Selective enhancement of the tumour necrotic activity of TNFα with monoclonal antibody

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Summary The binding and biological activity of human TNFα on endothelial and tumour cells has been studied in the presence of monoclonal antibodies (MAbs). In particular, one monoclonal antibody to TNFα (MAB 32) has been identified which failed to inhibit binding and cytotoxicity of TNFα on WEHI-164 tumour cells. However, the same antibody inhibited the activity on bovine aortic endothelial cells. The ability of MAB 32 to inhibit selectively the actions of TNFα on endothelial cells but not on tumour cells suggested a mechanism for enhancement of the anti-tumour action of TNFα in vitro when in complex with this antibody. Treatment of tumour-bearing mice with WEHI-164 and Meth A fibrosarcoma with TNFα-MAB 32 complex resulted in a 5- to 10-fold enhancement in the potency of the cytokine in comparison to free TNFα. Complexes between this cytokine and other MAbs generally resulted in either no effect or inhibition of TNFα activity in vivo and in vitro. Neither intact MAB 32 nor Fab' fragments of MAB 32 showed any tumour regressive activity in the absence of TNFα. The Fab' fragments were equipotent to the bivalent form of the antibody in enhancing TNFα activity. These data provide evidence that it is possible to segment the individual biological activities of TNFα with concomitant enhancement of the tumour regressive activity of the cytokine in vivo.

Tumour necrosis factor (TNFα) is a product of activated macrophages in response to infection and during malignancy. Systemic administration of this cytokine results in haemorrhagic necrosis of tumours in vivo (Carswell et al., 1975; Green et al., 1977) whereas in vitro, it has cytostatic and cytolytic activity on tumour cells (Helson et al., 1975). In addition to its 'host-protective' effects, TNFα has been implicated as the causative agent in the pathology associated with septicaemia, cachexia, cerebral malaria and cancer. Although recombinant TNFα has been used therapeutically in cancer patients, side-effects such as coagulopathy, thrombocytopenia, lymphopenia, hepatotoxicity and renal impairment have limited its application (Creaven et al., 1987; Kimura et al., 1987; Selby et al., 1987; Nawroth & Stern, 1986). The systemic toxicity associated with the administration of TNFα is believed to be, at least in part, a consequence of its interaction with the endothelium (Bevilaqua et al., 1986; Nawroth & Stern, 1986; Selby et al., 1987). Furthermore, reducing the ability of TNFα to bind to endothelial cells while preserving its tumour cytotoxic activity may have a beneficial outcome in the use of this cytokine therapeutically. We describe here a monoclonal antibody to human TNFα which significantly enhances the tumour regression activity of the cytokine (5-10-fold) whilst inhibiting some of the associated toxic side-effects. In particular, the antibody has been shown to inhibit the procoagulant activity of TNFα on endothelial cells whilst having no effect on the binding of the cytokine to WEHI-164 tumour cells. These observations may provide the basis for an improved approach to therapy with this cytokine.

Materials and methods

Animals and tumour cell lines

All experiments were performed with female BALB/c mice aged 10 - 12 weeks obtained from the CSIRO Division of Biomolecular Engineering animal facility. The WEHI-164 fibrosarcoma line was obtained from Dr Geeta Chauhdri (John Curtin School of Medical Research, Australian National University). The Meth A sarcoma lines were obtained from Dr Elizabeth Richards (Sloan Kettering Cancer Centre).

Fusions and production of monoclonal antibodies

A panel of 13 murine monoclonal antibodies (MAbs) against human recombinant TNFα was raised and characterised as previously described (Rathjen et al., 1991). Two monoclonal antibodies are described here; one is a potent inhibitor of all the activities of TNFα (MAB 47) and a second unique antibody (MAB 32) which selectively inhibits the effects of TNFα on endothelial cells (see text).

