European Journal of Surgical Oncology 1996; **22**: 152–157

Isolated limb perfusion with TNFα and melphalan in a rat osteosarcoma model: a new anti-tumour approach

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Isolated limb perfusion (ILP) with TNFα, IFNγ and melphalan causes impressive tumour reduction in patients with irresectable soft tissue sarcomas with a high limb salvage rate. Since this therapy could be of value in patients with progressive osteosarcoma, we performed a study in an osteosarcoma tumour model in the rat. The ROS-1 osteosarcoma was implanted s.c. in the hind leg of WAG rats. Rats were divided in four groups: rats that underwent ILP with perfusate alone, TNFα alone, melphalan alone or their combination. Almost all rats, treated with a sham ILP or a perfusion with 40 μg melphalan, showed progressive disease (PD) (6/6 and 5/6). After perfusion with 50 μg TNFα alone a varied response was observed: 2/6 PD, 2/6 no change (NC) and 2/6 a complete remission (CR). After combined perfusion: 3/6 rats had a partial remission and 3/6 a CR. The best and most consistent responses are obtained by combining TNFα and melphalan. The discrepancy with the *in vitro* sensitivity of ROS-1 indicates that indirect effects are important in this tumour model.

Key words: **oncology; chemotherapy; TNFα; osteosarcoma.**

Introduction

Osteosarcoma is a rare tumour (1 to 2 cases per million each year) and occurs mainly in patients in the second decade of life. Despite its rarity, osteosarcoma has attracted much attention, since pre- and post-operative chemotherapy increases survival rate considerably in patients with primary osteosarcoma.[1,2] Control of the primary tumour by pre-operative chemotherapy allows more conservative surgery[3] and the degree of tumour necrosis is an important prognostic factor.[4] Progressive disease in spite of chemotherapy is associated with a poor prognosis both with respect to local tumour control and survival. In most of these 'lost cases' ablative surgery is needed with no hope of a cure.

In these patients isolated limb perfusion (ILP) with TNFα, IFNγ and melphalan could be very beneficial, since this therapy often converts large tumours into necrotic, shrunken tumour remnants that can be resected at little functional cost of the extremity. This has been demonstrated for irresectable extremity soft tissue sarcomas (STS).[5-7] Rendering large tumours of the extremities resectable by loco-regional therapy has not only been described for STS but also for osteosarcoma: Vaglini *et al.* reported that large osteosarcomas became resectable after hyperthermic-antiblastic perfusion in combination with intra-arterial and intravenous chemotherapy in 11/18 of the patients.[8] ILP with TNFα, IFNγ and melphalan is less complex and avoids systemic administration of cytostatic agents and therefore needs to be evaluated in patients with irresectable osteosarcoma.

In a previous study in rats we found that ILP with a combination of TNFα and melphalan was effective against an aggressive soft tissue sarcoma in the Brown Norway ILP-rat model.[9] The aim of the present study is to investigate whether these effects can be found in the ROS-1 osteosarcoma tumour model. In addition, we are interested in the role of direct cytotoxicity of the agents both alone and combined. The presence of indirect effects of both agents in this tumour model may provide a rationale to use this combination therapy in clinic, despite chemoresistance, which is principally based on direct cytotoxic effects only.

Materials and methods

Animals

Male inbred WAG-Rij strain rats, weighing 250–300 g obtained from Harlan-CPB (Austerlitz, The Netherlands) were used. The rats were fed a standard laboratory diet delivered by Hope Farms (Woerden, The Netherlands) and kept under standard laboratory conditions of light and accommodation. The experimental protocols adhered to the

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This work was financed in part by the Dutch Cancer Society (Grant DDHK 93-659).

0748-7983/96/020152+06 $12.00/0

rules laid down in the 'Dutch Animal Experimentation Act' (1977) and the 'Guidelines on the protection of Experimental Animals' published by the council of the EC (1986). The protocol was approved by the 'Committee on Animal Research' of the Erasmus University Rotterdam, The Netherlands.

