0.1 µg of total RNA and by adhering to criteria for successful analysis [21]. The RT step was carried out at 42°C for 30 min followed by denaturation for 5 min at 95°C. PCR (30 cycles) was performed as follows: 1 min at 95°C, 1 min with the following primers and at an annealing temperature of 55°C, followed by extension for 1 min at 72°C. The primers used were as follows and the expected amplified bands were detectable at 230 bp for vascular cell adhesion molecule-1 (VCAM-1), 566 bp for intra-cellular adhesion molecule-1 (ICAM-1) and 437 bp for β–actin.

(VCAM-1-forward) 5′-TTGACTTGCAGCACCACAGGC-3′,
(VCAM-1-reverse) 5′-ATCTCTGGAGCTGGTAGACCC-3′,
(ICAM-1-forward) 5′-ACCTTCCTCACCGTGTACTGG-3′,
(ICAM-1-reverse) 5′-ATTACTGCACACGTCAGCCGC-3′,
(β-actin-forward) 5′-CCAGAGCAAGAGAGGCATCC-3′,
(β-actin-reverse) 5′-CTGTGGTGGTGAAGCTGTAG-3′.

RT-PCR products were visualised on a 1.5% (w/v) agarose gel, stained with SYBR Green I. Images were acquired using the GeneSnap imaging system and the bands analysed using the GeneTool program. The expression of each gene was normalised against the house keeping gene, β-actin. Absence of contaminating DNA was demonstrated by carrying out the above reaction but without the reverse transcriptase step.

Flow cytometric analysis of cell surface VCAM-1 and ICAM-1 antigen

To assess the surface expression of VCAM-1 and ICAM-1, cells (2×10^5) were resuspended in PBS (100 µl) containing FITC labelled murine monoclonal antibodies (10 µl) against VCAM-1 (clone MVCAM A (429)) and ICAM-1 (clone MEM-171) (1 µg/ml, ABD Serotec UK, Oxford, UK) and incubated for 30 min at 4°C in the dark. The cells were then centrifuged (400 *g* at 5 min), washed twice with PBS, and resuspended in PBS (100 µl) and then incubated with $F(ab')_2$ rabbit anti-mouse IgG:RPE (AbD Serotec) (10 µl) for 60 min at 4°C. Finally, the cells were centrifuged at 400 *g* for 5 min, the pellet washed twice, resuspended in PBS (300 µl) and measured on a FACSCalibur flow cytometer running CellQuest software version 3.3 (BD Biosciences, Oxford, UK).

Measurement of the cytotoxic activity of PBMC towards HT29 cells

The procedure was carried out as described previously [14]. HT29 cells were seeded out (5×10^4/well) in 12 well plates and transfected with pEGFP-TF_{wt} pEGFP-TF_{Ala253}, pEGFP-TF_{Ala258} and pEGFP-C3 (control) as before. Only successfully transfected cells, as determined by flow cytometry or ELISA for TF antigen, and the prothrombin time assays, were used for subsequent investigations. Transfected HT29 cells were labelled with red fluorescence

(SNARF-1) and placed in the wells in serum-free medium (200 µl) and incubated for 4 h. Ten random sectors within each well covering approximately 50% of the well, were imaged on a TS100 microscope (Nikon, Kingston-upon-Thames, UK) equipped with fluorescence attachments and camera. PBMC were added to the wells containing the target cells (HT29) at a ratio of 10:1 (PBMC: HT29) and incubated for 4 h at 37°C. Subsequently, ten random sectors were recorded as before. Analysis was carried out using the ImagePro Plus program (Media Cybernetics, Berkshire, UK) and the data calculated as the percentage cell loss after incubation with PBMC against the initial number of viable HT29 as follows.

$$\% \text{ cell loss} = (\text{No. of cells prior to incubation} - \text{No. of cells after incubation})/\text{No. of cells prior to incubation}$$

Finally, we tested the hypothesis that since adhesion molecules are a requirement for cellular recognition by PBMC, any downregulation or blocking of activity of these surface antigens would in effect confer protection to tumour cells against recognition and cytotoxicity. Sets of HT29 cells (5×10^4/well) were transfected with pEGFP-C3 or pEGFP-TF_{wt} and incubated with 50 mM cyclo(Arg-Gly-Asp-D-Phe-Val) (Merck Biosciences, Nottingham, UK) [22, 23] to block VCAM-1 interactions, or a random pentamer peptide, prepared as before, for 1 h before analysis of cytotoxic evasion, as above.

