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High Dose Recombinant Tumour Necrosis Factor (rTNFα) Administered by Isolation Perfusion for Advanced Tumours of the Limbs: A Model for Biochemotherapy of Cancer*

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INTRODUCTION

TUMOUR NECROSIS FACTOR (TNFα) was discovered as a serum factor in mice treated with BCG and endotoxin, producing haemorrhagic and coagulative necrosis of tumours in recipient mice [1]. In fact, TNFα is the first cytokine which is able to produce very fast and effective necrosis of tumours more efficiently than chemotherapy itself. Therefore, efforts were made to clone the gene. In 1985, the human TNFα gene [2] was cloned and expressed in *E. coli*, followed in the same year, by the murine TNFα gene [3, 4]. It is commonly accepted that the human TNFα structure is a non-glycosylated trimer of 157 amino acids with several cysteine bounds [5, 6]. The trimer has three receptor binding sites apparently situated between each part of the trimer. There are two membrane receptors for TNFα of different molecular weights currently named p55 and p75 [7].

The recombinant TNFα (rTNFα) was made available for clinical trials, but unfortunately it was found, at that time, to cause fatal septic shock in humans (reviewed in ref [8]). It is not surprising, therefore, that phase I and II studies in humans were hampered by high levels of toxicity and seldom showed antitumour effects [9–15].

In 1988, we considered using isolated limb perfusion for administering efficient high dose TNFα combined to interferon-γ and melphalan. The results in melanoma-in-transit-metastases and soft tissue sarcoma of the limbs have been astonishingly high in terms of complete response rate [16]. During the past 5 years, we have treated, with our Dutch colleagues, approximately 200 cases. Side-effects were always acceptable and have been drastically reduced recently by improving the technique. A comprehensive study of the cases, including immunohistology, pathophysiology, immunology and biochemistry, allows us to propose that isolation perfusion of the limbs with high dose TNFα is a model for biochemotherapy of cancer.

WHAT MAKES TNFα A UNIQUE ANTICANCER AGENT?

Cancer growth depends on angiogenesis, which is promoted by angiogenic factors secreted by tumour cells. Old's group discovered that TNFα acted through selective destruction of tumour microvasculature [5]. TNFα is also moderately cytotoxic to tumour cells, and around 30% of cell lines tested so far have shown some sign of cytotoxicity (reviewed in [17]). Mouse tumour models and human tumour xenografts on nude mice have shown that TNFα alone has a transient antitumour effect, with regrowth of the tumour after necrosis. However, it has been shown by several authors that a definitive cure in animals could be obtained by combining TNFα with either chemotherapeutic agents or interferon-γ.

WHY WAS THE SYSTEMIC ADMINISTRATION OF rTNFα ABANDONED?

The efficacious dose of rTNFα in mice, either in syngeneic tumours or in nude mice carrying human tumour xenografts, is around 50 mg/kg [18, 19]. The MTD in human is 350 mg/m^2 or 5 mg/kg [13–15]. This 10-fold lower dose in humans produces only seldom and partial responses, sometimes accompanied by severe side-effects. It is worth emphasising that most of the phase I and II studies were designed as for chemotherapeutic agents, that is, with no special protocol for preventing the so-called "septic shock like syndrome". In fact, TNFα upregulates NO synthetase followed by the release of NO, which in turn produces a general vasoplegia leading to a reduction in the vascular resistance [20]. Therefore, it is not surprising that after either i.v. or even intratumoural TNFα, most reports indicate severe hypotension. With isolation perfusion using high dose TNFα, rTNFα may peak to more than 100 ng/ml in the peripheral blood from leakage during the procedure [21]. However, with appropriate measures, as will be described below, it is completely feasible to reverse the hypotension induced by TNFα.

As for chemotherapy (and probably more stringently), a good performance status of the patient is essential in order to cope with the side-effects of TNFα. Unfortunately, patients receiving the cytokine in phase I and II trials had a performance status which was very often lower than those of surgical patients submitted to isolation perfusion with TNFα. This difference seems unavoidable because of the difference in selection criteria between phase I–II and surgical trials.

