Effect of tumour necrosis factor on the uptake of specific and control monoclonal antibodies in a human tumour xenograft model

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Summary The investigations reported in this paper aim to exploit tumour necrosis factor (TNF)-induced vascular changes in an attempt to increase the tumour uptake of specific monoclonal antibody. The vascular permeability to monoclonal antibody of a human tumour xenograft increased 2.6-fold by 1 h post injection of 2.5 x 10^6 U of TNF, although this effect was lost by 3 h. The normal tissues also demonstrated increased vascular permeability to IgG, but to a lesser extent. Liver permeability increased 1.5-fold at 1 h but returned to the control value by 6 h. Lung permeability increased 1.4-fold at 1 h post injection and returned to normal by 3 h. Muscle values were not significantly increased compared with controls. The blood activity was cleared more quickly in the TNF-treated mice (T = 101 h, compared with 121 h in control mice). This was probably due to the increased vascular permeability in normal organs of treated mice. At 1 day and 3 days post injection, the tumour uptake of the specific, but not the control, antibody was significantly increased by 25% and 29% respectively. This resulted in an increase in the area under the tumour activity curve, and therefore tumour radiation dose, of 25% in treated compared with control mice. In addition, a consequence of the faster blood clearance of the isotope in the TNF-treated mice was a reduction in the area under the blood activity curve of 12%, thereby reducing systematic toxicity. The increase in vascular permeability to IgG following TNF injection resulted in both specific and control antibodies having improved access to the tumour antigens, and a transient increase in uptake was observed. Only in the case of the specific antibody was the increase maintained, since this antibody binds to the available antigenic sites, whereas the control antibody was cleared from the tumour without binding. No evidence of tumour necrosis was observed at the TNF doses given, nor was there any toxicity to the mice.

Keywords: antibody; tumour necrosis factor; vascular permeability; xenograft

Vascular parameters such as blood flow, vascular permeability and vascular volume are serious obstacles limiting delivery of cytotoxic agents, including radiolabelled monoclonal antibodies, to tumours. Antibody access to tumour cells may be limited by the tumour vasculature, with its abnormal vessels and poor blood flow, and by regions of high interstitial pressure (Jain and Baxter, 1988; Jain, 1991).

Tumour vascular permeability and blood flow have been shown to correlate well with the uptake of monoclonal antibody in two different human tumour xenografts, the tumour having twice the vascular permeability, resulting in a 5-fold increase in antibody uptake (Sands et al., 1988). However, few attempts have been made to alter tumour vascular parameters. Bomber et al. (1986) showed that the β-blocker propranolol increases tumour perfusion and 67Ga uptake in a mouse sarcoma. Smyth et al. (1987) have shown, in a xenograft model, that both non-selective and cardioselective β-adrenergic blocking agents increase the tumour blood and tumour–liver uptake ratios of 111In-labelled monoclonal antibody. Attard et al. (1991) used the corticosteroid dexamethasone to reduce tumour interstitial pressure and thereby increase the tumour–background ratios in patients being investigated by immunoscintigraphy. In one patient, two metastatic deposits which were not visible without dexamethasone administration became visible after dexamethasone. Tumour vessels differ from normal vessels, particularly in lacking sufficient smooth muscle to dilate or constrict in response to drugs which have these effects on normal blood vessels. By their selective action on normal blood vessels, vasoactive drugs can change the tumour–normal tissue perfusion ratio (Chan et al., 1984).

