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

Eric R. Manusama, Jeroen Stavast, Nicola M. C. Durante, Richard L. Marquet and Alexander M. M. Eggermont

Department of Surgery, University Hospital Rotterdam dr Daniel den Hoed Cancer Center, Rotterdam, The Netherlands

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. Control of the primary tumour by pre-operative chemotherapy allows more conservative surgery and the degree of tumour necrosis is an important prognostic factor. 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). 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. 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. 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
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 x 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. Moreover, in a previous study we measured the partial oxygen pressure (PaO2) 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 perfusion 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. 6 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: progressive 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 CO2 (5%) 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 microtitre 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) x 100%.
Table 1. Tumour response of ROS-I osteosarcoma after ILP.

<table>
<thead>
<tr>
<th>Response</th>
<th>Sham n=6</th>
<th>Melphalan n=6</th>
<th>TNFα n=6</th>
<th>Melphalan + TNFα n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progressive disease</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>No change</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial remission</td>
<td></td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Complete remission</td>
<td>2</td>
<td>3</td>
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</tr>
</tbody>
</table>

Fig. 1. Growth curves of ROS-I 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-I osteosarcoma cell line to TNFα and melphalan are depicted in Fig. 2. ROS-I appeared to be relatively resistant to TNFα, as evidenced by 60% growth at even very high concentrations (50 μg/ml) of TNFα. However, ROS-I was sensitive to melphalan with an IC₅₀ at 0.009 μg/ml.

In Fig. 3 the dose/response curves of ROS-I to melphalan is shown in the presence or absence of different concentrations of TNFα. The maximal growth of ROS-I, 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...
Isolated limb perfusion with TNFα and melphalan

Fig. 2. Dose/response curves of ROS-I osteosarcoma to TNFα (--) and melphalan (---) determined in the sulforhodamine 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-I osteosarcoma to melphalan in the absence or presence of various concentrations of TNFα, determined in the sulforhodamine 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-I 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.\(^{11,18}\) 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.\(^{22-24}\) 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
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