Statistical analysis

The data were presented as means ± SEM of the experiments. Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS Inc. Chicago, USA). One-Way ANOVA procedure was used for the analysis of variance of data against the control. Tukey's honesty significant difference test was performed as multiple comparison tests to highlight statistically significant differences.

Results

In this study we first investigated the importance of the two serine residues within the cytoplasmic domain of overexpressed TF in HT29 cells, on evasion from immune cytotoxicity. Active forms of human TF protein, carrying mutations at serine 253 (TF_{Ala253}) and serine 258 (TF_{Ala258}) were prepared from the pEGFP-TF expression construct and overexpressed in HT29 cells. These mutations did not significantly influence the procoagulant activity of the overexpressed TF forms ($TF_{wt} = 73 \pm 5$ s, $TF_{Ala253} = 78 \pm 4$ s, $TF_{Ala258} = 72 \pm 2$ s, control cells = >180 s; $n = 5$), indicating comparable levels of transfection.

In order to investigate the influence of the serine mutations on the ability of TF to confer cytotoxic evasion, the target cells were labelled with SNARF-1 and ten fields of view analysed in each sample, using fluorescence image microscopy, before and after challenge with PBMC. The percentage of surviving (labelled) cells was calculated in each sample following incubation with PBMC against the initial number of cells. Cells transfected to overexpress TF_{wt} exhibited a reduction in recognition by PBMC (37% ± 2.5 reduction in cell number) in comparison to untransfected cells (52% ± 4.5 reduction in cell number) ($n = 5$) (Fig. 1) which is in agreement with our previously reported data [14]. Moreover, overexpression of TF_{Ala253} conferred a slight loss in immune evasion (40% ± 2.5 reduction in cell number) but was more significant in cells overexpressing TF_{Ala258} (46% ± 3.5 reduction in cell number).

To assess the efficiency of the peptide transfection procedure, a fluorescein-labelled 20mer peptide corresponding to the C-terminal of human TF (244–263) was prepared and transfected into HT29 cells, using the Chariot™ protein transfection reagent. The transfection of the fluorescein-labelled peptide resulted in an increase in the mean fluorescence, confirming the usability of the transfection procedure (Fig. 2). HT29 cells were transfected

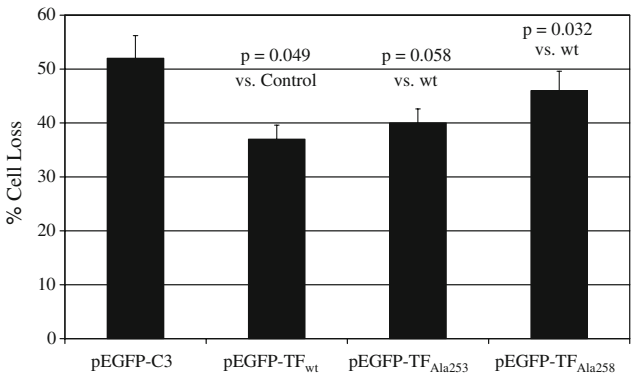

Fig. 1 The influence of mutation of serine residues within TF on cytotoxicity towards HT29 cells. HT29 cells (5×10^5/well) were seeded in a 12-well plate and transfected with pEGFP-C3, pEGFP-TF_{wt} pEGFP-TF_{Ala253} and pEGFP-TF_{Ala258} constructs (1 µg) and allowed to express the hybrid protein for at least 72 h prior to assaying. Cells were then labelled with 5 µM SNARF-1 in culture medium (200 µl final volume). Ten random views within each well were recorded using a fluorescence microscope. The cells were then challenged with five preparations of isolated PBMC at a ratio of 10:1 (PBMC:HT29). After 4 h incubation at 37°C, ten random sections were analysed as before. Analysis was carried out using the Image Pro Plus program and the data were calculated as the percentage cell loss after incubation with PBMC against the initial number of viable HT29 cells. The data represent the mean of five separate experiments, conducted in duplicate ± SEM

