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Short Communication

Tumour necrosis factor alpha increases melphalan concentration in tumour tissue after isolated limb perfusion

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Summary Several possible mechanisms for the synergistic anti-tumour effects between tumour necrosis factor alpha (TNF-α) and melphalan after isolated limb perfusion (ILP) have been presented. We found a significant sixfold increase in melphalan tumour tissue concentration after ILP when TNF-α was added to the perfusate, which provides a straightforward explanation for the observed synergism between melphalan and TNF-α in ILP. © 2000 Cancer Research Campaign

Keywords: TNF; melphalan; tissue concentration; isolated limb perfusion; rats

With isolated limb perfusions (ILP) high drug concentrations can be achieved in the vasculature of a limb with no or negligible leakage into the systemic circulation. With the addition of high dose tumour necrosis factor alpha (TNF-α) to melphalan high response rates were demonstrated in patients with melanoma and irresectable soft tissue sarcomas (Liénard et al, 1992; Eggermont et al, 1996*a*, 1996*b*). Similarly, in rat sarcoma models synergy has been demonstrated between melphalan and TNF-α (Manusama et al, 1996; de Wilt et al, 1999).

The exact mechanisms for synergistic anti-tumour effects between melphalan and TNF-α, however, are not clear. Several possible mechanisms have been suggested such as selective destruction of tumour vasculature, which is accompanied by thrombus formation and haemorrhagic necrosis of the tumour (Shimomura et al, 1988; Renard et al, 1995). This process is accompanied by an inflammatory response that seems to be leucocyte-dependent (Yi and Ulich, 1992; Renard et al, 1994; Manusama et al, 1998). Moreover, TNF-α increases the permeability of tumour vasculature (Folli et al, 1993; Umeno et al, 1994) and has been reported to lower interstitial pressure in the tumour (Kristensen et al, 1996), which could both increase leakage of melphalan in tumour tissue and explain the observed synergy.

To demonstrate this hypothesis we analysed melphalan concentrations in tumour and limb tissue after melphalan isolated limb perfusions with and without the addition of TNF-α.

MATERIALS AND METHODS

Chemicals

Melphalan (Alkeran, Wellcome, Beckenham, UK) was diluted in 0.9% sodium chloride and stored at –20°C. Recombinant human TNF-α was provided by Boehringer (Ingelheim, Germany), with specific activity of 5.8×10^7 U mg^{-1} and endotoxin levels < 1.25 endotoxin units (EU) per mg protein and stored at –80°C. During perfusion 40 μg melphalan with or without 50 μg TNF-α was used.

Animal tumour model and perfusion setting

Male inbred BN rats, weighing 250–300 g, obtained from Harlan-CPB (Austerlitz, The Netherlands) were used. The perfusion technique was performed as described previously (Manusama et al, 1996). Briefly, small tumour fragments of the non-immunogenic BN-175 soft-tissue sarcoma were implanted in the right hind limb. ILP was performed at a tumour diameter of 13 mm ± 3 mm at least 7 days after implantation. Animals received 50 IU of heparin and the hind limb was kept at a constant temperature of 38–39°C. The femoral artery and vein were cannulated and collaterals were occluded by a groin tourniquet. An oxygenation reservoir was included into the circuit, and melphalan and TNF-α were added as boluses herein. A roller pump recirculated the perfusate at a flow rate of 2.4 ml min^{-1} for 30 min. A washout with 5 ml oxygenated Haemaccel was performed at the end of the perfusion. The committee on Animal Research of the Erasmus University Rotterdam, The Netherlands, approved the experimental protocol.

Tumour growth after perfusion was daily recorded by calliper measurement. Tumour volume was calculated as $0.4(A^2B)$, where A is the minimal tumour diameter and B the diameter perpendicular to A. Tumour volumes were compared 5 days after perfusion.

Assessment of melphalan concentrations in tissue

Immediately after ILP the perfused tumour and hind limb tissues were excised, homogenized in 2 ml acetonitrile (PRO 200 homogenizer, Pro Scientific, CT, USA), centrifuged at 2500 ***g*** and stored at –80°C. Melphalan was measured by gas chromatography-mass spectrometry (GC-MS), as described previously (De Boeck et al, 1997). P-[Bis(2-chloroethyl)amino]-phenylacetic acid methyl