WEHI-164 cytotoxicity assay

Bioassay of recombinant TNFα activity was performed according to the method described by Espevik and Nissen-Meyer (1986). Briefly, WEHI-164 cells were cultured in RPMI-1640 supplemented with 10% foetal calf serum, 10 mM hapes and penicillin-streptomycin. Prior to use in the assay of TNFα activity the cells were harvested, washed in culture medium once and placed in wells of a 96 well microtary (2 x 10⁴ cells/well). TNFα (Bissendorf Biochemicals 3.2 x 10⁶ units mg⁻¹) at varying concentrations was then added to each well. Actinomycin D (5 µg ml⁻¹) was used to enhance the cytotoxic action of TNFα. Monoclonal antibodies were used at 1 µg well. After 20 h in the presence of TNFα and/or MAB MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 10 µl of 5 mg ml⁻¹ stock) was added to each well. The cultures were incubated for a further 4 h before the supernatant was carefully removed and the insoluble precipitate dissolved by the addition of 100 µl of acidified propan-2-ol to each well. The optical density was then read at 570 nm with a reference wavelength of 630 nm.

Tumour regression experiments

Subcutaneous tumours were induced by the injection of approximately 5 x 10⁵ cells (WEHI-164 or Meth A fibrosarcoma). This produced tumours of diameters of 10 to 15 mm approximately 14 days later at which time experiments commenced. Mice were injected i.p. for four consecutive days with recombinant human TNFα (0.1 µg - 10 µg) and MAB (50 µg as ascitic globulin fraction prepared by sodium sulphate precipitation) mixed 60 min prior to administration.

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Control groups received injections of PBS alone, MAb alone or control MAb (MAb against bovine growth hormone) with TNFα. Tumour size was measured daily throughout the course of the experiment. Statistical significance of the results was determined by unpaired t-test.

Radioceptor assays

WEHI-164 cells grown to confluence were scrape harvested and washed once with 1% bovine serum albumin in Hanks balanced salt solution (HBSS, Gibco) and used at 2 × 10⁶ cells per assay sample. Bovine aortic endothelial cells (passage 6) were seeded (4 × 10⁶ cells per well) into 24 well culture dishes and grown to confluency (3–4 days) in McCoys 5A medium supplemented with 20% FCS, L-glutamine and penicillin/streptomycin (growth medium). For the radioceptor assay, the cells were washed once in growth medium and then incubated with varying amounts of either unlabelled TNFα (1–10⁶ ng per assay sample) or MAb (10-fold dilutions commencing 1/10 to 1/10⁵ of ascitic globulin) and 125I-TNFα (50,000 c.p.m.) labelled using the lactoperoxidase method as previously described (Aston et al., 1985) for 3 h at 37°C in a shaking bath (WEHI-164 cells) or 1 h at 37°C in a humidified CO₂ incubator (endothelial cells). At the completion of the incubation 1 ml of HBSS/BSA was added to the WEHI-164 cells, the cells spun and the bound 125I in the cell pellet counted. For the endothelial cell assay, 1 ml of growth medium was added to each well and aspirated followed by the addition of 0.1 ml of 0.1 M sodium hydroxide to lyse the cells. The cell lysate was then transferred to tubes for counting of bound 125I-TNFα. Binding that could not be displaced by an excess (1 μg) of unlabelled TNFα was considered to be non-specific. Specific binding was calculated from total binding minus non-specific binding of triplicate assay tubes. One hundred per cent specific binding corresponded to 1,500 c.p.m.

Endothelial cell clotting assays

Endothelial cell procoagulant activity (PCA) induction by TNFα was determined using bovine aortic endothelial cells (BAE) according to the procedure of Bevilacqua et al. (1986) with the following modifications: BAE cells were propagated in McCoys 5A medium supplemented with 10% FCS, penicillin, streptomycin and L-glutamine in standard tissue culture flasks and 24-well dishes. TNFα treatment of cultures (3 μg ml⁻¹) was for 4 h at 37°C in the presence of growth medium after which the cells were washed and scrape-harvested before being frozen, thawed and sonicated. Total cellular PCA was determined in a standard one-stage clotting assay using normal donor platelet poor plasma to which 100 μl of CaCl₂ and 100 μl of cell lysate was added. The time taken for clotting to occur was then measured. Statistical significance was determined by unpaired t-test.