Recently, it has been proposed that the side-effects induced by rTNFα do not completely mimic those of septic shock. Indeed, the latter requires other components of sepsis such as endotoxin, which was shown to have a very strong synergism with TNF for toxicity, presumably because of differences in the

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cytokine cascade. The systemic symptoms occuring after TNFα in patients devoid of infection is better termed "Systemic Inflammatory Response Syndrome" (SIRS) [22]. This syndrome can be easily controlled with vasoactive amines and fluid loading.

Since the phase I and II trials used only low dose TNFα, generally in patients sometimes in poor general condition, it is not surprising that the conclusions of most reports were pessimistic, and that TNFα was abandoned by most of the medical oncology investigators. Disappointed with the poor results obtained with the systemic administration of TNFα, Genentech (San Fransisco) and Boehringer Ingelheim (Ingelheim) who had purchased the licence from the latter for producing rTNFα decided to stop the production of rTNFα for clinical trials in 1988. At that time, we decided to try rTNFα in isolation perfusion of the limbs (ILP), using remaining supplies of the cytokine.

RATIONALE FOR USING rTNFα IN ISOLATION LIMB PERFUSION (ILP) AND IN COMBINATION WITH INTERFERON-γ AND CHEMOTHERAPY

In 1988, we routinely performed isolation perfusion (ILP) of the limbs for melanoma and sarcoma. This method involves isolation of the diseased limb, its connection to a heart–lung machine and the administration of high dose chemotherapy. The rationale is to improve the response rate by increasing the drug concentration in the limb, with a dose dependent cytotoxic agent, while abolishing systemic toxicity which is dependent upon the efficiency of the isolation. The dose limitation will then be the regional toxicity. In fact, ILP with melphalan was, at the time, the best treatment for melanoma-in-transit-metastases, with a 50% complete remission rate, compared with a rate of less than 1% when the same drug was administered systemically (reviewed in [23]). We were prepared to try the highly toxic TNFα molecule in ILP. To determine whether TNFα would be superior to melphalan in the same setting, we designed a protocol with an effective dose of rTNFα, 10 times the maximum tolerated dose (MTD) in humans, and equivalent to the effective dose in animals, that is around 50 mg/kg [8]. There are three types of tumours that can be located on the limbs but are irresectable, either because of multiplicity or large volume and tissue invasion: in-transit-melanoma-metastases (stage IIIA or IIIAB), soft-tissue sarcomas and squamous cell carcinomas.

The isolation perfusion system consists of a circuit made of a heart–lung machine which provides the circulation of a total of around 2 litres of perfusate, under hyperthermic conditions. The perfusate is heated and oxygenated by a membrane or bubble oxygenator, and cutaneous tissues of the perfused limb are given extra heat from a heating blanket, with the aim of reaching hyperthermia without any temperature gradient.

The first three cases were treated with rTNFα only, at total doses of 2, 3 and 4 mg in the perfusate. This pilot study was intended to determine the pharmacokinetics in the perfusate, and to verify whether the side-effects observed in the systemic setting could be abrogated. The pharmacokinetics of rTNFα showed a plateau for the whole 90 min and there was no significant leakage into the systemic circulation. The plateau levels found both by immunoassay [16] and by bioassay [21] were between 1.5 and 6 μg/ml, which is close to the optimal concentration observed to be efficient in experimental models, both *in vivo* and *in vitro*. This feasibility study showed that ILP allows the application of rTNFα in conditions where saturation of the receptors can be expected, because of the high dose. Moreover, these conditions mimic the *in vitro* systems. That is not the case with chemotherapies, which usually show a bimodal disappearance curve, the first phase representing distribution within the vascular bed, and the second, extraction from the tissue together with hydrolysis.

In addition to our 3 patients treated with 1, 2 and 3 mg of TNFα alone, respectively, 3 other patients were treated by Posner and associates [24] with 4 mg. Partial response (PR) of less than 1 month was seen in 2 patients, with no response noted in 3. One patient had a complete response (CR) of 7 months duration and then progressed. 3 patients have been re-perfused with triple drug ILP which produced 2 CR and 1 PR. We concluded that ILP with TNF alone has inadequate activity to warrant further investigation [16, 24]. From the various publications on experimental models using either syngeneic tumours or human tumour xenografts, it appears that rTNFα alone is rarely able to induce tumour regression of long duration and with no regrowth.