Tumour

The ROS-1 osteosarcoma (transplantable to WAG/Rij rats) was used. This osteosarcoma originated spontaneously in the tibia of a rat.[10] Cells from this tumour were maintained in tissue culture and from these cultures new tumours were produced by inoculation in the flank.

Melphalan

Melphalan (Alkeran, 50 mg per vial, Wellcome, Beckenham, UK) was diluted in 10 ml diluent solvent. Further dilutions were made in 0.9% NaCl to give a volume of 0.2 ml in the perfusion circuit.

TNFα

Recombinant human TNFα was provided by Boehringer (Ingelheim, Germany) with a specific activity of 5.8×10^7 U/mg as determined in the murine L-M cell assay.[11] Endotoxin levels were <1.25 EU/mg protein.

Tumour model

Fragments of 3–5 mm were implanted in the right hind limb s.c. just above the ankle. Perfusion was performed at a tumour diameter of 15 mm ± 5 mm at least 7 days after implantation. Tumour growth was recorded by calliper measurement. The mean of two perpendicular diameters was obtained. Tumour diameters were measured at least three times a week.

Isolated limb perfusion

We used a perfusion technique originally described by Benckhuijsen *et al.*[12] with some modifications.[13] Briefly, Hypnorm (Janssen Pharmaceutica B.V., Tilburg, The Netherlands) was given as an anaesthetic and 50 i.u. of heparin were injected i.v. To keep the rat's hind leg at a constant temperature of 38–39°C, a warm water mattress was applied around the leg. Temperature was monitored by a temperature probe (Ellab, Copenhagen, type DU-3) fixed at the convexity of the tumour. The femoral artery and vein were approached through an incision parallel to the inguinal ligament. Collaterals were temporarily occluded by the application of a tourniquet in the groin. The tourniquet was fixed at the inguinal ligament. The femoral artery and vein were cannulated with silastic tubing (0.30 mm ID, 0.64 mm OD; 0.64 mm ID, 1.19 mm OD, respectively, Degania Silicone, Degania Bet, Israel). An isolation time of 30 min commenced when the tourniquet was tightened. An oxygenation reservoir and a roller pump were included in the circuit. The perfusion commenced with 5 ml Haemaccel (Behring Pharma, Amsterdam, The Netherlands) and the haemoglobin (Hb) content of the perfusate was 1.45 g/dl (0.9 mmol/l). Melphalan and TNFα were added as boluses to the oxygenation reservoir. The roller pump (Watson Marlow, Falmouth, UK; type 505 U) recirculated the perfusate at a flow rate of 2.4 ml/min. A washout of 2 ml oxygenated Haemaccel was performed at the end of the perfusion. The collateral circulation via the internal iliac artery towards the leg is so extensive that it allows ligation of the femoral vessels without detrimental effects. Despite ligation of the femoral artery, back-flow from the femoral vein is seen in all rats immediately after release of the tourniquet. Moreover, in a previous study we measured the partial oxygen pressure (PaO_2) to be similar after ligation of the femoral vessels to that prior to perfusion.[13]

Tumour response studies

The limbs of 24 rats were perfused. There were four experimental groups: sham perfusion ($n=6$), perfusion with 50 μg TNFα ($n=6$), 40 μg melphalan perfusion ($n=6$) and pefusion with both 40 μg melphalan and 50 μg TNFα ($n=6$). The concentrations used are equivalent to those used in an efficacy study against the BN 175 fibrosarcoma in the BN rat.[9] Since systemic administration of TNFα in clinic is limited by severe toxicity, it makes no sense to perform i.v. dose–effect studies in the rat.

The classification for tumour response was: progresive disease (PD), increase of tumour diameter >25% within 10 days; no change (NC), tumour diameter equal to diameter during perfusion ± 25%; partial remission (PR), >25% decrease of tumour diameter; complete remission (CR), no palpable tumour.