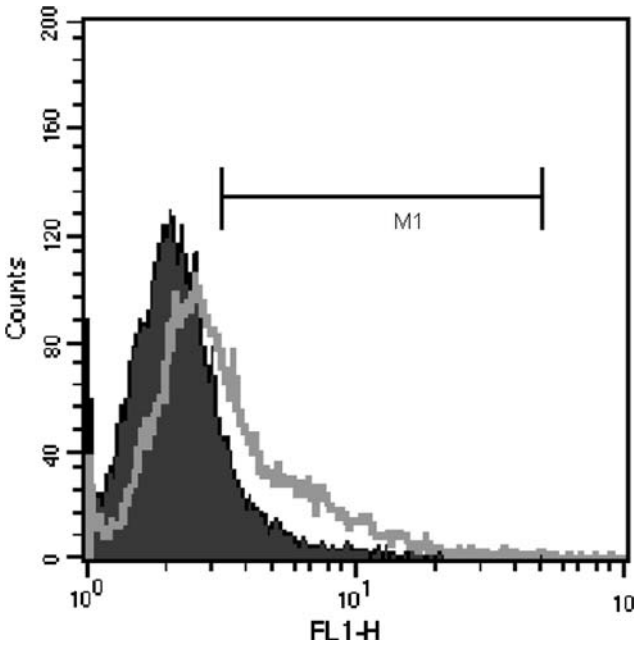

Fig. 2 Assessment of the efficiency of peptide transfection using the Chariot reagent. HT29 cells were transfected with a fluorescein-labelled synthetic peptide corresponding to the Lys244-Ser253 within the cytoplasmic domain of TF using the Chariot reagent. The analysis of transfected (*grey line*) and un-transfected cells (*solid area*) was carried out by flow cytometry. A marker was set containing 12% of the control sample, 38% for peptide-transfected cells were in this region. Mean cell fluorescence intensity for the two samples were 4.7 and 6.3. The data presented are typical of three separate transfections

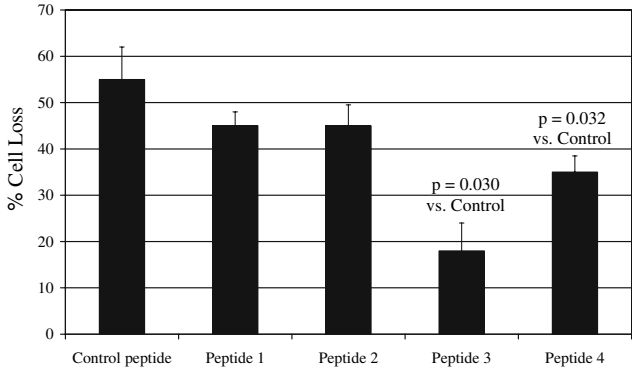

Fig. 3 The influence of phosphorylation of TF-derived peptides on cytotoxicity towards HT29 cells. HT29 cells were transfected with a control random peptide, or four 20-mer peptides corresponding to the C-terminal of TF in four different phosphorylation states (p) as follows: *Peptide 1* KCRKAGVGQSWKENSPLNVS, *Peptide 2* KCRKAGVGQ**p**SWKENSPLNVS, *Peptide 3* KCRKAGVGQSWKEN**p**SPLNVS, *Peptide 4* KCRKAGVGQ**p**SWKEN**p**SPLNVS. A control sample was transfected with a random peptide (not shown). Cells were then labelled with 5 μM SNARF-1 in culture medium (200 μl final volume). Ten random views within each well were recorded using a fluorescence microscope. The cells were then challenged with five preparations of isolated PBMC at a ratio of 10:1 (PBMC:HT29). After 4 h incubation at 37°C, ten random sections were analysed as before. Analysis was carried out using the Image Pro Plus program and the data were calculated as the percentage reduction in cell number after incubation with PBMC against the initial number of viable HT29 cells. The data represent the mean of five separate experiments carried out in duplicates ± SEM

with four 20-mer peptides corresponding to the C-terminal of TF in four different phosphorylation states (p) at serine residues 253 and/or 258, or the random peptide, as below:

Peptide 1: KCRKAGVGQSWKENSPLNVS
Peptide 2: KCRKAGVGQ**p**SWKENSPLNVS
Peptide 3: KCRKAGVGQSWKEN**p**SPLNVS
Peptide 4: KCRKAGVGQ**p**SWKEN**p**SPLNVS

Transfection of the peptides conferred protection against immune cytotoxicity, resulting in 45% ± 30, 45% ± 4.5, 18% ± 5 and 35% ± 3.5 cell loss ($n = 5$ in duplicate), for cells transfected with peptides 1–4 respectively (Fig. 3). Transfection of a random 20-mer peptide had no detectable influence, while the control cells exhibited a 55% ± 7 reduction in surviving cells.