Chemotherapeutic agents, such as alkylating agents or 5 fluorouracil (5FU) or anthracyclines, are synergic to TNFα in human tumour xenografts such as melanoma, colon, ovarian, gastric cancers [25]. In an experimental isolation perfusion model on sarcoma of the rat, Eggermont's group demonstrated a synergistic effect with the association of melphalan and TNFα [26]. Interferon-γ, which has a very poor antitumour effect, has been shown to act synergistically with TNFα in sarcoma and melanoma models [18, 19, 25]. Moreover, interferon-γ has been demonstrated to upregulate TNFα receptors [27]. However, the synergy of TNFα and interferon-γ also results in higher systemic toxicity [15]. It is clear that the experimental models indicate that if TNFα has the unique property of destroying tumour associated vasculature, it needs to be used in combination with other agents in order to achieve a complete response.

In our protocol, we decided to combine rTNFα with rIFNγ and with melphalan, since no additional toxicity has been reported with combined rIFNγ and chemotherapy. However, synergistic toxicity has been reported for rTNFα and rIFNγ. We chose to use the gold standard, melphalan, a bifunctional alkylating agent, since it has been shown to produce a 50% complete response as a single agent, at high concentrations, in ILP for in-transit-metastases of melanoma [23]. The isolation perfusion was performed under mildly hyperthermic conditions since hyperthermia has been shown to potentiate the activity of both rTNFα and melphalan [28, 29].

PROTOCOL FOR THE TRIPLE ASSOCIATION OF rTNFα, rIFNγ AND MELPHALAN ADMINISTERED BY ILP

All the patients were subjected to a thorough clinical examination, which always included a thoraco-abdominal scan. For sarcomas, pretreated melanomas and in elderly patients, a selective angiogram was performed to verify the integrity of the vessels to be perfused, and for sarcoma and bulky melanoma metastases, the tumour vascularisation. Complete haematological examination and coagulation tests were performed, together with an assessment of kidney and liver functions.

The patients, who had given informed consent, received 0.2 mg rIFN-γ by subcutaneous injection in the evening of days 1 and 2, preceded by the administration of 500 mg of acetaminophen. The volume of the limb to be perfused was measured according to water displacement. ILP was performed under general anaesthesia. Before the procedure, an arterial and a pulmonary (Swan-Ganz®) catheter were installed by the anaesthesiologist. Typically, dopamine infusion with 3 mg/kg/

min was started just before the administration of rTNFα. The protocol has been described [16] and is summarized in Figure 1.

TOXICITY OR ILP WITH TNFα

It is well established that systemic toxicity from isolation perfusion with any drug is the result of leakage. The latter is monitored by the use of radioactive human serum albumin, injected into the perfusate. The only reliable determination of the leakage is obtained by continuous monitoring using a gamma detector placed above the heart [30]. The maximum side-effects of rTNFα that can be encountered with ILP were demonstrated when workers at Lausanne were using a high pump-flow.

The peak TNFα concentrations in the peripheral blood exceeded 100 μg/ml for 60–120 min. Using fluid loading and vasoactive amines, these haemodynamic acute effects all evolved into a hyperdynamic state. A complete recovery was obtained on day 3 (days 2–5) with no toxic death and no sequelae [31].

Our collaborative experience with A. Eggermont (Rotterdam), H. Schraffordt Koops (Groningen) and B. Kroon (Amsterdam) is that both keeping the flow to 40–45 ml/l of perfused limb, and injecting rTNFα when radioactivity over the heart reaches a plateau, provide the safest perfusion conditions with minimal systemic toxicity. Currently, most patients only show transient and mild distributive shock during the first afternoon and night after ILP.

TNFα PHARMACOKINETICS AND SOLUBLE RECEPTORS

The pharmacokinetics of TNFα release show that the patients who did not experience significant side-effects during and after the perfusion did not demonstrate more than 5% leakage. However, no correlations were found between the maximum TNFα concentration in the peripheral blood of an individual and the side-effects, indicating that patients vary in their sensitivity to TNFα. Moreover, the greater than 100 ng TNFα levels found in our patients [21] contrast with the current literature on septic shock, which indicates that people dying of multi-organ failure show only pg levels of TNFα. Infected patients have significant levels of endotoxin, which has been shown to be synergistic with TNFα for toxicity. Another hypothesis to explain why our patients tolerated high plasma concentrations of TNFα, is that there is a very early and efficient production of soluble p75 and p55 receptors in the peripheral blood [32], presumably due to peri-operative and even low leakage of TNFα into the systemic circulation. Soluble p75 receptors are thought to play a major role in TNFα elimination in urine [33]. However, our patients had a mean p75 plasma level, peaking at 11.5 mg/ml, a value much lower than the systemic TNFα concentration found in the same series of patients [21]. Thus, it is difficult to believe that soluble p75 receptors alone account for TNFα tolerance in our setting.