The cytokine interleukin 2 (IL-2) has been shown to increase vascular permeability in normal organs, either alone (Rosenstein et al., 1986) or in combination with lymphokine-activated killer cells (Ettinghausen et al., 1988). Tumour necrosis factor (TNF) is another cytokine which has been shown to increase endothelial permeability directly, as measured in endothelial cell monolayers (Royall et al., 1989). In vivo, TNF is known to be a mediator of the inflammatory response, and can lead to diffuse intravascular coagulation with consequent changes in vascular permeability. This damage to tumour vasculature induces ischaemia and haemorrhagic necrosis of the tumour within 24 h of administration, both in animal models (Palladino et al., 1987; van de Wiel et al., 1989) and in a patient (Robertson et al., 1989). This phenomenon was first exploited by Coley (1891), who treated sarcoma patients by injecting bacterial cultures containing Streptococcus erysipelas, and noted tumour regressions, some complete, in many patients. The principal active constituent of Coley's toxins was undoubtedly lipopolysaccharide (LPS), which is a potent inducer of TNF. Endotoxic shock is associated with acute vascular endothelial injury resulting in oedema (Brunson et al., 1955). Administration of TNF to rats, in doses similar to those produced endogenously in response to endotoxin, produces many of the symptoms of LPS toxaemia, including hypotension, pulmonary inflammation and haemorrhage (Tracey et al., 1986). Pulmonary vascular leakage has also been induced in sheep by injection of TNF (Horvath et al., 1988). The barrier function of the endothelium has been shown to be altered directly by TNF (Sato et al., 1986). Aicher et al. (1990) have detected changes in capillary permeability to gadolinium-conjugated albumin in Meth A sarcomas in mice following TNF administration by means of contrast-enhanced magnetic resonance imaging. van de Wiel et al. (1989) suggested that the broad interference of tumour blood supply was the major cause of necrosis of Meth A sarcomas in mice, since TNF did not affect the Meth A cells in vitro. TNF has been shown to increase vascular permeability in vivo immediately after injection (Kallinowski et al., 1989), and to affect the functional and structural vascular volume in solid murine tumours (van

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Vascular studies

Vascular volume and vascular permeability to IgG were determined for groups of control and TNF-treated mice bearing s.c. HT29 xenografts. In the treated groups of mice, 2.5 × 10³ U of TNF was injected i.v. at 1, 3, 6 and 24 h before injection of radioactivity. Vascular volume and vascular permeability were determined by the method of Song and Levitt (1970), as modified by Sands et al. (1985), who replaced ⁵¹Cr labelling of erythrocytes in vitro with radiolabelling in vivo using ⁹⁹mTc (Pavel et al., 1977).

Groups of tumour-bearing mice were given i.v. injections of 1.2 µg of stannous fluoride in 100 µl of saline (Amerscan; Amersham International) via a lateral tail vein. This was followed after 30 min by an i.v. injection of a mixture of 25 µCi of ⁹⁹mTc technetium pertechnetate and 10 µCi (5 µg) of ¹²⁵I-labelled HMFG1 antibody. Exactly 1 h post injection of radioactivity, mice were killed by cervical dislocation, and then tumour, liver, lung, muscle and a small sample of blood were removed, weighed and their ⁹⁹mTc activity counted in a γ-counter. The low energy of the ¹²⁵I emission does not interfere with the detection of the ⁹⁹mTc activity. After allowing sufficient time for total decay of the ⁹⁹mTc (t½ = 6 h), the tissues were counted again to determine their ¹²⁵I content. The vascular volume (VV) in units of ml blood per g of tissue was calculated according to the formula:

\[
VV = \frac{{\text{⁹⁹mTc activity g}^{-1} \text{in tissue}}}{{\text{⁹⁹mTc activity g}^{-1} \text{in blood}}}
\]

The vascular permeability (VP) to IgG was determined by calculating the amount of ¹²⁵I-labelled irrelevant antibody extravasated in 1 h, defined as the total plasma ¹²⁵I c.p.m. g⁻¹ of tissue minus the intravascular plasma ¹²⁵I c.p.m. g⁻¹ tissue. This was calculated in units of ml h⁻¹ g⁻¹ tissue as:

\[
VP = \frac{{\text{¹²⁵I c.p.m. g}^{-1} \text{in tissue}}}{{\text{¹²⁵I c.p.m. g}^{-1} \text{in blood}}} - VV (1 - haematocrit)
\]

The haematocrit (the percentage red cell volume in the blood) was measured for several mice and found to agree with the published value for mice of 40% (Green, 1979), which was therefore used in all calculations. It has been found, however, that the haematocrit in tissues is less than that in the systemic circulation (O’Connor and Bale, 1984).

Since, in the present study, vascular permeability is compared in the model in tumour and normal organs without TNF administration, then if the haematocrit were different from the systemic value this would result in a systematic error in both measurements, as it is unlikely that TNF administration alters the haematocrit.

The efficiency of red cell labelling was estimated by injecting a group of mice with the same reagents at the same time intervals as the experimental animals, and killing them by exsanguination 1 h post injection of radiosotopes. The blood was then centrifuged for 15 min at 2000 r.p.m. in a bench centrifuge to isolate the red cells, which were washed twice with phosphate-buffered saline (PBS). The ¹²⁵I and ⁹⁹mTc activities in the erythrocytes and plasma were measured.