Preparation of FAb' monoclonal antibody fragments

Univalent antibody fragments were prepared by digestion of MAb 32 with agarose immobilised papain (Pierce) according to the manufacturer's instructions. FAb' and Fc antibody fragments were separated by protein A-Sepharose affinity chromatography (Pharmacia) and tested for size and binding to 125I-TNFα by electrophoresis and radioimmunoassay respectively.

Results

Binding of TNFα-MAb complexes to tumour and endothelial cells

The binding of TNFα to cultured WEHI-164 tumour cells and bovine endothelial cells in the presence of anti-TNFα MAbs is shown in Figure 1. Unlike MAb 47, which was found to inhibit TNFα binding to both cell types, MAB 32 only inhibited 125I-TNFα binding to endothelial cells. This correlated with the corresponding effects of MAB 32 and MAB 47 on the cytotoxic effects of TNFα on cultured WEHI-164 tumour cells (Figure 2a). The activity of MAB 47 was found to be typical of 'inhibitory' antibodies; that is, the inhibitory activity was consistently observed in both the endothelial and tumour cell assays. Examination of the activation of cultured endothelial cells by TNFα in the presence of MABS 32 and 47 is shown in Figure 2b. Both antibodies significantly inhibited the TNFα-induced production of procoagulant as determined in the single stage clotting assay (P < 0.01). Treatment of endothelial cells with MABS 32 and 47 in the absence of TNFα failed to induce procoagulant activity and therefore had no effect on the clotting time in the single stage clotting assay (data not shown). Similarly, neither TNFα nor the antibodies themselves were cytotoxic to the endothelial cells under the conditions employed; however, TNFα clearly stimulated the induction of procoagulant at these doses.

Enhancement of TNFα-induced tumour regression of MAB 32

TNFα, at a dose of 10 μg per injection, co-administered with a control MAB daily for 4 days to WEHI-164 tumour-bearing mice caused 50% reduction in tumour size (Figure 3). The degree of tumour regression observed was dose related such that animals treated with only 1 μg of TNFα per day failed to show any reduction in tumour size. The tumour regressive activity of TNFα was completely inhibited by precomplexing the cytokine with anti-TNFα MAB 47 before injection. In contrast, significant enhancement of tumour regression (P < 0.01) was observed in mice treated with 10 μg TNFα in complex with MAB 32 when compared to control mice (receiving 10 μg TNFα in the presence of a control MAB). Enhanced tumour regression was also observed in mice treated with lower doses of TNFα (1 μg) in the presence of MAB 32; indeed, at this dose of TNFα in complex with MAB 32, an equivalent degree of regression was only observed following the treatment of mice with 10 μg of non-
Differences control 47, 164

Control MAb MAb 32 MAb 47

Figure 2 Effect of MAb 32 on TNFα-mediated tumour cell killing in vitro with cultured WEHI-164 fibrosarcoma cells a, and induction of endothelial cell procoagulant activity b. (MAb 32, ■; MAb 47, □; Control MAb ○). Results are the mean ± s.e.m. of quadruplicate determinations.

Figure 3 Enhancement of TNFα-mediated regression of WEHI-164 tumours in vivo by monoclonal antibody. MAb 32, ■; MAb 47, *; Control MAb, ○. Mice were treated daily with TNFα either pre-complexed with MAb 32 (50 μg) or after mixing with control MAb (50 μg). Tumour size was determined daily during the course of the experiment. The results show the mean ± s.d. % change in tumour size at the completion of treatment (day 4). Differences observed between control MAb-TNFα and MAb 32-TNFα treated groups are significant (P<0.01, unpaired t-test).