When we analysed the phamacokinetics of TNFα in relation to leakage, it was obvious that even in the absence of detectable leakage, and after intense wash-out (2 to 3 l) of the intravascular TNFα, there was always a release of TNFα into the systemic circulation. The data can be explained by the fact that TNFα is retained and slowly released from the perfused tissues after restoration of the physiological circulation. We recently found (unpublished data) that more intense wash-out with 5–7 l Dextran and limb massage drastically reduce TNFα systemic release.

Our observations indicate that the acute response (SIRS) to TNFα observed in ILP is tolerable. In contrast to sepsis, ILP involves only one bolus injection of rTNFα given at a preset time to patients normally devoid of circulating endotoxin. In fact, one patient died of genuine septic shock 3 weeks after perfusion with rTNFα because his tumour was heavily infected, and the treatment resulted in a mobilisation of bacteria. Since then, an infected tumour has been considered a contraindication to isolation perfusion with TNFα.

THERAPEUTIC EFFICACY OF TRIPLE ASSOCIATION WITH TNFα ILP IN MELANOMA AND SARCOMA

In melanoma, the pilot study started in 1989 and was closed to patient entry at the end of 1993. The objective was to improve

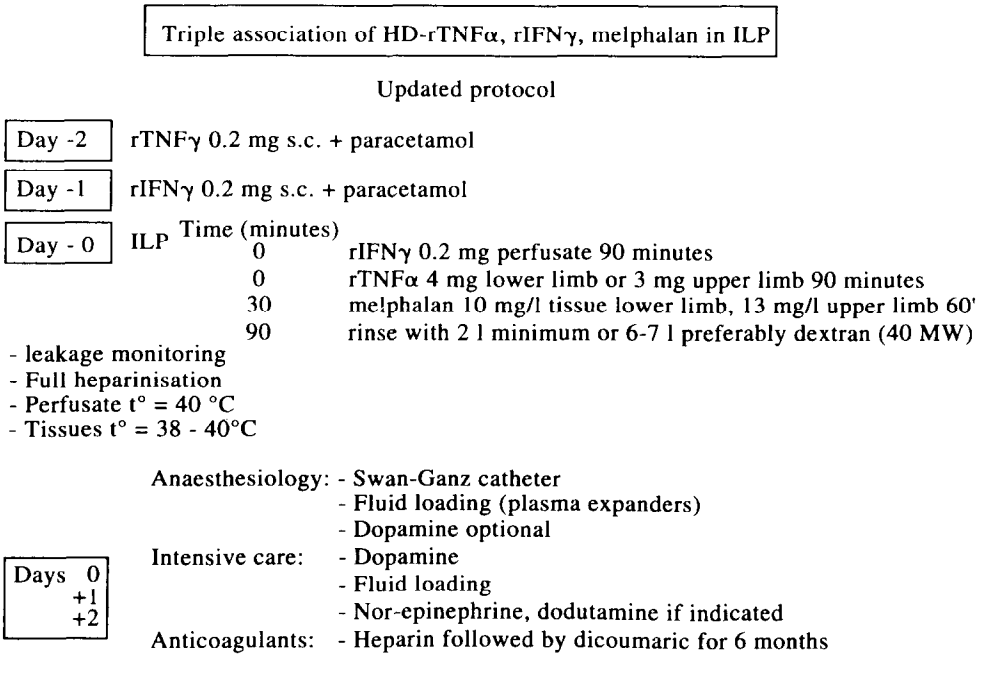

Figure 1. Triple association of HD-rTNFα, rIFNγ, melphalan in isolated limb perfusion. Updated protocol.

upon the 50% response rate predicted for ILP with melphalan alone, and to evaluate the duration of the response. The first report [16] on the Brussels cases was followed by a multicentric study with the addition of cases from Lausanne, Rotterdam, Groningen and Amsterdam [34]. The responses of 53 evaluable patients were compared to standard melphalan ILP. For that purpose, a database was built up of 103 patients, with the same stage III regionally recurrent melanomas, who were treated between 1980 and 1988 by the same four teams [35].

