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A 20 amino acid synthetic peptide of a region from the 55 kDa human TNF receptor inhibits cytolytic and binding activities of recombinant human tumour necrosis factor in vitro

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SUMMARY

Tumour Necrosis Factor (TNF) and Lymphotoxin (LT) can exert a wide range of effects on cells and tissues and they are important effector molecules in cell mediated immunity. All these effects are induced subsequent to the binding of these cytokines to specific membrane receptors. Recently, two of these membrane receptors of 55 and 75 kDa, have been identified which share some amino acid (AA) homology in their N-terminal extracellular domains but differ in their intracellular domains. We synthesized two synthetic 20 AA peptides from hydrophilic regions of the N-terminal extracellular domains of the 55 kDa receptor; peptide A shares homology with both 55 and 75 kDa receptors, peptide B is unique. We found peptide B inhibits both the binding and cytolytic activity of recombinant human TNF when tested on murine L929 cells in vitro. Polyclonal antiserum generated against peptide B will block binding of 125I-labelled TNF to these cells in vitro. However, peptide A and antiserum prepared against peptide A are without effect in these same assay systems. These data suggest that the 20 AA sequences from AA 175 to 194 in the N-terminal extracellular domain of the 55 kDa TNF receptor are expressed on the cell surface and are involved in the binding of TNF.

1. INTRODUCTION

Tumour necrosis factor (TNF) is a cytokine produced by activated monocytes, macrophages (Old 1985) and T-lymphocytes (Dett et al. 1990). TNF causes haemorrhagic necrosis of certain transplantable tumours in mice (Carswell et al. 1975) and is also involved in a spectrum of important immunological and inflammatory reactions (Goeddel et al. 1986; Beutler & Cerami 1988). Although the molecular mechanisms whereby TNF induces these diverse responses is still unknown, it is necessary that TNF first bind to specific cell membrane receptors (Bajzer et al. 1989; Watanabe et al. 1989).

Two cell membrane TNF receptors have been recently isolated, cloned and sequenced; one is 55 kDa (Loetscher et al. 1990; Schall et al. 1990), the other is 75 kDa (Smith et al. 1990). These TNF receptors both have an N-terminal extracellular domain (55 kDa: positions 39 to 212, 75 kDa: positions 23 to 257) with some amino acid (AA) homology, a short transmembrane region and non-related C-terminal intracellular domains. Espevik et al. have shown that binding of monoclonal antibodies to the 55 kDa receptor can mimic biological activities of TNF in vitro, including cell cytotoxicity, stimulation of fibroblast growth and interleukin 6 secretion by endothelial cells (Brockhaus et al. 1990; Espevik et al. 1990). In contrast, monoclonal antibodies against the 75 kDa receptor did not exhibit biological activity (Espevik et al. 1990). Although it is possible that the 55 kDa receptor is physiologically more important, it is also possible that only the monoclonal antibody against the 55 kDa receptor binds to the active sites while the one against the 75 kDa receptor does not.

Soluble forms of these receptors have been identified in the urine of patients with chronic inflammatory disease (Engelmann et al. 1989; Olsson et al. 1989) and in the serum of cancer patients (Gatanaga et al. 1990). The AA sequences, derived from purified materials, indicate these are the extracellular N-terminal domains of the receptors (Engelmann et al. 1989; Olsson et al. 1989; Gatanaga et al. 1990). These soluble receptors retain the capacity to bind to and block TNF and LT biological activities in vitro and in vivo (Engelmann et al. 1989; Olsson et al. 1989; Gatanaga et al. 1990). However, studies of the TNF receptor sites which interact with TNF molecules have not yet been

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reported. We constructed two 20 AA synthetic peptides identical to AA sequences in the hydrophilic portions of the N-terminal extracellular domain of the 55 kDa receptor. In this report, we present data indicating that a sequence of 20 AA in the region from AA 175 to AA 194 in the 55 kDa TNF-R may be directly involved in the binding site.