Finally, to examine the mode of action of TF in the induction of immune evasion, we examined alterations in the expression of the adhesion molecules VCAM-1 and ICAM-1, and also the influence of blocking the binding site within VCAM-1 on the cellular cytotoxicity. Prior to analysis, the expression of VCAM-1 and ICAM-1 in HT29 cells was confirmed and it was shown that these cells expressed measurable amounts of VCAM-1 mRNA (270 bp band) and antigen. However, neither RT-PCR amplification nor flow cytometry could detect the presence of any ICAM-1 although, the expression of this receptor by HT29 cells has been reported previously [24]. Hence, the latter was not assessed further. Initially, the expression of VCAM-1 mRNA in HT29 cells, transfected with pEGFP-C3 and pEGFP-TF_{wt}, was measured by semi-quantitative RT-PCR amplification and agarose gel electrophoresis. The amount of mRNA was normalised against *β*–actin amplified in each sample, and the ratio of the mRNA expression, as a percentage of that of the control cells, was calculated (Fig. 4). Our data clearly demonstrates the downregulation of VCAM-1 expression by approximately 85% in cells overexpressing wild type TF. Furthermore, to analyse the influence of serine mutations on the surface expression of the VCAM-1 protein, cells were transfected with pEGFP-C3, pEGFP-TF_{wt}, pEGFP-TF_{Ala253} and pEGFP-TF_{Ala258} and analysed for surface VCAM-1 by flow cytometry, at 72 h post-transfection. A marker was set to detect the presence of VCAM-1 on the control plasmid (pEGFP-C3)-transfected cells. This region contained 63% of the control sample; 32% of pEGFP-TF_{wt} transfected cells, 44% of pEGFP-TF_{Ala253} transfected cells and 78% of pEGFP-TF_{Ala258} transfected cells (Fig. 5). Cell transfected to overexpress wild type TF exhibited a reduction ($\Delta = -31\%$) in VCAM-1 surface antigen expression which is in

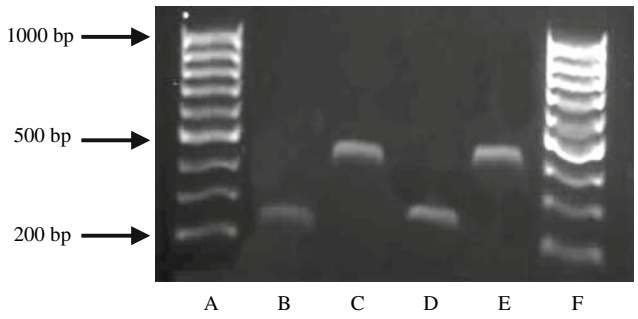

Fig. 4 The influence of overexpression of TF on VCAM-1 mRNA expression. The expression of VCAM-1 mRNA in HT29 cells (5×10^4/well), transfected with pEGFP-C3 and pEGFP-TF_{wt}, was measured at 72 h post-transfection, by semi-quantitative RT-PCR amplification. Volumes of the treated and control amplified samples (5 and 15 µl) were analysed by agarose gel electrophoresis to identify VCAM-1 bands. The amount of mRNA was normalised against *β*–actin amplified in each sample, and the ratio of the mRNA expression, as a percentage of that of control cells, was calculated. The data presented are typical of three separate experiments. *A* and *F* size markers, *B* and *C* VCAM-1 and *β*–actin respectively from the TF overexpressing cells, *D* and *E* VCAM-1 and *β*–actin respectively from control cells

agreement with data obtained from the RT-PCR analysis. Moreover, the overexpression of pEGFP-TF_{Ala253} resulted in reduced VCAM-1 surface expression ($\Delta = -19\%$) while interestingly, the mutation of serine 258 resulted in an increase in surface VCAM-1 ($\Delta = +15\%$).