Melphalan ILP alone produced the predicted 52% CR compared with the 91% with the combined rTNFα, rINFγ and melphalan treatment (Table 1). When subgroups were analysed, the difference remained highly significant. When the cases presenting only with in-transit-metastases without lymph-node involvement (stage III A) were considered, the complete remission rate was 62% with mephalan alone, compared with 100% for the combination treatment. However, when the patients with both in-transit- and lymph-node-metastases were analysed (stage III AB), the difference remained highly significant, that is 41% compared with 87%. The difference between the treatments remained highly significant ($P < 0.001$) when the bivariate logistic model was used for analysis after adjusting for sex, age, stage, site, number of lesions and time since primary [36].

In systemic chemotherapy of cancer, when combination therapy is used to increase the response rate, it can be found that the response duration diminishes. To address that question, the durations of CRs for melphalan ILP and for the combined rTNFα, rINFγ and melphalan treatments were compared. The median duration has not yet been reached, but the means are 3.9 years for the 103 melphalan alone patients and 3.6 years for the 53 TNFα–IFNγ and melphalan patients, a difference that is not significant (logrank test: $P = 0.35$). These results confirm that the quality of this never before, previously reached high complete remission rate, obtained with the triple combination, is equal to that of the 50% complete remission obtained with melphalan alone ILP. With a median follow-up time of 26 months, there have so far been 12 (23%) regional recurrences, 15 (29%) distant metastases and 9 cases (17%) of both regional and distant recurrence. The overall median survival time has been 28 months [34]. We are currently analysing the 103 melphalan alone patients according to prognostic criteria using a statistical model. This should allow us to accurately compare the disease-free survival of our patients.

These results further confirm that ILP with high-dose rTNFα, rIFNγ and melphalan is a highly efficient therapy of in-transit-melanoma metastases. Soft-tissue sarcomas of high volume are usually found to be invading several anatomical compartments, a clinical situation leading to the indication of amputation or disarticulation. However, survival rates of patients with soft-tissue sarcomas treated with salvage surgery seem to be similar to those obtained after multilating surgery. This observation suggests that any regional treatment that increases limb salvage would not be determined to the survival expectancy of the patient. Attempts to increase the local operability of soft-tissue sarcomas have been made using systemic neo-adjuvant chemotherapy. The literature shows that the overall response rate is around 38% with 6% complete response. Alternatively, ILP with melphalan with or without other drugs gives a 5–10% CR of short duration [37].

Table 1. Complete response in malignant melanoma with in-transit metastases

Stage	ILP with melphalan alone	P	ILP with TNFα + melphalan + IFNγ
AII	54/103 (52%)	$P < 0.001$	48/53 (91%)
IIIA + IIIAB	48/89 (54%)	$P < 0.001$	46/48 (96%)
IIIA	34/55 (62%)	$P < 0.001$	33/33 (100%)
IIIAB	14/34 (41%)	$P < 0.003$	13/15 (87%)

A preliminary study was done on the efficacy of ILP with the triple-drug regimen on irresectable soft-tissue sarcomas of the extremities. Although the results were very encouraging [16] in terms of response, local problems due to tumour necrosis and local recurrence were noticed. This prompted us to design a neo-adjuvant study for unresectable soft-tissue sarcomas with high-dose rTNFα, rIFNγ and melphalan. The objective was to assess limb salvage and to evaluate the clinical, angiographic and histological response [38]. 56 patients received 59 ILP and were evaluable. Complete remission was achieved in 39%, a PR occurred in 52%, minimal response in 1 and there was no change in 1 patient. Amputation had to be carried out for 6 patients and limb salvage was therefore obtained in 89% of the patients [39]. It can be concluded that ILP with rTNFα, rIFNγ and melphalan produces, in soft-tissue sarcoma, a significant improvement over previous treatment modalities, with a high limb salvage rate.