Biodistribution

Nude mice bearing s.c. HT29 tumours were given a mixture of 5 µg each of ¹²⁵I-labelled AUA1 and ¹²⁵I-labelled HMFG1 antibodies i.v. In half the mice 2.5 × 10³ U of TNF was combined with the injected antibody. Groups of four treated and control mice were dissected at times between 2 h and 6 days after antibody administration.

Statistical analysis

The statistical significance of the difference between means was determined using the Student’s t-test. A P-value <0.05 was considered to be significant.
**Results**

**Effect of TNF on tumour vascular parameters**

The efficiency of red cell labelling *in vivo* was measured as described. The washed cells contained 98% of the $^{99m}$Tc activity, while the plasma contained 99.5% of the $^{125}$I activity, confirming the validity of the method. No evidence of tumour necrosis was observed at the TNF doses given, nor any toxicity to the mice.

Figures 1 and 2 show the effect of i.v. TNF (2.5 x $10^4$ U) on the vascular volume and vascular permeability to IgG in treated and control mice bearing s.c. HT29 tumours. There was no significant difference in the vascular volume in any tissue at any time after i.v. TNF injection, except for liver at 1 and 3 h post injection. In contrast, the vascular permeability of tumour was increased 2.6-fold by 1 h post injection of TNF ($P<0.001$), although this effect was lost by 3 h. The normal tissues also demonstrated increased vascular permeability but to a lesser extent. Liver permeability increased 1.5-fold at 1 h ($P<0.02$), but returned to the control value by 6 h. Lung permeability increased 1.4-fold at 1 h post injection ($P<0.02$) and returned to normal by 3 h. Muscle values did not increase significantly from controls.

**Effect of TNF on antibody biodistribution**

Tables I and II show the uptake of co-injected specific (AUAI) and control (HMFG1) antibodies, with or without the inclusion of 2.5 x $10^4$ U of TNF, in mice bearing s.c. HT29 xenografts. The blood activity was cleared more quickly in the TNF-treated mice (for AUAI and HMFG1, respectively, $t_90=101$ h and 101 h in TNF-treated mice compared with 121 h and 115 h in control mice). Although there are few time points used to calculate these values, since the same result is observed for both antibodies, it may be a genuine effect caused by increased vascular permeability in normal organs of treated mice. Two hours post injection, the tumour uptake of both antibodies was increased 2-fold, however this

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**Table I** Percentage of administered dose of specific (AUAI) antibody per gram of tissue at the following times post injection. In half the mice, 2.5 x $10^4$ U of TNF was included in the injectate. Each value represents the mean ± s.d. of four mice.

|                | 2 h | 1 day | 3 days | 6 days |
|----------------|-----|-------|--------|--------|
| **With 2.5 x $10^4$ U TNF** |     |       |        |        |
| Blood          | 28.4±3.7 | 15.4±0.6 | 13.7±2.7 | 7.4±1.5 |
| Tumour         | 4.9±1.9 | 5.8±0.9 | 4.8±0.3 | 3.6±0.6 |
| Stomach        | 2.0±0.2 | 1.8±0.2 | 1.7±0.1 | 0.9±0.2 |
| Intestine      | 2.9±0.2 | 1.6±0.2 | 1.4±0.1 | 0.9±0.4 |
| Kidney         | 6.4±0.6 | 3.6±0.2 | 2.9±0.3 | 1.7±0.5 |
| Spleen         | 5.0±0.5 | 2.9±0.3 | 2.6±0.5 | 1.3±0.2 |
| Lungs          | 7.9±2.0 | 4.8±0.1 | 5.3±1.2 | 2.7±0.5 |
| Liver          | 7.4±0.7 | 3.7±0.2 | 3.4±0.9 | 1.6±0.4 |
| Muscle         | 0.6±0.1 | 0.8±0.1 | 0.7±0.1 | 0.4±0.1 |
| **Without TNF** |     |       |        |        |
| Blood          | 25.3±2.7 | 12.1±1.1 | 8.1±0.5 | 5.3±0.6 |
| Tumour         | 6.6±2.9 | 3.9±0.6 | 3.1±0.3 | 1.8±0.4 |
| Stomach        | 3.7±0.6** | 2.5±1.4 | 1.4±0.7 | 0.7±0.0 |
| Intestine      | 4.8±2.3 | 1.8±0.4 | 1.0±0.2 | 0.7±0.0 |
| Kidney         | 6.7±0.7 | 3.1±0.4 | 2.2±0.4 | 1.2±0.2 |
| Spleen         | 12.2±4.9 | 3.1±0.5 | 1.5±0.2 | 1.0±0.1 |
| Lungs          | 7.4±0.7 | 5.3±1.1** | 2.7±0.3** | 1.7±0.4 |
| Liver          | 11.5±0.9* | 3.3±0.8 | 1.8±0.2 | 1.1±0.1 |
| Muscle         | 0.4±0.1 | 0.6±0.1 | 0.5±0.0 | 0.3±0.0 |