Finally, pre-incubation of the cells with cyclo(Arg-Gly-Asp-D-Phe-Val) (50 nM) to interfere with cell surface VCAM-1, prior to cytotoxicity assay resulted in up to 29% reduction in cell loss, in the presence or absence of over-expressed TF (1 U/ml) ($n = 3$) (Fig. 6).

Discussion

Recently, we demonstrated that TF has a direct protective influence on the ability of tumour cells to escape immune recognition by PBMC. Hence, TF may allow tumour cells to be protected from cellular cytotoxicity, increasing the possibility of successful tumour dissemination and metastasis. Moreover, this influence was independent of fibrin coat formation, but instead involved the cytoplasmic domain of TF. In this investigation, an attempt was made to elucidate the mechanisms by which the cytoplasmic domain of TF participates in tumour cell evasion of cytotoxic activity of the immune system. Although the HT29 cells are known to express very low levels of active TF, the overexpression of the various TF variants and the comparison to control cells allows the determination of effects on the rate of survival. Additionally, since the majority of the untransfected cells are likely be eliminated, the difference in percentages measured only reflect the minimum amount of change as a result of the overexpression of TF. In agreement with our previous data, the overexpression of wild type TF resulted in a significant reduction in cellular recognition ($\Delta = 15\%$). It has been suggested that a 10% alteration in immune recognition of circulating tumour cells could be sufficient to promote metastasis [15]. The level of suppression observed here is over this limit and therefore may be crucial in the promotion of metastasis in cells that express TF.

In particular, this study focused on the implications of serine phosphorylation within the cytoplasmic domain of TF. Alanine substitutions of both serine 253 and 258 resulted in the reduction of the effectiveness of TF in protecting tumour cells from immune cytotoxicity. Moreover, the latter serine residue seems to be of greater consequence to the ability of TF to confer protection. Full phosphorylation of the cytoplasmic domain of TF is thought to be a two-step process in which the activation of protein kinase C results in the phosphorylation of serine 253 [10]. The subsequent signalling mechanisms, possibly arising from the phosphorylation of this residue, induce the phosphorylation of serine 258, mediated via a proline-directed kinase mechanism [25]. A possible explanation for the reduced rate of immune evasion is that mutation of serine 253 decreases the rate of the downstream serine 258 phosphorylation. Conversely, mutation of serine 258 results in a more profound influence, preventing the mechanism that confers immune evasion in TF-over-expressing cells. This hypothesis was further supported by the use of peptides, corresponding to the various phosphorylation forms of the cytoplasmic domain of TF. The use of these peptides clearly demonstrated that the cytoplasmic domain alone was capable of inducing the mechanisms that confer immune evasion in tumour cells. Phosphorylation of serine 253 alone had little additional influence, over and above that of the unphosphorylated peptide. However, it was clear that the phosphorylation of serine 258 in particular, was most effective in conferring cytotoxic evasion. Finally, cells transfected with the double-phosphorylated peptide exhibited a lower level of evasion than those transfected with the serine 258 phosphorylated peptide alone, suggesting that a possible serine 253 de-phosphorylation step is necessary for full immune evasion. These data are also in agreement with those obtained using alanine-substitutions of the two serine residues, indicating that the transient phosphorylation of serine 253 may be an intermediate step in the formation of phospho-serine 258 TF. In conclusion, it appears that the mechanism of induction of immune evasion by TF follows a set pathway, mediated through transient phosphorylation of serine 253 and ultimately resulting in the phosphorylation of serine 258 and possible de-phosphorylation of serine 253 to confer maximum immune system evasion.