Four distinct other groups have reported their preliminary results using either the triple combination or a double combination with the omission of rIFNγ (Table 2). The overall response rate has always been 100% with the exception of that of Vaglini and associates [40], who reported 69% CRs. Due to small series of patients, the results for melanomas, sarcomas and even carcinomas had been pooled by other investigators. Thomas' group [41] treated an equal number of melanomas and sarcomas with a double combination treatment. Although they reported CRs in all patients, the regional toxicity was so high that it required above-knee amputation in 3 patients because of the large soft-tissue deficit, which failed to heal after tumour necrosis.

Thom and colleagues [42] at the NCI, undertook a randomised phase III trial where they compared the triple combination with melphalan alone for melanoma and sarcoma. Out of the 9 melanoma and sarcoma cases, only 44% showed a CR, and the response of the rest was partial. More time will be needed to observe enough evaluable patients and to make comparisons with the melphalan control. Lev and associates [43] obtained complete remission in 3 out of 4 patients with extensive or recurrent soft-tissue sarcomas.

These results are comparable with our series for the overall response. However, the CR rates vary considerably. Moreover, comparison is made difficult by the fact that there were two different sources of rTNFα and that some groups withdrew rIFNγ from the therapeutic regimen. The small series from other groups will certainly expand, allowing a valid comparison in the future.

UNRESOLVED QUESTIONS: THE OPTIONAL DOSAGE OF rTNFα AND THE ROLE OF rIFNγ

Despite the fact that ILP allows the delivery of ten times the MTD of rTNFα, and that the efficacy seems to be maximal in melanoma, it has not been established whether the rTNFα dosage of our protocol is optimal, in terms of toxicity- and cost-benefit ratios. As stated above, we chose the 3–4 mg rTNFα which approaches the optimal dose found in animals. However,

Table 2. ILP with rTNFα, rIFNγ and melphalan or rTNFα and melphalan in melanoma and sarcoma

First author [ref.]	Year	Evaluable patients	Histology			rTNFα* (IFNγ)	Tumour response†			
			Melanoma	Sarcoma	Carcinoma		CR (%)	PR (%)	SD	ORR
Liénard [16]	1992	23	19	4	–	BI (+)	21 (91)	2	0	100%
Hill [41]	1993	9	4	4	1	K (−)	9 (100)	0	0	100%
Vaglini [44]	1993	13	11	1	1	K (+)	9 (69)	0	4	69%
Thom [42]	1993	9	8	1	–	K (+)	4 (44)	5	0	100%
Lev [43]	1993	5	–	4	–	BI (−)	3 (75)	1	0	100%
Liénard [34]	1994	53	53	–	–	BI (+)	48 (91)	5 (9)	0	100%
Eggermont [39]	1994	32	–	32	–	BI (+)	16 (50)	16 (50)	0	100%

*BI: Boehringer Ingelheim; †CR: complete response; PR: partial response; K: Knoll; SD: stable disease; ORR: overall response rate.

two other groups have recently tried lower doses. Hill and colleagues [41] used a descalation schedule with 500, 250 and 125 mg rTNFα without IFNγ for only 30 min preceded by an injection of melphalan at doses similar to ours. In their small series of 9 patients, they observed 9/9 CR. Although the authors claimed that they had used a low dose of rTNFα, the levels measured in the perfusate were at least 25% of those that we found in our patients [41]. However, they reported a highly regional toxicity that required amputation in 3 patients. This unusual toxicity might have been due to the large amounts of corticosteroids administered during the perioperative period. Vaglini and associates [44] also used a descalating dosage of 1.5 mg, 1 mg and 500 μg with no IFN-γ in 10 patients that they compared to 12 other patients who had received the triple combination with high dose rTNFα. The overall response rate after low dose double combination was 100%, with 65% CR. Surprisingly, there were 55% CR and no PR after the triple high dose combination. Regional toxicity was minimal, but the general toxicity was extraordinarily high, all patients experiencing a septic shock like syndrome. Patients treated with lower dose experienced only transient side-effects.

From these two independent but small experiences, it is not yet possible to conclude that lowering the dosage of rTNFα does not reduce response rate. Indeed, melphalan alone provides 50% CR and this cannot be ruled out in these two series. It is worthwhile emphasising that the systemic 10-fold lower dose of rTNFα was inefficient and still toxic in the reported phase I and II trials.

Because responses in sarcomas were lower that in melanoma and that the MTD of rTNFα in ILP has not been determined, Fraker and associates [45] decided to escalate the dose, in a triple combination ILP regimen. This study has already indicated that the regional MTD for TNFα is only 6 mg (Fraker, personal communication, 1994).