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**Table II** Percentage of administered dose of control (HMFG1) antibody per gram of tissue at the following times post injection. In half the mice, 2.5 x $10^4$ U of TNF was included in the injectate. Each value represents the mean ± s.d. of four mice.

|                | 2 h | 1 day | 3 days | 6 days |
|----------------|-----|-------|--------|--------|
| **With 2.5 x $10^4$ U TNF** |     |       |        |        |
| Blood          | 25.7±2.8 | 11.6±0.6 | 10.5±2.5 | 5.6±0.6 |
| Tumour         | 3.3±0.8 | 3.5±0.3 | 2.8±0.2 | 1.0±0.1 |
| Stomach        | 2.7±0.4 | 1.7±0.2 | 1.3±0.1 | 0.7±0.1 |
| Intestine      | 3.1±0.2 | 1.4±0.3 | 1.1±0.1 | 0.7±0.2 |
| Kidney         | 5.9±0.5 | 2.9±0.2 | 2.3±0.3 | 1.3±0.2 |
| Spleen         | 7.7±1.1 | 2.7±0.4 | 2.2±0.6 | 1.0±0.1 |
| Lungs          | 7.5±1.7 | 3.7±0.2 | 3.9±0.9 | 2.1±0.2 |
| Liver          | 9.0±0.8 | 3.1±0.1 | 2.7±0.9 | 1.2±0.2 |
| Muscle         | 0.5±0.1 | 0.6±0.1 | 0.6±0.2 | 0.3±0.0 |

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*P<0.05, **P<0.01 compared with untreated mice.
was not significant owing to the large errors on the data points. The tumour uptake of the control antibody (Table II) was within the range of the normal organs at all time points studied, while the tumour uptake of the specific antibody (Table I) was higher than all normal organs by 1 day post injection. At 1 day and 3 days post injection, the tumour uptake of the specific, but not the control, antibody was significantly increased by 25% and 29%, respectively, compared with that in untreated mice. This results in an increase in the area under the tumour activity curve, and therefore tumour radiation dose, of 25% in treated compared with control mice, as shown in Figure 3. In addition, a consequence of the faster blood clearance of the isotope in the TNF-treated mice is a reduction in the area under the blood activity curve of 12%, thereby reducing systemic toxicity.

Discussion

The increase in tumour vascular permeability following TNF injection (Figure 2) resulted in both specific and control antibodies having improved access to the tumour antigens, and a transient increase in uptake was observed. As seen in Tables I and II, only in the case of AU1 was the increase maintained, since this antibody binds to the available antigenic sites, whereas HMFG1 is cleared from the tumour without binding. Liver and lung also showed a transient increase in vascular permeability after TNF treatment, but this did not result in increased antibody uptake, since the antibodies do not bind to normal organs.

