Improvement of potential therapeutic value of tumor necrosis-α (TNF-α) by charge modulation in the tip region

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ABSTRACT. Analysis of published data reveals that the introduction of more basic amino acid residues in the flexible N-terminal region of the human tumour necrosis factor alpha (TNF) molecule indicates a weak but consistent trend towards increased *in vitro* cytotoxicity, especially when the effect of N-terminal length is taken into account. In our laboratory, a series of TNF analogues with a charge modification in the tip region of the molecule was prepared, and cytotoxicity measured. Similar trends in cytotoxicity with increasing basicity of the TNF analogue were found in this study for two mouse cell lines, L929 and WEHI-164 clone 13-1, as well as for the human line KYM-1D4. For the series of analogues as a whole, a general increase in *in vitro* cytotoxicity with increasing pI values was not apparent, but some analogues with charge reversal in the tip region, for example, the LK-805 analogue (E107K), exhibited significantly increased cytotoxicity in comparison to native TNF in a range of cell lines, including L929, KYM-1D4-K, WEHI-164 clone 13-1, HEPA 1-6 and EAhy926 cell lines. Experiments with heparinase-pre-treated cells demonstrated that the increased *in vitro* cytotoxicity of LK-805 is most probably due to interactions with cell surface heparan sulphates that effectively concentrate it before binding to TNF receptors occurs. Examination of structural models of TNF bound to soluble TNF receptor 1 (TNFR1) indicates that simple mutations in the tip region most probably cannot interact with receptor binding sites, and therefore do not directly modulate cytotoxicity.

Keywords: TNF, basic analogues, isoelectric point, heparan sulphate, L929, KYM-1D4

Tumour necrosis factor alpha (TNF) is a physiologically important, pleiotropic cytokine with many diverse roles in health and various pathologies. It acts in a complex cytokine network, working agonistically or antagonistically with other cytokines. TNF activity is associated with two different TNF receptors, which can be up-regulated, down-regulated or shed depending on cell type, environment and physiological conditions. TNF receptor 1 (TNFR1), which is responsible for most TNF-mediated cytotoxic effects, is present on the surface of virtually every cell type in the body. Besides cytotoxicity, TNF can trigger several other biological responses, including cell survival and differentiation (maturation), immunomodulatory and antitumour effects, and synthesis of other cytokines. All this makes understanding the true role of TNF *in vivo* difficult to ascertain [1, 2].

An interesting and potentially useful effect of TNF, its antitumour activity, have evoked strong interest in this protein. Unfortunately, most attempts to use TNF systemically as an antitumour agent in human malignant diseases have induced severe side effects [3-5]. To overcome the high systemic toxicity of TNF, which appears to be the major obstacle for its broader use in the treatment of various malignancies, different strategies have been used [6-11]. For example, TNF is particularly successful when applied locally as in isolated limb or organ perfusion techniques, especially in combination with chemotherapeutic melphalan and conditions of mild hyperthermia [12, 13]. Experiments in animal models, where a combination of different strategies was used, e.g., limb or organ perfusion with selected TNF analogues, have shown great promise [14].

There have also been numerous attempts to prepare TNF analogues in order to reduce or abolish its systemic toxicity and increase its specific antitumour activity [14-18].

At the N-terminal of the molecule, single or multiple amino acid residues were mutated, introduced or deleted [14, 17, 19-23]. Observations that introduction of basic amino acid residue(s) in the N-terminal region of the TNF molecule leads to increased, specific, *in vitro* activity have stimulated more detailed studies of this region [16, 17, 24]. Consequently, the N-terminal of TNF was recognised as a good target for different modifications. In contrast, the C-terminal, hidden in the compact structure, was not used for this purpose.

Although many analogues have increased *in vitro* cytotoxicity levels, only a few analogues were actually tested *in*

vivo for antitumour potential using animal tumour models [14, 18, 25]. For the determination of *in vitro* cytotoxicity, various, susceptible mouse and human cell lines have been used [26]. For example, the mouse cell line L929 has been widely used, and one can find large amounts of data for this cell line, including *in vitro* cytotoxicity of various TNF analogues [15-17, 19, 20, 27].

We have designed new TNF analogues based on the known 3D structure of human TNF-α [28] and TNF-β-TNFR1 complex [29]. The exposed tip of the trimeric TNF molecule, with relatively flexible loops, which are not part of the receptor-binding site, is an especially attractive region for introducing new structural variations. Moreover, we have previously shown a very pronounced improvement in the therapeutic index for the analogue LK-805, in which glutamic acid at position 107 is replaced by lysine [18]. Several other analogues with different mutations in this region have been designed and studied with respect to surface charge modulation and variation in *in vitro* biological response. In particular, we report the outcome of cytotoxicity evaluation of these analogues in a range of susceptible murine and human cell lines.

MATERIALS AND METHODS

Reagents

All chemicals used were of analytical grade and were purchased from Sigma unless otherwise stated.