In vitro *assessment of antitumour activity*

We determined the *in vitro* sensitivity of ROS-1 osteosarcoma for melphalan and TNFα. This cell line grows as a monolayer in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and glutamic acid (0.3 mM), all obtained from Gibco (Paisley, UK), in a humidified atmosphere of CO_2/air (5:95) at 37°C. We used the sulphorhodamine B (SRB) protein stain assay according to the method of Skehan *et al.*[14] Briefly, cells were isolated from cultures in the exponential growth phase by trypsinization, and counted, and plated in 96-well micro titre plates (Costar, Cambridge, MA). Each well contained 100 μl 24 h after plating, 100 μl culture medium, or culture medium containing drug, was added to the wells. Seventy-two hours after drug addition cells were incubated with trichloroacetic acid (200 μl/well) at 4°C for 1 h by means of protein precipitation and washed five times with tap water. The cells were stained for at least 15 min with 0.4% SRB dissolved in 1% acetic acid and subsequently washed thoroughly with 1% acetic acid to remove superfluous dye. After drying the plates, the bound protein stain was solubilized with 150 μl 10 mM unbuffered TRIS. The optical density was read at 540 nm. All experiments were performed eight times. Tumour growth was calculated using the formula: tumour growth = (test well/control) × 100%.

Table 1. Tumour response of ROS-1 osteosarcoma after ILP.

Response	Sham $n=6$	Melphalan $n=6$	TNFα $n=6$	Melphalan + TNFα $n=6$
Progressive disease	6	5	2	
No change		1	2	
Partial remission				3
Complete remission			2	3

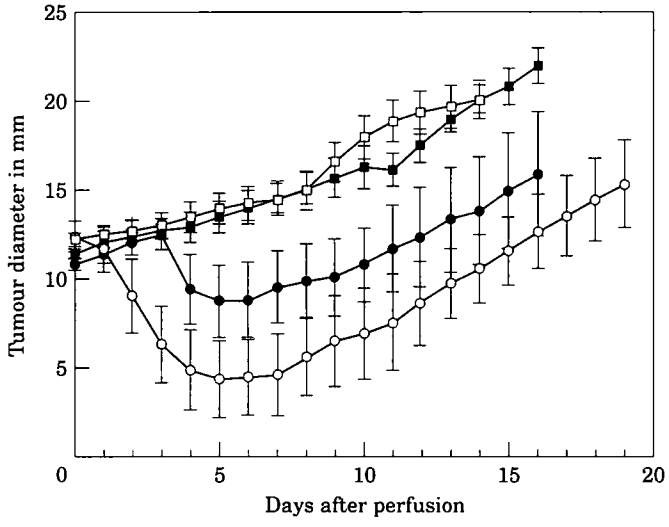

Fig. 1. Growth curves of ROS-1 osteosarcoma in the hind limb after sham ($n=6$, —■—), melphalan 40 μg ($n=6$, —□—), TNFα 50 μg ($n=6$, —●—) and melphalan + TNFα treatment ($n=6$, —○—). The mean (±SEM) of the tumour diameters are depicted. Only statistically significant differences exist between the combined and sham group at day 3–15 after perfusion (SNK test: $P<0.05$).

Statistical analysis

Tumour diameters are given as means ± SEM. Differences in results between the treatment groups were tested by Student Newman-Keul's (SNK) test, after one-way analysis of variance.

Results

ILP response studies

In Table 1 the tumour responses of the different groups are summarized. Only in rats that were perfused with TNFα alone or with TNFα in combination with melphalan, was tumour regression observed in 33% and 100% respectively. Sham perfusions or perfusions with melphalan alone did not result in tumour regression. In Fig. 1 the curves of the mean tumour diameters in the different groups are depicted. Only the group that underwent TNFα + melphalan perfusion showed a statistically significant (SNK test: $P<0.05$) difference to the group that received sham perfusion at 3–15 days after perfusion.

The recurrence rate was 100%. Tumours reappeared 7–13 days after perfusion. After recurrence tumours grew as fast as tumours in rats that had received sham perfusion.