Figure 4 Enhancement of TNFα-mediated regression of Meth A tumours in vivo by monoclonal antibody. MAb 32, ■; Control MAb, ○. Mice were treated daily with TNFα either pre-complexed with MAb 32 (50 μg) or after mixing with control MAb (50 μg). Tumour size was determined daily during the course of the experiment. The results show the mean ± s.d. % change in tumour size at the completion of treatment (day 4). Differences observed between control MAb-TNFα and MAb 32-TNFα treated groups are significant (P<0.01, unpaired t-test).

Discussion

The exploitation of genetic engineering technology has provided many protein hormones and mediators which may have clinical application in man; however, it is becoming progressively apparent that particular immunologically active recombinant molecules (e.g. TNFα, IL-1, IL-2, γ-IFN etc) retain high levels of toxicity in vivo. Reduction of the level of toxicity of such molecules may be a prerequisite for their more general therapeutic use. Since the toxicity of TNFα may manifest as a direct result of its interaction with a variety of receptors on different tissues following systemic administration, we have examined the possibility of 'restricting' the specificity of this cytokine to particular receptor subsets with monoclonal antibodies (MAb). By employing this approach it is shown that the binding of TNFα to different receptors can be selectively modulated by a particular MAb (MAb 32). These findings suggest that different regions of the cytokine are associated with its binding with different receptors and may account for the significant antibody-mediated enhancement of its activity described here.

Out of a panel of 13 monoclonal antibodies (MAbs) defining at least six distinct antigenic regions on TNFα, one specificity (MAb 32) has been identified which permits binding of the cytokine to tumour cell receptors (WEHI-164 cells) but not to sites on bovine aortic endothelial cells. The specificity of this MAb is unusual in that other inhibitory antibodies were characteristically found to block both the binding of 125I-TNFα to WEHI-164 tumour cells and to bovine endothelial cells (see typically MAb 47). In view of the unique nature of MAb-antigen interactions (i.e. binding to a single site), interpretation of the distinct binding charac-

complexed TNFα (i.e. with control antibody). At the 0.1 μg dose of TNFα there was no apparent beneficial enhancement of tumour regression. Enhancement of TNFα-induced tumour regression by MAb 32 was also observed following the treatment of Meth A solid tumours in vivo (Figure 4). In contrast, however, the treatment of a Meth A tumour subline grown as ascites failed to give the observed enhancement response (data not shown).

FAB' fragments of MAb 32, prepared by papain digestion and purified on Protein A Sepharose as described in the Materials and methods, were found to enhance TNFα induced tumour regression to the same degree as intact, bivalent MAb 32 (Figure 5). Neither intact MAb 32 nor FAB' MAb 32 caused tumour regression in the absence of TNFα.
The endothelial cell receptor, still uncharacterized, may represent a specific "carrier" molecule. The recent identification of a natural TNF receptor binding protein (Engelmann et al., 1989), which may have regulatory effects on the cytokine in vivo and the existence of at least two structurally distinct receptors (Hohman et al., 1989; Engelmann et al., 1989; Loetscher et al., 1990; Espevik et al., 1990), would also lend support to the above hypothesis. Endothelial cells appear to express two TNF receptors which mediate different biological effects of TNF (Brett et al., 1989). It appears that expression of tissue factor in response to TNF is signalled by a non-G protein linked receptor while increased vascular permeability in response to TNF occurs via a G protein-linked receptor. More recent evidence (Tartaglia et al., 1991) has further indicated that the two TNF receptors initiate distinct signalling pathways that result in the induction of different cellular responses namely thymocyte proliferation and LM cytokotoxicity. The antibody described here may not only enable further clinical trials with TNF, but may have application in cancers where circulating TNF levels are high and evidence of coagulopathy is apparent.

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