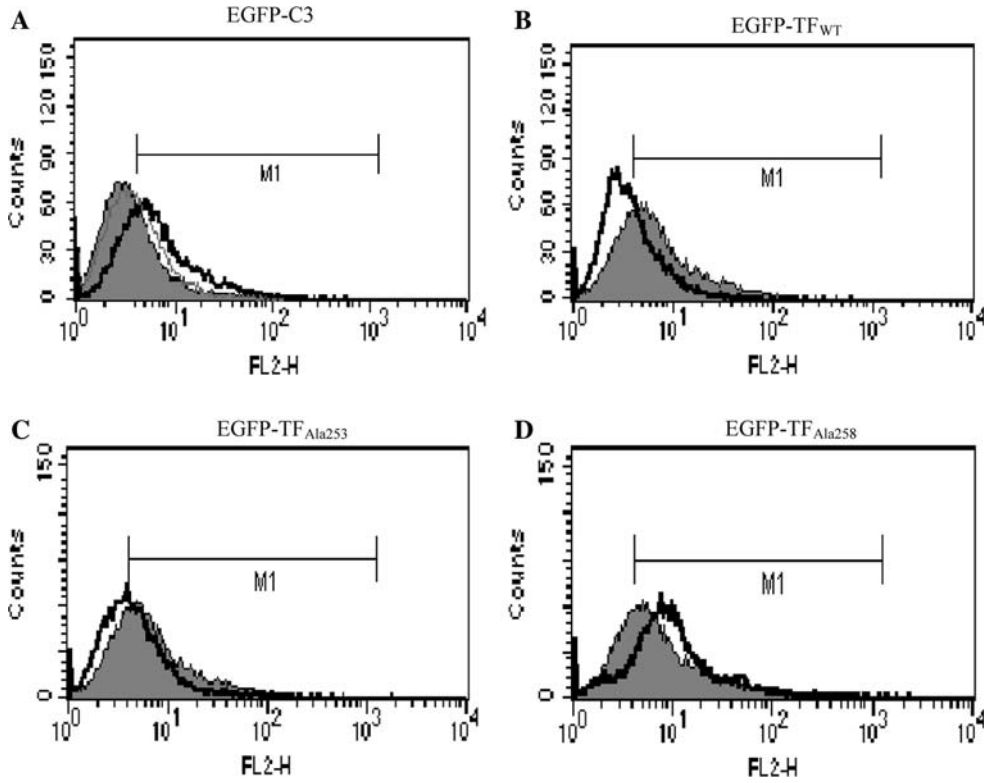

Fig. 5 The influence of mutations of serine residues within TF on VCAM-1 antigen expression. HT29 cells (5×10^5/well) were transfected with pEGFP-TF$_{wt}$, pEGFP-TF $_{Ala253}$ and pEGFP-TF$_{Ala258}$ constructs and allowed to express the protein for 72 h prior to assaying. Cells were harvested and resuspended in 300 µl PBS, incubated with FITC-labelled murine monoclonal antibodies against VCAM-1 for 1 h in the dark, then incubated with F(ab′)$_2$ rabbit anti-mouse IgG:RPE (2nd antibody) (10 µl) in the dark for 1 h. The cells were analysed on a FACSCalibur flow cytometer running CellQuest software. (**a**) The presence of surface VCAM-1 was confirmed in cells transfected with pEGFP-C3 (control cells) and probed with anti-VCAM-1 (*line*), and compared to unlabelled cells (*solid area*) and IgG control (*grey line*). (**b**) The change in surface VCAM-1 was measured against control (pEGFP-C3-transfected) cells (*solid areas*), in cells transfected with pEGFP-TF$_{wt}$ (**b**), pEGFP-TF$_{Ala253}$ transfected cells (**c**), pEGFP-TF$_{Ala258}$ transfected cells (**d**). A marker was set containing 63% of the control sample (**a**); 32% of pEGFP-TF$_{wt}$ transfected cells (**b**), 44% of pEGFP-TF$_{Ala253}$ transfected cells (**c**) and 78% of pEGFP-TF$_{Ala258}$ transfected cells (**d**), measurement of VCAM-1 were in this region. Mean cell fluorescence intensity for the four samples were 11.75, 8.25, 8.6 and 13.1, respectively. The data are typical of three separate experiments

We also tested the hypothesis that the suppression of the recognition of tumour cells by PBMC is mediated through the downregulation of adhesion molecules on the surface of tumour cells. This hypothesis was based on reports which demonstrated that the recognition of tumour cells by NK cells requires the presence of adhesion molecules [16, 17] in addition to the well-defined Ly69 and KIR family of proteins. To confirm the functional importance of the adhesion molecule VCAM-1, we examined the influence of blocking this interaction, using cyclo(Arg-Gly-Asp-D-Phe-Val), on the cytotoxic activity of PBMC towards HT29 cells. Our data indicate that the presence of functional VCAM-1 is at least partially responsible for the recognition of these tumour cells by PBMC (Fig. 4). This function was unaffected by the overexpression of wild type TF in these cells. The finding is in agreement with studies that describe the recognition of ICAM and VCAM on tumour cells by NK cells through an interaction with the leukocyte-function associated antigen (LFA-1) on NK cells [16].