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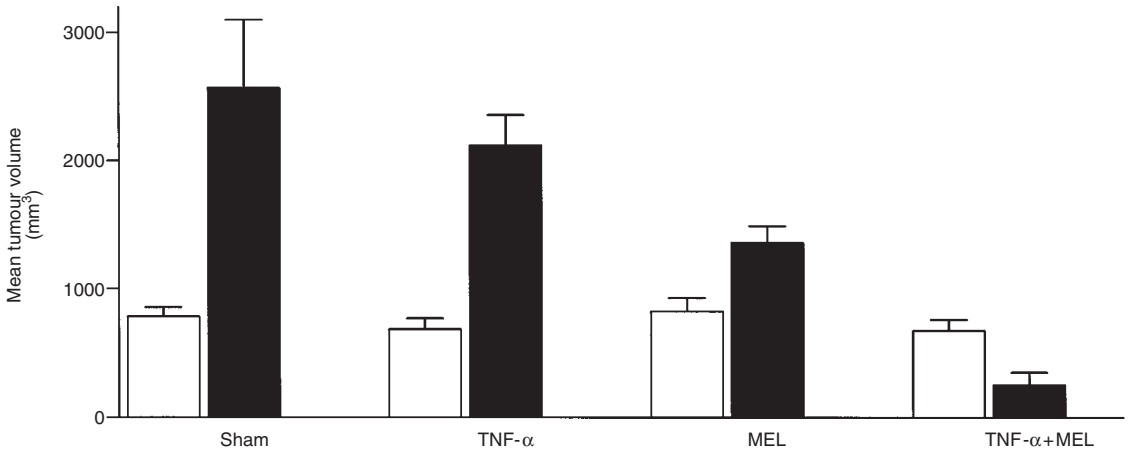

Figure 1 Mean tumour volumes (± s.e.m.) of BN-175 sarcoma before (□) and 5 days after (■) ILP

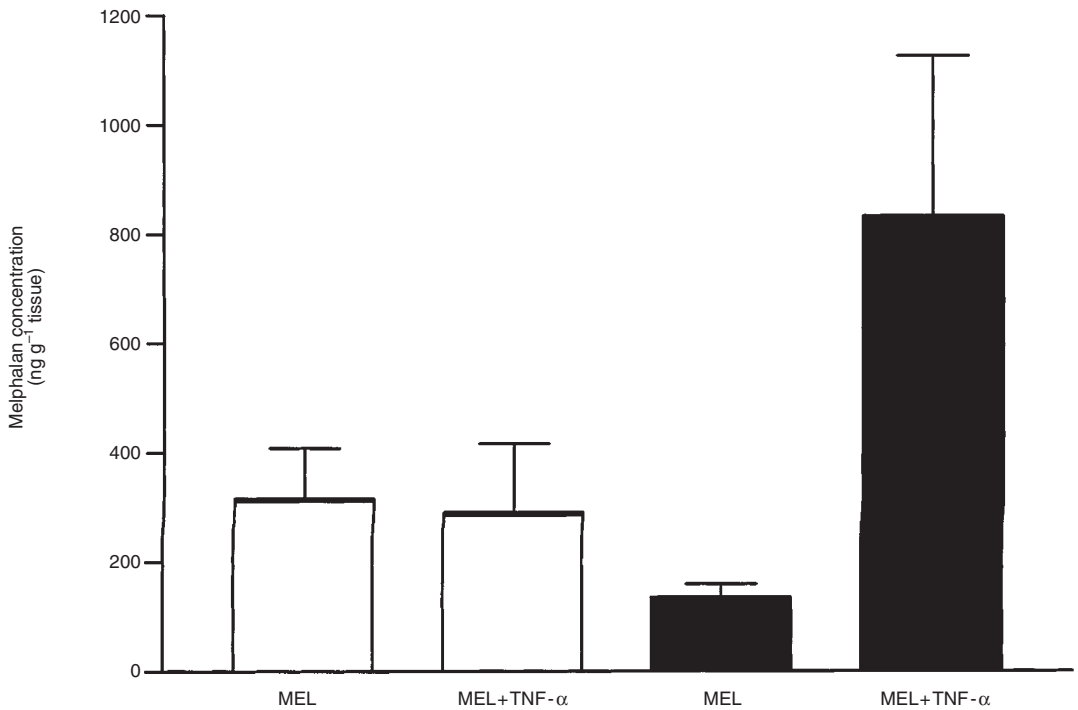

Figure 2 Melphalan concentration (± s.e.m.) in skin/muscle tissue (□) and tumour tissue (■) immediately excised after ILP with melphalan with or without TNF-α

ester was used as an internal standard. Samples were extracted over trifunctional C18 silica columns. After elution with methanol and evaporation, the compounds were derivatized with trifluoroacetic anhydride and diazomethane in ether. The stable derivatives were separated on a methyl phenyl siloxane GC capillary column and measured selectively by single ion monitoring GC-MS in the positive EI mode.