Cell lines and culturing conditions

In this study we used mouse L929 fibroblast cell line (ATCC), mouse WEHI 164 clone 13 fibrosarcoma cell line (ATCC), mouse WEHI 164 clone 13-1 fibrosarcoma cell line (a kind gift from Dr. Terje Espevik), mouse HEPA 1-6 hepatocyte cell line (ATCC), human KYM-1D4 and KYM-1D4-K rhabdomyosarcoma cell lines [30] and human EAhy926 endothelial cell line (a kind gift from Dr. Cora Edgell [31]). WEHI164 clone 13, WEHI164 clone 13-1, L929, HEPA 1-6 and EAhy926 cells were maintained in DMEM, KYM-1D4 and KYM-1D4-K cells were grown in RPMI 1640. All media were supplemented with 10% foetal bovine serum. Cultures were grown in a humidified atmosphere of 5% CO_2 at 37 °C. To allow the cells to enter their logarithmic growth phase, cells were subcultured for 20-24 hours prior to each cytotoxicity assay.

Model proteins

In this study, native TNF and the following TNF analogues were used: LK-804, LK-820, LK-822, LK-803, LK-801, LK-805 and LK-823. Their experimentally determined and calculated pI values are shown in *table 1*.

Cloning and expression

Recombinant TNF proteins were prepared by subcloning a synthetic TNF gene with *Escherichia coli* (E. coli)-optimised codons (British Biotechnology) into suitable expression vectors. Mutations were introduced by oligonucleotide-directed mutagenesis on ssDNA according to Kunkel *et al.* [32].

Table 1
Experimentally determined and calculated isoelectric point (pI) of TNF analogues

Analogue	Experimentally determined pI	Calculated pI
TNF	6,85	6,83
LK-804	7,27	6,90
LK-820	7,40	6,96
LK-822	7,55	7,06
LK-803	7,81	7,80
LK-801	7,84	7,82
LK-805	8,65	8,56
LK-823	9,10	8,91

Purification of TNF and TNF analogues

All TNF proteins were expressed intracellularly in the soluble and biologically active form using various *E. coli* strains at 30 °C in the LB medium [33]. Cells were collected at the end of logarithmic phase and centrifuged. Bacterial pellets were resuspended in 50 mM TRIS/HCl, 30 mM NaCl, pH 8.0 and homogenized with a high-pressure homogeniser EmulsiFlex®-C5 (Avestin). Nucleic acids were precipitated with 0.1% polyethyleneimine. After centrifugation at 15 000 rpm, the supernatants containing TNF proteins were precipitated at 65% saturated ammonium sulphate. The aliquoted precipitates were stored at + 4 °C and dissolved in appropriate buffers prior to chromatographic purification.

Chromatographic isolation of TNF proteins

All chromatographic procedures were carried out using either the FPLC system (Amersham Pharmacia Biotech) or the Knauer HPLC system, both equipped with two pumps, variable UV-Vis wavelength monitor, and a fraction collector FRAC-100 (Amersham Pharmacia Biotech). TNF was isolated by a two-step chromatographic procedure consisting of an anionic step performed on DEAE Sepharose FF (Amersham Pharmacia Biotech) and an HIC step performed on Phenyl Sepharose HP (Amersham Pharmacia Biotech).

TNF analogue LK-805 was isolated by a two-step chromatographic procedure consisting of a cationic step performed on SP Sepharose FF (Amersham Pharmacia Biotech) and an HIC step performed on Macro-prep® Methyl (Bio-Rad).

For all other TNF proteins containing histidine mutations, IMAC was used as the main chromatographic step. Purifications were performed on Chelating Sepharose (Amersham Pharmacia Biotech) charged with Zn^{++}, Cu^{++} or Ni^{++} ions. For polishing steps, ion exchange chromatography on DEAE Sepharose (Amersham Pharmacia Biotech) or HIC on Phenyl Superose (Amersham Pharmacia Biotech) were employed.

N-terminal amino acid sequence analyses were performed on a Procise protein sequencing system 492A (PE Applied Biosystems).

Protein concentration determination

The protein concentration of purified TNF and TNF analogues was spectrophotometrically determined at 280 nm

using an extinction coefficient of 1.62 for 0.1% solution [34]. Relative standard deviations (RSD) of these determinations, which were always made in triplicate, fall within the range of ± 0.14 to ± 0.34 % for individual TNF proteins. This is negligible in comparison to variations of *in vitro* cytotoxicity measurements, therefore, errors in protein determination have not been included in the error bars of the presented graphs.

Storage

Purified TNF and TNF analogues were prepared at 1 mg/mL concentration in phosphate-buffered saline with 0.5 M NaCl, aliquoted and stored at -80° C.

Calculation and experimental determination of isoelectric point

Calculation

Isoelectric points were determined with the Internet service ExPASy[6] – Compute pI/MW tool, which allows the computation of the theoretical pI (isoelectric point) and MW (molecular weight) for a user entered sequence: http://www.expasy.org/tools/pi_tool.html

Experimental determination

For experimental pI value determination, a Multiphor II Electrophoresis system (Amersham Pharmacia Biotech) was used: 0.4 mm-thick gels, with preblended Ampholine Carrier Ampholyte for isoelectric focusing in the wide pH range 3.5 – 9.5, were cast according to the instructions of the manufacturer. After prefocusing and focusing, the protein bands were fixed and stained with Coomassie blue according to the procedure described in the PhastSystem Development Technique File No. 200 (Amersham Pharmacia Biotech). For pI determination, calibration curves using a wide range, standard pI 4.6 – 9.6 (Bio-Rad), a composed standard with pI values 3.6, 4.6, 5.1, 5.9, 6.6 (all from Sigma) and a composed standard with pI values 8.2, 8.6 and 8.8 (Sigma) were used. For pI determination, the main protein band, in most cases representing more than 75% of the signal (densitometrically evaluated by Imaging Densitometer, Model GS-670 (Bio-Rad), data not shown), was taken into consideration. pI values of TNF and its analogues, determined from calibration curves on three different gels, were found to be very reproducible, displaying an average, relative standard deviation (RSD) of ± 0.14 %. Since variation of *in vitro* cytotoxicity testing is much higher, errors in pI value determination have not been included in the error bars of the presented graphs.