In vitro *cytotoxicity assay*

The dose/response curves of the ROS-1 osteosarcoma cell line to TNFα and melphalan are depicted in Fig. 2. ROS-1 appeared to be relatively resistant to TNFα, as evidenced by 60% growth at even very high concentrations (50 μg/ml) of TNFα. However, ROS-1 was sensitive to melphalan with an IC_{50} at 0.009 μg/ml.

In Fig. 3 the dose/response curves of ROS-1 to melphalan is shown in the presence or absence of different concentrations of TNFα. The maximal growth of ROS-1, shown as a plateau at the lower concentrations melphalan, is reduced in the presence of TNFα in a concentration-dependent manner, which can be explained by addition of effects. The dose/response curves bend towards total growth inhibition at the same dose of melphalan independent of what concentration of TNFα is used. Thus, these experiments could not reveal synergism in the direct tumour cytotoxic effects of both agents.

Discussion

The present study demonstrated that an experimental osteosarcoma responded in all rats treated with the

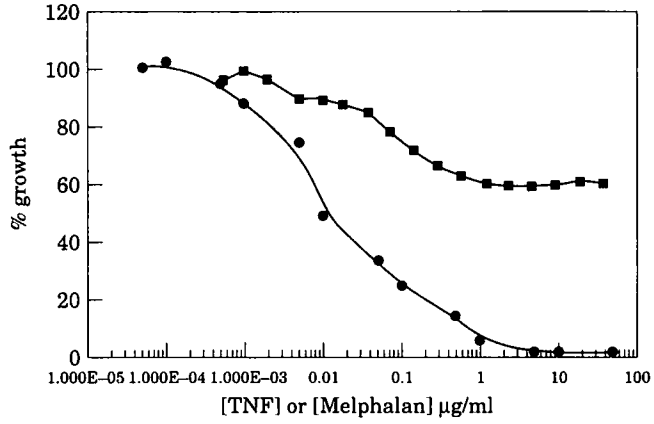

Fig. 2. Dose/response curves of ROS-1 osteosarcoma to TNFα (—■—) and melphalan (—●—) determined in the sulphorhodamine B assay. Cell number measured as absorbance in the colorimetric assay is represented as a percentage of the control cell growth.

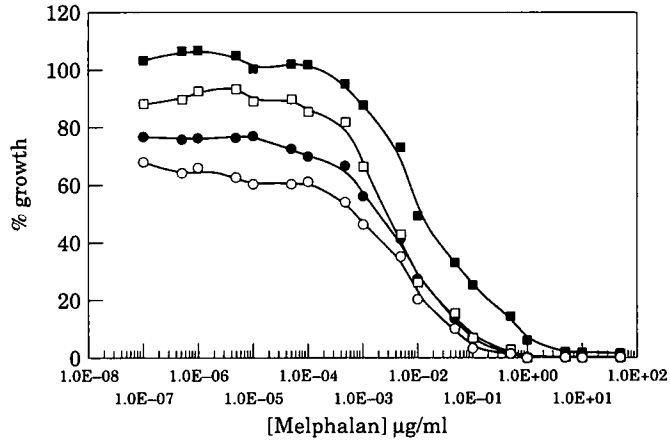

Fig. 3. Dose/response curve of ROS-1 osteosarcoma to melphalan in the absence or presence of various concentrations of TNFα, determined in the sulphorhodamine B assay (—■— = melphalan (M) only; —□— = M + 0.1 μg/ml TNFα; —●— = M + 1 μg/ml TNFα; —○— = M + 10 μg/ml TNFα.)

combination of TNFα and melphalan in an ILP model. No statistically significant tumour response was noted in groups that were sham perfused or perfused with melphalan alone or TNFα alone. Perfusion with melphalan alone and sham perfusion was followed by progressive disease. After ILP with TNFα alone the response varied, but no consistent antitumour activity was observed. In spite of the absence of consistent antitumour effects of TNFα, the combination of TNFα with melphalan showed clear synergistic antitumour activity resulting in a 100% response rate. These observations are in line with the synergy observed between TNFα and melphalan in the Brown Norway soft tissue sarcoma model.[9] Regarding the varied response to TNFα alone it should be kept in mind that anoxia in the tumour may be a critical determinant for its propensity to respond to TNFα alone,[13,15,16] and thus a variation in size or structure may explain why responses after TNFα alone may vary.