In the next part of the investigation, any alteration in VCAM-1 mRNA expression in response to TF was investigated. Overexpression of EGFP-TF$_{wt}$ in HT29 cells resulted in a marked reduction in VCAM-1 expression ($\Delta = -96\%$) compared with control cells (Fig. 5). This reduction was also observable by analysis of cell surface VCAM-1 using flow cytometry ($\Delta = -31\%$) (Fig. 6). Interestingly, alanine-substitution of serine 258 restored VCAM-1 expression ($\Delta = +15\%$) at a level above that of the control cells, while alanine-substitution of serine 253 did not markedly influence the VCAM-1 suppression. It is therefore clear that serine 258 is essential for the repression of VCAM-1 expression by TF, while serine 253 has little influence. This finding supports the above data which suggest that the phosphorylation of serine 258

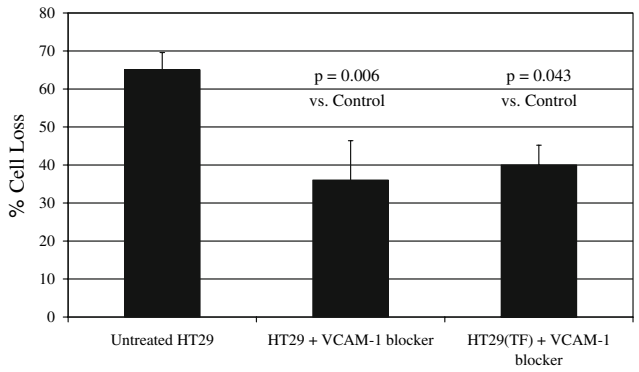

Fig. 6 The influence of blocking of VCAM-1 on cell cytotoxicity towards HT29 cells. Sets of HT29 cells (5×10^4/well) were transfected with pEGFP-C3 or pEGFP-TF_{wt} and incubated with 50 mM cyclo(Arg-Gly-Asp-D-Phe-Val) or a random peptide for 1 h prior to assessment Cells were then labelled with 5 μM SNARF-1 in culture medium (200 μl final volume). Ten random views within each well were recorded using a fluorescence microscope. The cells were then challenged with five preparations of isolated PBMC at a ratio of 10:1 (PBMC:HT29). After 4 h incubation at 37°C, ten random sections were analysed as before. Analysis was carried out using the Image Pro Plus program and the data were calculated as the percentage reduction in cell number after incubation with PBMC against the initial number of viable HT29 cells. The data represent the mean of duplicates from three separate experiments ± SEM

is a crucial step in conferring TF-mediated immune evasion in tumour cells. At present, there is no definite explanation as to why the level of VCAM-1 increased beyond that of the control cells. However, our preliminary data from a separate study suggest that the phosphorylation state of TF may also influence cellular microparticle formation which may in turn interfere with the incorporation of VCAM-1.

The initiation of the coagulation cascade by the TF-factor VIIa complex and subsequent activation of factor X and thrombin result in the induction of cell signalling through the protease activated receptors (PAR) 1 and 2 [26–28]. Moreover, it is now known that the signals arising from the activation of PARs can result in the phosphorylation of the cytoplasmic domain of TF [29]. Therefore, it is feasible that the expression of TF on the surface of tumour cells that disseminate into the bloodstream, can initiate local activation of the coagulation factors, that consequently results in the activation of PARs and serine phosphorylation within TF. Ultimately, the tumour cells downregulate the surface proteins required for recognition by the NK cells, and attain a covert state that is not detectable by the immune system. This mechanism could account for the reported requirement for both the cytoplasmic serine residues and also, the interaction of factor VIIa with TF for full metastatic potential in tumour cells [30].

In conclusion, we have built on our previous study, demonstrating the ability of TF to suppress recognition of tumour cells by the immune system. We have explored the function of the cytoplasmic domain of TF in the suppression of tumour cell recognition by the immune system and have demonstrated the essential role of serine 258 phosphorylation in this mechanism. Furthermore, we suggest that the mechanism of immune evasion involves the suppression of adhesion molecules such as VCAM-1. Therefore, the presence of TF on the surface of tumour cells results in a better likelihood of immune evasion and hence, confers a higher capacity to metastasis to distal tissues in cells that express high levels of TF.

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