Biological activity of TNF proteins - cytotoxicity assay

Suspension or semi-attached cell lines

Biological activity was measured as cytotoxic activity against WEHI 164 clone 13, WEHI 164 clone 13-1, KYM-1D4 and KYM-1D4-K cell lines according to the procedure of Meager [30]. Off-plate dilutions of TNF standard (1st WHO international standard of TNF, 87/650) and TNF derivatives were prepared from aliquots (c = 1. 0 mg/mL) independently for every assay run. After that, serial, 2-fold dilutions of TNF proteins were prepared with medium in 96-well microtitre plates. One µg/mL of actinomycin D was added for the assay with WEHI cell lines and none for the assay with KYM cell lines. After that, $2x10^4$ cells per well in 50 µl culture medium were added. After incubation in a 5% CO_2 atmosphere at 37 °C for 18-20 hours (WEHI cell lines) or 40-44 hours (KYM cell lines), cell survival was estimated with MTT assay [35]. The insoluble formazan product of MTT formed in metabolically active cells was dissolved with 10% SDS in 0.02 M HCl after 1 hour at 37 °C. Absorbances were determined at 590 nm.

Adherent cell line

Biological activity was measured as cytotoxic activity against L929, HEPA 1-6 and EAhy926 cell lines according to the procedure of Flick and Grifford [36]. Briefly, $2x10^4$ cells in 100 µL culture medium were seeded into 96-well microtitre plates and incubated for 24 hours (37 °C, 5% CO_2).

Off-plate dilutions of TNF proteins were prepared as described above. Serial, 2-fold dilutions of TNF standard and TNF derivatives were prepared and added to the wells in the presence of 2 µg/mL actinomycin D. After incubation at 37 °C, 5% CO_2 for 18-20 hours (L929, HEPA 1-6) or 40-44 hours (EAhy926), cell survival was estimated as above with the MTT assay, or viable cells were fixed with 25% glutaraldehyde and stained with 0.5% crystal violet in 20% methanol. After solubilising the cells in 1% SDS, the absorbance was measured at 570 nm.

Biological activity of TNF proteins - the addition of heparinase

The method was basically the same as described above, with the following modification: cells were treated either with 0.5 or 1 U/mL Heparinase I or with the mixture of 0.15 U/well Heparinase I, 0.15 U/well Heparinase II and 0.15 U/well Heparinase III, for 1 or 4 hours, prior to the addition of serial dilutions of TNF standard, native TNF and of the analogue LK-805.

In all methods used, biological activity was measured in three independent assay runs, each with 8 replicates on separate assay plates. The potency of the TNF and its analogues was determined by comparison of the dilutions of the TNF standard and the TNF analogue yielding 50% of maximal cytotoxicity, for every assay plate separately. The average of the potencies, standard deviations and coefficient of variance were determined for each assay run separately. The intra-assay coefficient of variance ranged from 5 to 12%. The specific activity plotted on the graphs represents the average of the three, independently determined potencies. The error bars represent the inter-assay variation (coefficient of variance ranged from 6 to 23%).

TNF-α solubleTNFR1 structure modelling

Models of the TNF analogue LK-805·solubleTNFR1 complex were produced with the computer program Swiss-Pdb Viewer V3-7b2 on the basis of structures from Protein data bank 1 TNR and 1TNF.

RESULTS AND DISCUSSION

TNF analogues with charge modification at the N-terminal - analysis of selected, published data

In the past, much effort was invested into research involving mutating various amino acid residues near the natural

N-terminal of TNF, the main goal being to obtain an analogue with improved antitumour activity [16, 17, 19-22, 24, 27, 37-40]. It was generally recognised that shortening of the N-terminal, as well as introduction of basic amino acid residues in this region, resulted in higher *in vitro* cytotoxicity, which was also suggested to correlate with more efficient antitumour activity [17, 19, 20].

Systematic evaluation of these data has not so far been performed. Therefore, we collected relevant published data into a graph showing normalized cytotoxic activities versus calculated pI values of TNF and various analogues *(figure 1)*. All results collected of *in vitro* cytotoxicity testing were obtained in the mouse L929 cell line. The use of this cell line has an historical background and was chosen because of its high susceptibility to TNF in the presence of actinomycin D, which made it conducive to measurement of low TNF levels in biological fluids [26]. Since most laboratories engaged in *in vitro* biological activity testing of TNF, use the L929 cell line, relevant comparison of the published results is possible.