Statistical analysis

Mann–Whitney *U*-test was used to compare tumour volumes in different animal groups and to compare melphalan concentrations in different groups.

RESULTS

Tumour response after ILP

Mean tumour volumes were compared to demonstrate the efficacy of ILP with TNF-α and melphalan. Four groups of rats were perfused with sham (n = 10), TNF-α alone (n = 10) melphalan alone (n = 10) and the combination of TNF-α and melphalan (n = 10). A synergistic anti-tumour response was observed with the combination of melphalan and TNF-α as demonstrated before (Manusama et al, 1996; de Wilt et al, 1999) (Figure 1). A significant decrease in mean tumour volume after perfusions with the combination of melphalan and TNF-α was observed ($P < 0.001$), whereas in all other perfusions tumour volume increased.

Tissue concentrations of melphalan

Figure 2 demonstrates a sixfold increased melphalan concentration was found in tumour tissue after perfusion with the combination of TNF-α and melphalan ($n = 6$) in comparison with perfusions with melphalan alone ($n = 6$) ($P = 0.01$). TNF-α had no effect on skin and muscle tissue since melphalan concentrations after ILP were comparable with or without the addition of TNF-α.

DISCUSSION

In the present study we demonstrate an increased accumulation of melphalan in tumour tissue after ILP with the combination of melphalan and TNF-α as compared to melphalan alone. The increased melphalan accumulation could not be demonstrated in skin and muscle tissue, suggesting that TNF-α has no effect on normal tissue. The increased melphalan concentration in tumour tissue correlates very well with the observed tumour response.

The observed responses in this rat soft-tissue sarcoma model are comparable to patients, where TNF-α (Posner et al, 1994) or melphalan alone is not or only marginally active (Klaase et al, 1989). The combination of TNF-α and melphalan, however, results in high response rates (Liénard et al, 1992; Eggermont et al, 1996*a*, 1996*b*). Addition of TNF-α seems crucial in the observed synergy with melphalan and several mechanisms could be responsible for this. A direct effect of TNF-α on the anti-tumour activity of melphalan on BN-175 tumour cells was previously ruled out in vitro (Manusama et al, 1996).

Fajardo et al (1992) previously demonstrated that low-dose TNF-α has a proliferative effect on angiogenesis, whereas higher doses can cause destruction of newly formed blood vessels. It has been demonstrated that this destruction of blood vessels is the result of apoptosis and detachment of angiogenic endothelial cells (Ruegg et al, 1998) which may lead to thrombocyte aggregation, erythrostasis and haemorrhagic necrosis (Shimomura et al, 1988; Renard et al, 1994, 1995; Nooijen et al, 1996).

Whereas previous studies focus on tumour regression resulting from TNF-α-mediated destruction of the vasculature we show that augmented melphalan concentrations in tumour tissue after ILP with TNF-α correlates very well with tumour response and provides an elegant and straightforward explanation for the observed responses. Similarly, drug accumulation in tumour tissue has been shown after systemic pretreatment with TNF-α in mice treated with liposomal doxorubicin (Suzuki et al, 1990; Maruo et al, 1992). An explanation for this phenomenon can be the increased vascular permeability or decreased interstitial pressure that was demonstrated after administration of TNF-α (Folli et al, 1993; Umeno et al, 1994; Kristensen et al, 1996). Alexander et al (1998) demonstrated an increased capillary leakage during isolated hepatic perfusions (IHP) and an increased uptake of I-131 albumin in tumour tissue compared to liver tissue. However, addition of TNF-α did not affect melphalan concentrations in tumour tissue after IHP. Several reasons for this discrepancy are possible such as concentration of TNF-α used, sampling method and duration of perfusion. Another reason can be the difference in tumour vasculature, since colorectal metastases are usually hypovascular and largely necrotic, whereas soft-tissue sarcoma are usually hypervascular.

In conclusion, we hypothesise that increased tumour concentration of melphalan could very well be the main mechanism by which TNF-α enhances the anti tumour response. This finding is not only important for further TNF-α-based limb perfusions using melphalan or other cytostatic agents, but also for other perfusions settings such as isolated liver (Borel Rinkes et al, 1997; Alexander et al, 1998), lung (Pogbreniak et al, 1994) or kidney perfusions (van der Veen et al, 1999).

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