In contrast to the *in vivo* data the observations *in vitro* show the relative resistance of ROS-1 in culture to TNFα and its sensitivity to melphalan. Furthermore, no synergistic effects were observed in the *in vitro* experiments. Also, in previous studies the lack of correlation between direct tumour-cytotoxicity of TNFα and the *in vivo* tumour response has been shown.[17,18] The opposite picture of the *in vivo* results to the *in vitro* results obtained in the present study makes it clear that indirect, host-mediated effects must be important in the tumour response of ROS-1, observed after ILP with TNFα + melphalan.

The effect of TNFα on the neo-vasculature of the tumour has been the subject of many preclinical studies.[19-21] The tumour response with evident haemorrhagic necrosis within 24 h is characteristic for TNFα.[23,24] Also in man vascular effects have been associated with the response on TNFα: in patients with soft tissue sarcoma, treated with an ILP with TNFα, IFNγ and melphalan, the tumour response was associated with the angiographic disappearance of the tumour's neo-vasculature[25] and with the histopathological findings of vascular occlusion and haemorrhagic necrosis.[26,27] In the present study, the tumour regression within 3 days is typical for the TNFα tumour response with a target role of the tumour's neo-vasculature. Also, the involvement of the immune system is considered to be important in the

TNFα tumour response and believed to be executed by the following mechanisms: (i) invasion of PMNs in the acute phase enhances the detrimental effects on the neo-vasculature[27-29] and (ii) stimulation of inflammatory cells, e.g. lymphocytes, results in tumoricidal activity. The latter effect can result in a prolonged tumour control and is only described in immunogenic tumours.[30] Since recurrences were found in all rats that showed tumour regression, we believe that the immune-mediated responses may not play an important role in our ILP model.

The synergism between TNFα and melphalan, observed in our tumour model, can be first explained by an enhancement by melphalan of the TNFα-induced vascular effects, since melphalan may well induce vascular damage, similar to that reported for the alkylating agent cyclophosphamide.[31] A second underlying mechanism of the synergism could be an improved penetration of the cytostatic agent in the tumour by the TNFα effects on the tumour's neo-vasculature. In previous studies vascular leakage and an enhanced accessibility of the therapeutical agent has been associated with a better tumour response.[32,33] Both mechanisms of synergism may well be relevant in the chemo-resistant osteosarcoma in man and therefore it seems justified that in a setting of ILP TNFα is combined with melphalan.

To mimic the clinical situation of advanced osteosarcoma, we waited to treat tumours until they were relatively large. In the presence of an ineffective dose of melphalan, TNFα induced its typical quick tumour response, based on vascular effects. Thus, the concepts of targeting neo-vasculature also seems valid for experimental osteosarcoma. Since all large tumours are dependent on their neo-vasculature, irrespective of their histological type, we anticipate that ILP with TNFα and melphalan will be effective in all advanced osteosarcomas and thus may overcome the problem of chemoresistance. However, the early recurrences observed in all rats is a warning against too high an expectation of this combination therapy in the long term, and indicate that ILP with TNFα and melphalan should always be followed by dissection of the shrunken tumour remnants.

Thus, in line with the experience in patients with locally advanced soft tissue sarcomas, ILP with TNFα and melphalan could be of great therapeutical benefit in patients with chemoresistant osteosarcoma to obtain local tumour control.

Acknowledgements

This work was financed in part by the Dutch Cancer Society (Grant DDHK 93-659). We acknowledge Boehringer Ingelheim GmbH for generously providing TNFα.

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Accepted for publication 12 November 1995