Inspection of data in *figure 1* indicates no significant correlation, but all results fall into a defined zone (within dashed lines), which shows some evidence of a consistent trend. We have to take into account that analogues, summarised in *table 2*, and used for constructing this graph, possess N-termini of different lengths, which also influences their *in vitro* cytotoxicity [19, 20, 41]. In our previous study we had already demonstrated that the extension of the N-terminal, e.g., by histidine tags, significantly reduced the specific activity in the L929 cell line [41]. On the other hand, it is well documented that shortening of the N-terminal up to the eighth amino acid residue enhances the specific activity [19, 20]. The influence of extension as well as shortening of the N-terminal on *in vitro* cytotoxicity can be ascribed to steric hindrance of the receptor binding site, which is roughly described in terms of radius of gyration of the structurally undefined N-terminal chain [41]. Therefore, cytotoxicity results are probably influenced by at least two different factors, N-terminal length and its basicity.

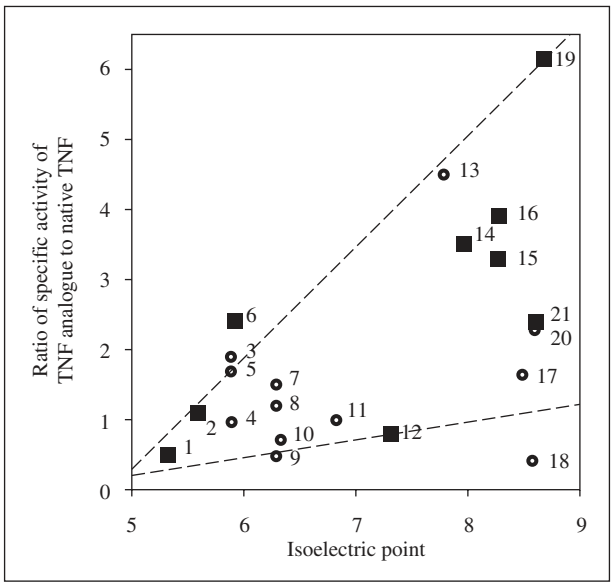

Figure 1
Analysis of published data. Relative specific cytotoxicity in relation to isoelectric point (pI) of TNF analogues being modified at the N-terminal as determined in the L929 cell line (for explanation see text and *table 2*). The values obtained in individual laboratories were normalized by us in such a way that the value for TNF cytotoxicity was defined as 1 and the activities of analogues were expressed relative to TNF cytotoxicity as higher or lower values.

To show separate effects of the N-terminal length and pI value on cytotoxicity, we analysed the reduced subset of data for nine analogues having the same N-terminal length

Table 2
Selected examples of TNF analogues with charge and length modification at the N-termina

	Analogue	N terminal part of the TNF analogue or TNF -alpha																Theoretical Ip	Ratio of SA of TNF analogue to TNF-alpha	Reference
								1	2	3	4	5	6	7	8	9	10	11		
1	Mutant 609														D	D	D	K	5,32	0,5	17
2	Mutant 604														P	D	D	K	5,58	1,1	17
3	Deletion 6													T	P	S	D	K	5,88	1,9	20
4	F4168									S	S	S	R	G	D	S	D	K	5,89	0,96	22, 45
5	Deletion 8															S	D	K	5,89	1,7	19
6	Deletion 7														P	S	D	K	5,92	2,4	19
7	Deletion 4											S	R	T	P	S	D	K	6,28	1,5	19
8	Deletion 2									S	S	S	R	T	P	S	D	K	6,28	1,2	20
9	F4236	M	D	G	Y	I	G	S	R	S	S	S	R	A	P	S	D	K	6,29	0,49	46
10	Deletion 10																	K	6,33	0,7	20
11	TNF - alpha						M	V	R	S	S	S	R	T	P	S	D	K	6,83	1	
12	Mutant 467														P	S	R	K	7,31	0,8	17
13	TNF-b									M	V	R	K	R	P	S	D	K	7,78	4,5	37
14	Mutant 475														R	S	R	K	7,97	3,5	17
15	Mutant 473														P	K	R	K	8,27	3,3	17
16	Mutant 603														P	R	R	K	8,28	3,9	17
17	rTNF-S_{AM2}							V	R	S	C	T	R	T	P	S	R	K	8,49	1,64	34, 35
18	TNF-M5						M	V	R	S	S	S	R	T	P	S	R	K	8,58	0,4	36
19	Mutant 471														R	K	R	K	8,59	6,0	17, 20
20	rTNF-S_{AM1}							V	R	S	S	T	R	T	P	S	R	K	8,60	2,3	34, 35
21	Mutant 608														R	R	R	K	8,60	2,4	17

(squares in *figure 1*). We observe a stronger correlation between pI and cytotoxicity for this more homogeneous series of various ΔN7 TNF analogues. Synergistic overlapping of these two effects, N-terminal length and its basic character, is most apparent in Mutant 471 (point 19 in *table 2* and *figure 1*) having highest specific activity, which is most probably the result of optimal N-terminal length and high basic character. On the other hand, in analogue TNF-M5 (point 18 in *table 2* and *figure 1*) having equal pI, the cytotoxicity is much lower, most probably due to the longer N-terminal. However, in any of the cases considered we cannot exclude the possible contributions on the increased or decreased activity, of specific interactions of individual N-termini with receptor-binding sites lying nearby.

Considering these « trends », it should be borne in mind that large data dispersion also arises from different methods and different sublines of the L929 cells used by various research groups.