2. MATERIALS AND METHODS

(a) L929 cytolytic assay

Inhibition of cytolytic activity of recombinant human TNF against L929 mouse fibroblast cells was assayed by the method of Gatenaga et al. (1985). Briefly, synthetic peptides (80 µg per well) and human TNF-α (Genentech Corporation, California) were pre-incubated at 37 °C for 1 h. They were then added to L929 cells (8 x 10⁴ cells per well) and actinomycin D (0.2 µg per well) in 96 well flat-bottomed microcytotoxicity plates in 200 µl per well RPMI–1640 with 10% Fetal calf serum (FCS). The control group was treated with the same volume of phosphate buffered saline (PBS) pH 7.2 instead of synthetic peptides. After incubation for 18 h at 37 °C, the supernatants were aspirated and the adherent viable cells stained with 1% crystal violet for 5 min at room temperature. Excess dye was removed by rinsing with water, the plate was allowed to dry and cells were solubilized with 100 µl acidified methanol (100 mM HCl in methanol). Absorbance of each well was measured at 600 nm in an EAR 400 AT microplate reader (SLT-Lab-Instruments, Austria).

(b) TNF binding assay

One million L929 monolayer cells were first incubated with binding buffer (RPMI–1640 with 10% FCS) and peptides or antibodies in 24-well flat-bottomed Gibcoware culture plates for 1 h. They were then incubated with 0.5–15 ng of ¹²⁵I labelled human recombinant TNF (Amersham, Uppsala, Sweden). After a 3–4 h incubation at 4 °C, binding buffer was removed and the cells were washed two to four times with ice-cold PBS. Cells were solubilized in 1 ml of 0.3 N NaOH and radioactivity was determined in a Pharmacia Clinigamma counter (Uppsala, Sweden).

(c) Synthetic peptides

Peptides were made on a Milligen 9050 peptide synthesizer (Milligen Biosearch, Bedford, Massachusetts) using Fmoc chemistry. Twenty percent piperidine in DMF was used to remove protecting groups. Cleavage and deprotection were done by ‘Reagent R’ (Milligen Biosearch). Peptides were extracted with diethyl ether five separate times. The crude peptides made were purified by passage through a Vydac C4 reverse phase column in Waters 6000A high-performance liquid chromatography system (Waters Associates, Milford, Massachusetts).

(d) Antibody production

Peptides were conjugated with keyhole limpet haemocyanin (Sigma, Missouri) by the method of Fisher et al. (1989) and 200 µg of protein was injected into rabbits with Freund’s complete or incomplete adjuvant each time by the method of Yamamoto et al. (1978). After six injections, the antisera in different dilutions were used in TNF cytolytic and binding assays. Control sera were obtained from non-immunized rabbits.

3. RESULTS

We (Schall et al. 1990) and others (Loetscher et al. 1990) recently reported the complete amino acid sequence of the human 55 kDa cell membrane TNF receptor. Shortly thereafter the complete AA sequence of the 75 kDa human TNF receptor was also reported (Smith et al. 1990).

Sequence analysis and comparison of the 55 kDa and 75 kDa receptor with the Eugene computer program shows four regions (Domain I–IV) of at least 40% homology in the N-terminal extracellular region (figure 1a). Domain IV is the proposed transmembrane domain. Two 20 AA synthetic peptides were synthesized representing sequences 75 to 94 (peptide A) and 175 to 194 (peptide B) of the 55 kDa receptor (figure 1b). Peptide A shares 17 amino acids with Domain I of the 55 kDa receptor and 7 with that of the 75 kDa receptor. The homology between peptide A and the corresponding region of the 75 kDa receptor is approximately 40%. Peptide B is identical to a stretch of sequence between Domain III and Domain IV in the 55 kDa receptor. No homology was evident between peptide B and any region of the 75 kDa receptor.

(a) Synthetic peptides

| Domain I. | 55 KD | 78 | DTDCRECESG | SFTPASENHLR | HCLSC | |||| || || ||* ||* ||* ||* ||* |
| 75 KD | 72 | DTVCDCSEDS | TVQQLNWNP | ECLSC |

| Domain II. | 55 KD | 111 | QVEISSCTVD | RDVTGCGKLN | QY | ||* ||* ||* ||* |
| 75 KD | 104 | QYEQACTRE | QNRICTCPG | WY |