In this paper, intravenous TNF has been shown to increase the vascular permeability of tumour and normal tissue, resulting in a sustained increase in uptake of specific, but not control, radiolabelled antibody in tumour but not normal tissue. Russell et al. (1990) found that murine TNF increased the level of antibody in a murine thymoma model, and increased the cytotoxicity of an aminopterin--antibody conjugate in established tumours. Similarly, Melton et al. (1993) have shown that co-administration of human TNF increases uptake of an antibody--carboxypeptidase G1 conjugate in a human tumour xenograft in nude mice. As in the present investigation, decreased blood activity was also observed in the latter study. No vascular parameters were measured in either of these studies. In contrast, Pimm et al. (1991) found that, 4 h after i.v. injection of human TNF, blood flow was reduced in treated tumours compared with controls. In their study, TNF did not significantly alter the uptake of antibody in human osteosarcoma and gastric cancer xenografts, although the antibody and TNF were not administered simultaneously, and the number of mice was small. Changes in vascular permeability were not measured. The importance of tumour vascular permeability in relation to antibody uptake has been demonstrated by Sands et al. (1988), who showed that a renal cell carcinoma xenograft having twice the permeability of a breast tumour xenograft accumulated five times the amount of monoclonal antibody by 24 h post injection. In the present study, the TNF-induced elevation in tumour vascular permeability was short-lived, returning to the untreated value by 3 h (Figure 2), so that the concurrent administration of TNF and antibody is probably a major factor in the success of TNF in increasing the antibody uptake by tumour.

Other properties of tumour necrosis factor may make it a useful component to radioimmunotherapy. The half-life of the radiolabelled antibody in the circulation was reduced in the TNF-treated mice, which would reduce the radiation dose to bone marrow, and therefore myelotoxicity. A different inflammatory cytokine, IL-1, has been shown to be a radioprotector (Neta et al., 1986). Old (1987) proposed that this may also be true of TNF, since TNF is one of the main mediators of endotoxin lipopolysaccharide action, and bacterial endotoxins have been shown to protect mice against lethal doses of X-rays (Smith et al., 1957). Neta et al. (1988) have also shown that human recombinant TNF promotes lethally irradiated mice from death, but not as effectively as IL-1 on a dose per mouse basis. In addition, they demonstrated that administration of the two cytokines together resulted in additive radioprotection, implying that they each act through different radioprotective pathways. The same group has also shown that administration of anti-TNF antibodies reduces survival in irradiated mice, suggesting that natural levels of TNF contribute to radioreistance of normal mice (Neta et al., 1991). In addition, the radioprotective effect of systemically administered TNF could be blocked not only by anti-TNF antibody, but also by an antibody against the IL-1 receptor. Slordal et al. (1989) have demonstrated TNF-dose-dependent enhancement of haematological recovery after irradiation in TNF-treated mice. A possible mechanism for this radioprotection is the induction by TNF of mSOD (and superoxide dismutase) (Wagen et al., 1988). MnSOD is an enzyme which protects against oxidative damage by potentially toxic free radicals, which can be produced by irradiation. Since the haematopoietic system is much more radiosensitive than the tumour cells, then this property of TNF and the more rapid blood clearance of radioactivity should selectively protect against myelosuppression during radioimmunotherapy, and allow a higher dose of radiation to be delivered to the tumour.

The mice in the studies presented in this paper tolerated 2.5 x 10³ U (175 µg m⁻²) of TNF well, although the T-cell deficiency of nude mice may have accounted for the lack of an inflammatory response. In addition, human TNF has been shown to react with the murine TNF receptor 1 on tumour and endothelial cells, but not with the murine TNF receptor 2 on thymocytes and cytotoxic lymphocytes (Tartaglia et al., 1991). Phase I clinical trials have shown that the maximum tolerated single dose of TNF in humans is 350 µg m⁻² i.v. (Abbruzzese et al., 1989). Thus, it should be possible to increase the antibody uptake in tumours in patients with cancer by the concurrent administration of a tolerable dose of TNF. The additional inflammatory response in patients, not observed in athymic mice, may also facilitate antibody access to tumour.

The effect of TNF could be increased by targeting it directly to the tumour, resulting in an increased local concentration. Therefore, although TNF may be more toxic in...
humans than in nude mice, targeting an antibody-TNF fusion protein to the tumor should allow lower doses to be given systemically to achieve the same tumor dose. Hoogenboom et al. (1991) have produced a hybridoma capable of secreting an antibody-TNF fusion protein which retains the biological activities of both constituent molecules in vitro. This type of fusion protein may be capable of specifically increasing tumor vascular permeability without the increase in normal tissue permeability observed in the studies reported in this paper. A recombinant fusion protein of a single-chain Fv region and IL-2 which retains the biological properties of each constituent protein has been produced (Savage et al., 1993). This method should also be suitable for TNF-sfV fusions, since the gene for human TNF has been cloned and expressed in Escherichia coli (Shirai et al., 1985).

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