Tip region

To further explore the possible influence of increasing pI of TNF analogues on the cytotoxicity, another interesting region, the tip of the TNF trimer was studied.

It should be pointed out that the tip of the trimer is oriented towards the cell surface when interacting with the receptors [29]. Further, in contrast to the completely unstructured and flexible N-terminal, one can expect to see the influence of charge more clearly by studying the less flexible and more localised tip region, where no extreme variations in length of the peptide loop are made.

Several new analogues have been designed, all mutated in the exposed tip region, of the trimeric TNF molecule.

Two of these analogues, LK-801 (E107H, G108H) and LK-805 (E107K), having increased pI values, exhibited significantly reduced systemic toxicity in tumour-bearing mouse models compared to the native TNF [18]. Interestingly, analogue LK-801 exerted *in vivo* antitumour activities comparable to the native TNF molecule, but the analogue LK-805 with even more pronounced charge reversal in the tip region, also showed significantly higher antitumour activity in the same animal model. Furthermore, analogue LK-805 showed approximately 2-fold higher specific cytotoxicity in comparison to unmodified TNF in the mouse L929 cell line [18]. Thus, in this case, a relatively small increase of *in vitro* cytotoxicity in the L929 cells was accompanied by a large effect on therapeutically important properties in the *in vivo* mouse model.

This somehow « paradoxical effect », i.e., of having higher *in vitro* toxicity and higher *in vivo* antitumour activity but reduced *in vivo* systemic toxicity, is, although rare, the most desirable one, leading to potentially safer and more effective therapeutic applications. However, we have observed other situations, e.g. some analogues show even higher *in vitro* toxicity, but also much higher systemic toxicity, which is less attractive for therapy (unpublished results). Currently there are not enough experimental data for a sound scientific explanation for this. Systemic toxicity experiments on healthy and tumour-bearing mice are underway and should help in understanding our observations.

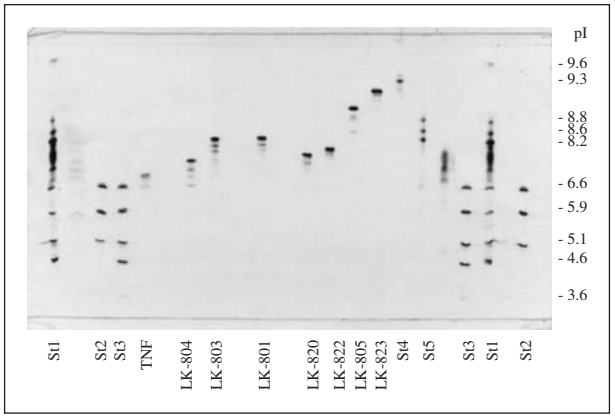

Figure 2

Isoelectric focusing gel. Samples prepared in PBS pH 7.3-7.4 with 0.5 M NaCl and frozen at -80 °C have long-term stability and pI values are constant (for interpretation of pI values see *table 1*).

The tip region has also been studied in the mouse TNF molecule and reported to exert lectin-like affinity towards trypanosomes [42]. A series of alanine substitutional mutants in the tip region of mouse TNF was produced. Interestingly, the mouse TNF triple alanine mutant (T104A-E106A-E109A) lacking the lectin-like affinity was reported to have also significantly reduced systemic toxicity and pro-metastatic activity in a mouse model compared to the native mouse TNF [43, 44].

Provoked by literature data about the impact of basic mutations in the N-terminal region of human TNF, and considering the fact that increased pI values of LK-801 and LK-805 analogues were of therapeutic significance, we directed subsequent experiments to charge modulation – activity studies, confined to the tip region of TNF.

A series of analogues mutated in the tip region was prepared, all possessing higher pI values than the native TNF molecule. A typical IEF gel representing various analogues is depicted in *figure 2*. TNF and its analogues have been prepared in a highly purified form and stored in an appropriate buffer at -80° C, resulting in a relatively homogeneous appearance and high reproducibility of IEF gels. For the majority of analogues, the main electrophoretic band accounts for more than 75% of total protein. Furthermore, in most cases a relatively good agreement between the calculated and experimentally determined pI values has been confirmed *(table 1)*. This represents a good basis for reliable charge modulation – activity studies in the particular part of TNF molecule.

Comparison of cytotoxic activity of TNF analogues, with increasing pI, in vitro in mouse and human tumour cell lines

Comparison of biological effects in mouse and human cells *in vitro* is of interest in the development of new pharmaceuticals, principally because, for preclinical testing, the mouse and/or its cells are widely used. There is a general trend to reduce *in vivo* testing and collect as much as possible data using *in vitro* cultures. In this respect, similarities in the biological response *in vitro* for human and murine cells would be desirable and should be explored.

In general, it appears reasonable to compare murine and human cell lines of similar tissue origin, e.g., murine and human fibroblasts, hepatocytes, etc.

While the results of *in vivo* biological testing are very complex and include different effects on various tissues and organs, it can be assumed that biological testing on matching pairs of mouse and human cell lines should show certain effects more clearly. In this way, the impact on hepatocytes, endothelial cells or tumour cells can be evaluated separately.