| Domain III. | 55 KD | 158 | COEKKTVTC | CHAGPF |
| 75 KD | 110 | CTREQNICT | CRPWW |

| Domain IV. | 55 KD | 214 | LPLVIFFGLC | LLSLFFGLM |
| 75 KD | 260 | LPVGLLVGVT | AGLLIIGVV |

(b) Peptide A

| 55 KD | 75 | PQQ7TCRDC | EGSFPASEN |

Peptide B

| 55 KD | 175 | RENECVCSN | CKKSLCTKL |

Figure 1. Sequences of homologous domains (I–IV) of 55 kDa and 75 kDa human TNF receptors were compared by Monte analysis in Eugene program. The minimal length for homology domains was set at 15 AA. The threshold s.d. score is 3.0. The number at the beginning of each domain is the amino acid residue position with respect to the initial methionin. The bars mark positions occupied with identical amino acids. Stars mark identical positions occupied by similar amino acids. (b) AA sequence of synthetic peptides A and B.
The effects of synthetic peptides A and B on TNF-induced lysis of murine L929 cells were tested as described in Materials and methods.

The results shown in figure 2 reveal that peptide B significantly blocked TNF activity while peptide A showed no blocking activity. The effect of these peptides on 125I-labelled TNF binding assay was also examined.

The results in figure 3 show that peptide B at the level tested blocked about 50% of TNF binding to the TNF receptor of L929 cells. In contrast, peptide A had no effect on TNF binding in this system. PBS(-) was substituted for synthetic peptides as a control. Peptide A (○), peptide B (□), control (○).

Rabbits were immunized with both peptides as described in Materials and methods. Antisera from animals immunized with peptide B showed blocking activity in the TNF cytolytic assay (data not shown).

The data suggested that anti-peptide antibodies bound to the TNF cell surface receptor and blocked the subsequent binding of TNF molecules.

The effect of these antisera on the 125I-labelled TNF binding assay was examined. The data in figure 4 show that the serum from animals immunized against peptide B blocked 71% of TNF binding compared with control serum.

4. DISCUSSION

It is not clear which regions on the TNF molecule and their corresponding cell surface receptor(s) interact. However, binding of TNF with its specific cell surface receptor is the necessary first step in inducing subsequent effects (Bajzer et al. 1989; Watanabe et al. 1989). The ultrastructure of TNF has been elucidated down to 2.6 Å (Eck & Sprang 1989). The putative receptor binding site on TNF, proposed by these authors, was a concave surface. They suggested that the binding site of the membrane receptor for TNF should be an extended structure on a hydrophilic domain.

Synthetic peptides have been very important in elucidating structure–function relation studies. Baird et al. (1987) used a series of synthetic peptides to identify two functional domains in basic human fibroblast growth factor (FGF) that can bind both heparin and the human FGF receptor. Antibodies to synthetic peptides have also been used to identify specific regions on proteins involved in functional activity in human interleukin–1 (Boraschi & Tagliabue 1989) human TNF (Socher et al. 1987), IgE (Roberston & Liu 1988) and the human insulin receptor (Pessino et al. 1989).

We produced 20 AA synthetic peptides identical to two hydrophilic regions of the N–terminal extracellular
domain of the human 55 kDa TNF receptor. We assumed that these hydrophilic regions are exposed portions of the TNF receptor and thus accessible to and perhaps involved in TNF binding. The data we presented suggest that peptide B is probably within or near the binding domain of the human 55 kDa TNF receptor.

Both 55 kDa and 75 kDa TNF human receptors belong to a family of membrane proteins having cysteine-rich regions (Loetscher et al. 1990; Schall et al. 1990; Smith et al. 1990). In our previous report, computer analysis revealed that the 55 kDa TNF receptor has four internal cysteine repeats (Schall et al. 1990). Further analysis revealed that homologous domains between 55 kDa and 75 kDa TNF receptors are located at the junctions of these internal cysteine repeats. Peptide A represents a 20 AA sequence from the first homologous domain and is located at the junction between the first and second internal cysteine repeats. Peptide B represents 20 AA residues found in the central portion of the fourth internal cysteine repeat. The finding that peptide B is important for binding supports the idea that these internal cysteine repeats may be important to form the stereochemical structures necessary for receptor functions. The central portions of these internal cysteine repeats may be a good target area for further structure-function studies.

It is important to point out that our antisera were made against human 55 kDa TNF receptors and we demonstrated they could bind to murine TNF receptors on L929 cells. These results suggest that this region in the 55 kDa TNF receptor has been conserved in evolutionary development. This is logical because human TNF can cross species and bind to and kill susceptible cells from various animal species. The data presented here suggest that AA residues from 174 to 195 in the amino terminal extracellular domain of the 55 kDa TNF receptor are involved in the actual site for TNF binding.

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