Current knowledge on the therapeutic value of TNF ascribes its beneficial effect mainly to increasing permeability of tumour endothelia, thus enabling higher penetration of chemotherapeutic agents, e.g., melphalan into the tumour tissue [45]. One of our goals is to develop new analogues with increased direct antitumour activity. Considering TNF or its analogues also as potential chemotherapeutic agents having direct cytotoxic effect on tumour cells, experimentally determined biological effects *in vitro* might also be expected to occur *in vivo* under appropriate circumstances, e.g. in intratumoural or peritumoural applications.

In the determination of sensitivity or resistance to analogues in comparison to native TNF, a series of different tumour cell lines has often been used, e.g. KYM, Hela, MIA, PANC-1, MCF-7, HT-29 ME180, T24, A549, G-401 [17, 19, 27].

In this study, human and mouse cell lines were used for assessing cytotoxic responses. These cells were selected on the basis of their relatively high susceptibility to TNF-mediated cytotoxicity and their similar tissue origin.

Besides the most widely used L929 cell line (circles in *figure 3*), another mouse cell line, a fibrosarcoma line WEHI 164 clone 13-1 (squares in *figure 3*) and a human rhabdomyosarcoma-derived cell line KYM-1D4 (triangles in *figure 3*) were employed.

In preliminary experiments with homogeneous series of basic analogues, we observed some evidence of correlation of increased *in vitro* cytotoxicity with increasing pI values, although with a larger panel of tip region analogues, this was not demonstrated. However, some of our basic analogues exhibit very promising properties and deserve further evaluation.

Comparing cytotoxicity data presented in *figure 3*, it can be concluded that modulating of pI in the tip region influences *in vitro* cytotoxicity similarly in both mouse and human cell lines. This also leads to an assumption that similar interaction mechanisms are involved. Thus the response obtained in mouse cell lines appears representative for certain human cell lines, which is useful when human cells are difficult to obtain or as an indication that results from mouse "model" cell lines can be extended to human cell lines.

Cytotoxicity of analogue LK-805 in various cell lines

In addition to the traditionally-used mouse L929 cell line, WEHI 164 clone 13 (a mouse fibrosarcoma cell line), HEPA 1-6 (a mouse hepatocyte cell line), KYM-1D4-K (a human rhabdosarcoma cell line) and EAhy926 (a human endothelial cell line) were used to compare the cytotoxicity of the therapeutically most interesting analogue LK-805 with native TNF *(figure 4)*. These cell lines were chosen as representatives of three target cell groups, tumour, hepatocytic, and endothelial, which correspond to tissues that are most affected by TNF antitumour treatment *in vivo*.

In all cell lines studied, a statistically significant increase in cytotoxicity of the analogue LK-805 in comparison to native TNF was observed ($P < 0.01$ for L929, WEHI 164 clone 13, KYM-1D4-K, HEPA 1-6 and $P < 0.05$ for EAhy926).

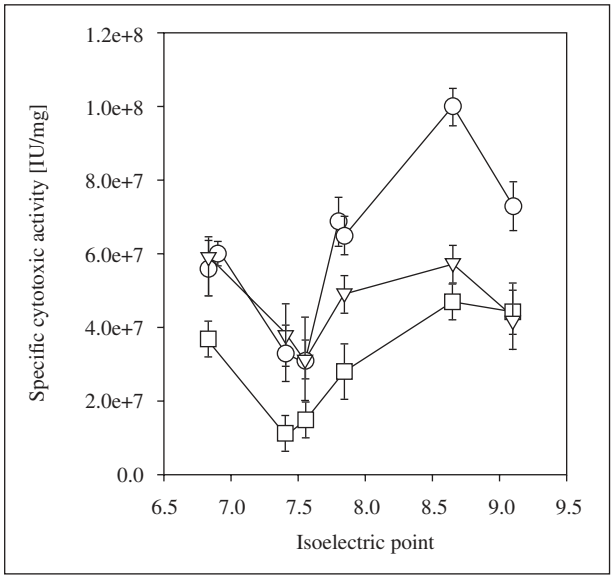

Figure 3
Comparison of cytotoxicity values as measured in mouse fibroblast L929 cell line (circles), mouse fibrosarcoma WEHI 164 clone 13-1 cell line (squares) and human rhabdomyosarcoma KYM-1D4 cell line (triangles) for a series of TNF analogues having increasing pI values. Similar biological response was found for human as well as mouse cell lines. From left to right: TNF, LK-804, LK-820, LK-822, LK-803, LK-801, LK-805 and LK-823.

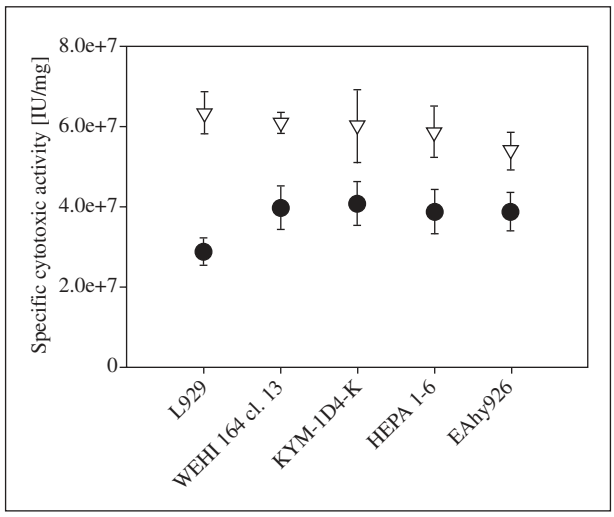

Figure 4
Specific cytotoxicity of TNF (circles) in comparison to analogue LK-805 (triangles) measured on different human (KYM-1D4-K; EAhy926) and mouse (L929; WEHI 164 clone13; HEPA 1-6) cell lines. A statistically significant increase in cytotoxicity of the analogue LK-805 in comparison to native TNF was observed ($P < 0.01$ for L929, WEHI 164 clone 13, KYM-1D4-K and HEPA 1-6 and $P < 0.05$ for EAhy926).

This finding, so far not reported for TNF or its analogues, might be due to additional, non-specific interactions with certain cell surface components. Glycosaminoglycans such as heparan sulphates, which are the most abundant negatively charged components, appear to be potential targets at the cell surface for binding of positively charged, basic TNF analogues, such as LK-805. To explore this possibility, we used specific, enzyme-mediated removal of heparin sulphates, as described in the next section.

Cytotoxicity of analogue LK-805 after treatment of cells with heparinase

Since a simple charge reversal in the tip region of the TNF molecule has such a strong impact on cytotoxicity, we assumed that the newly introduced positive charge on the tip of analogue LK-805 was influential in initiating interactions at the cell surface that could more effectively target the molecule to TNF receptors. We have previously shown that the positively charged, three lysine-containing tip of the analogue LK-805 is responsible for additional electrostatic interaction with negatively charged heparan sulphates exposed on the L929 cell surface [46]. To test the generality of this observation, we treated L929, WEHI 164 clone 13, KYM-1D4-K and HEPA 1-6 cells with heparinase, which enzymatically removes heparan sulphates.

According to our previous study [46], cytotoxicity in heparinase pre-treated cells should be reduced in comparison to the value measured on untreated cells. Indeed, in the case of L929 ($P < 0.01$), WEHI 164 clone 13 ($P < 0.01$) and HEPA 1-6 ($P < 0.05$) cell lines heparinase pre-treatment resulted in significantly reduced cytotoxicity *(figure 5)*. In contrast, heparinase treatment did not affect native TNF-induced cytotoxicity in any of these cell lines (results not shown).

Further, following heparinase pre-treatment, the cytotoxicity of LK-805 in these cells was reduced to the level induced by native TNF. This result strongly suggests that the E107K mutation has no effect on the affinity of LK-805 towards TNF receptors.

In addition, pre-incubating LK-805 with increasing concentrations of negatively charged heparan sulphates, or pre-incubation of cells with positively charged poly-L-lysine, was shown to decrease cytotoxicity proportionally in L929 cells (results not shown). Therefore, we conclude that increased *in vitro* cytotoxicity of LK-805, as well as other basic analogues, at least in these cell lines, is largely due to additional binding interactions with cell surface heparan sulphates leading to increased local concentrations of basic analogues, effectively bringing greater amounts to bind to TNF receptors. This "co-receptor" role of heparan sulphates has also been described for other cytokines and growth factors, e.g., basic fibroblast growth factor (bFGF) and others [47].

Although these interactions appear favoured for the tip region, they might also apply to other parts of the TNF protein, especially the N-terminal, where some correlation between basic character and *in vitro* cytotoxicity is indicated (see above).

Exceptionally, in the case of KYM-1D4-K cell line, heparinase pre-treatment seemed not to influence the cytotoxic activity of LK-805 *(figure 5)*. A possible explanation is that hyaluronic acid, secreted in large amounts into the medium by these cells [48], interferes and masks the effect of heparinase. It is known that excess of other negatively charged polymers such as heparin, completely inhibits heparinase activity [49].

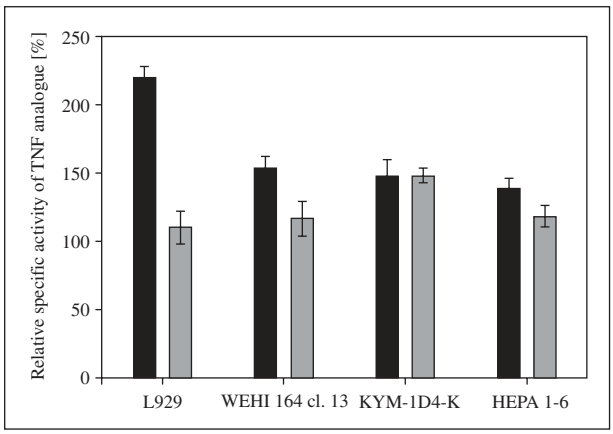

Figure 5

Cytotoxicity of TNF analogue LK-805 measured on untreated cells (black) or heparinase pre-treated cells (grey). Heparinase treatment of L929 ($P < 0.01$), WEHI 164 clone 13 ($P < 0.01$) and HEPA 1-6 ($P < 0.05$) cells resulted in significantly reduced cytotoxicity of LK-805. L929 cells were pre-treated for 1 hour with a mixture of 0.15 mU/mL Heparinase I, 0.15 mU/mL Heparinase II and 0.15 mU/mL Heparinase III. WEHI 164 clone 13 cells were pre-treated for 4 hours with 0.5 mU/mL Heparinase I. KYM-1D4-K were pre-treated for 4 hours with 1 mU/mL Heparinase I. HEPA 1-6 were pre-treated for 4 hours with a mixture of 0.15 mU/mL Heparinase I, 0.15 mU/mL Heparinase II and 0.15 mU/mL Heparinase III.

Structural model: TNF-α solubleTNFR1

To consider potential additional interactions of the mutated tip region in TNF with TNFR1, we constructed a simple model of the LK-805·soluble TNFR1 complex *(figure 6B)* based on the known 3D structure of the TNF-beta in complex with the soluble part of TNFR1 *(figure 6A)* [29]. By comparing the models it appears that the tip region, including the mutation Glu107Lys (marked in green in *figure 6B*), has no interaction with the receptor, simply because it is too distant. Therefore, we assume that mutations in the tip region of TNF do not influence its affinity for TNF receptors. Furthermore, the tip region contains a structurally ill-defined loop Pro106 - Glu110 [28], having highest flexibility in the region Glu107Gly108. It is highly improbable that single mutations to polar amino acid residues in this region of the molecule would force the loop to assume a defined configuration.

Therefore, modulation of biological activity, e.g., increased *in vitro* cytotoxicity as shown for these analogues, is most probably due to interactions with cell surface components that occur before binding to cognate TNF receptors.

Most evidence indicates only the type I receptor (TNFR1) is involved in mediating cytotoxicity [50]. Human TNF can only act on mouse L929 cells through TNFR1 [15, 51, 52]. Thus, there should be no effect on cytotoxicity due to the presence of type II receptors (TNFR2), which do not bind human TNF in L929.

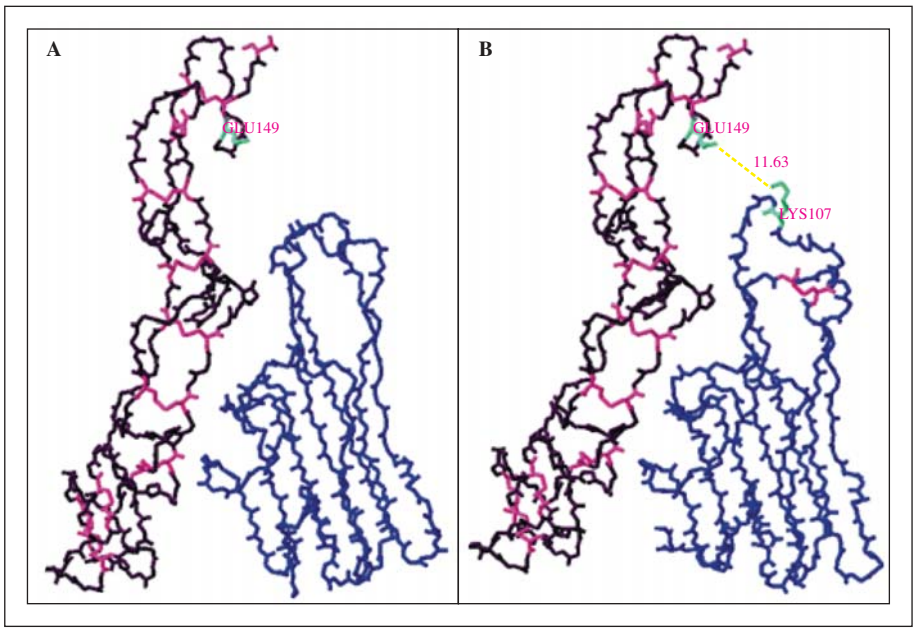

Figure 6
Comparison of a known model of TNF-beta (monomer) in complex with soluble TNFR1 (**A**) with a model of the TNF-alpha analogue LK-805 (monomer) in complex with soluble TNFR1 receptor (**B**). We suppose there is no increase in the affinity of the analogue towards the receptor. TNF-beta and LK-805: blue; TNFR1: black; cysteine bonds: red; Lys107 on LK-805 and Glu149 on TNFR1: green.

As already mentioned above, our experimental data with heparinase pre-treated L929 cells show that, when heparan sulphates are cleaved off the cell surface, cytotoxicity of the LK-805 analogue is reduced to the same level as for native TNF *(figure 5)*. This implies that the increased specific cytotoxicity of LK-805 arose from additional interactions with cell surface heparan sulphates and is not due to any changes in affinity for TNFR1.

Possibly a more complex situation occurs in human cells where TNFR2 might initially bind much of the TNF; it has been proposed that some TNF bound by TNFR2 can be passed to the higher affinity TNFR1 [52-54]. Some TNF analogues have been described by other groups that bind preferentially to one or other of the TNFR [15]. However, these analogues bear mutations in the loops or near the loops surrounding the cleft between two adjacent TNF monomeric subunits, at the broader half of the pyramidally shaped, trimeric molecule [15] and thus at a quite different location to the tip region. Currently we have no evidence that mutations in the tip region of TNF will affect the ratio of their specific binding to TNFR1 or TNFR2 compared to native TNF.

CONCLUSION

To summarise, our new findings as reported herein have confirmed the tip region of TNF to be an attractive site for the introduction of new structural variations that modulate biological activity, in particular cytotoxicity. Modifications in the tip region of other ligands of the TNF superfamily e.g., lymphotoxin-alpha, FasL, TRAIL and others, may therefore also enhance their biological activities and improve their safety and effectiveness when used for therapeutic applications.

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