The rationale for the triple regimen was based on experimental data, including human tumour xenografts (see above). However, neither the effect of IFNγ on TNFα receptors nor its antitumour effect has been proven in humans. In contrast, IFNγ was found to be synergistic for toxicity with TNFα [46, 47]. Moreover, since IFNγ up-regulates TNFα receptors (see above), it is possible that it might also increase soluble receptors, resulting in the inhibition of TNFα efficacy. As the triple-drug regimen contains two experimental drugs, it does not allow us to reach definitive conclusions about the impact of TNFα alone, in combination with chemotherapy. Therefore, we designed a randomised phase II trial, to establish whether withdrawal of IFNγ diminishes the complete remission rate and response duration observed with the triple-drug regimen. This trial was started in Spring 1992 and will close in 1994. Preliminary results from 50 patients suggest a reduction in CR rate when IFNγ is omitted. In addition, we can already rule out toxicity problems, since no difference between the two arms has yet been seen. Patients receiving rIFNγ did not express more circulating TNFα receptors. There has been no sign of tumour enhancement with IFNγ. An important issue will not only be the response rate but also the response duration. This is pertinent to both the role of IFNγ and the dosage of rTNFα.

ILP WITH HIGH DOSE TNFα AS A MODEL FOR BIOCHEMOTHERAPY OF CANCER

Experimental models have indicated that only tumours with organised micro-vascularisation can be intensively necrotised by rTNFα, as is the case for tumours implanted in subcutaneous tissues, as observed by Old [5]. This condition is fulfilled in melanoma-in-transit metastases which are located either in the subcutaneous tissue or in the dermis, and invade the epidermis. The 90% CR rate obtained in this condition is higher than the 39% seen in soft-tissue sarcomas, which are more deeply located. However, we observed dramatic necrosis of soft-tissue sarcomas that were invading subcutaneous or cutaneous tissues [16, 39].

Soft-tissue sarcomas are appropriate tumours for studying tumour vascularisation. Angiographies performed before rTNFα ILP and 1 week to 10 days later have shown, as was predicted from animal models, an extensive and fast destruction of the hypervascularisation associated with the tumour, leaving intact the normal small vessels in the limb including the small vessels surrounding the tumours [16, 39]. Using histology and immunohistochemistry, we were able to demonstrate that the first target of rTNFα is the tumour endothelial cells [48]. rIFN-γ is able to up-regulate adhesion molecules, but they are further increased a few hours after rTNFα, especially ELAM-1 (Endothelial Leucocytes Adhesion Molecule-1 or E-selectin), and VCAM-1 (Vascular Cell Adhesion Molecule-1). Moreover, signs of endothelial cell activation appear only in the tumour endothelial cells. They become swollen and are eventually lysed at a time when the tumour cells appear histologically normal, as do normal endothelial cells. Tumour endothelial cell destruction is preceded by polymorphonuclear cell sequestration and activation within the tumour vessels, and this is followed by an intense infiltration of the tumour [48]. At the same time, platelet aggregation on the tumour endothelium and within the tumour tissue is associated with a perivascular leaking of Von Willebrand

factor with no significant fibrin nor tissue factor staining [49]. This indicates that platelet aggregation is not due to TNF-induced procoagulant activity, but rather the result of induced endothelium damage.

The angiographic, histological and immunohistological observations allow us to conclude that the double or triple combination protocols work through a dual-targeting system (Figure 2). The first target is represented by the tumour vessels. rTNFα, with or without rIFNγ, activates and lyses the tumour endothelial cells. The second target is the tumour cells themselves, which are, at a later stage, subjected to melphalan.

The fact that our patients had a long-lasting CR prompted us to study some immunological parameters. Preliminary results on the systemic lymphocyte phenotyping indicate that 8 of 10 patients showed a sustained increase of HLADR-positive T-lymphocytes, of CD38, CD45RO and ICAM-1, which is a sign of T-lymphocyte activation [50]. It remains to be seen whether this T-lymphocyte activation corresponds to the induction of specific cytotoxic T-lymphocytes. These findings strongly suggest that future anticancer strategies could emerge from this biochemotherapy model.

WILL IT BE POSSIBLE TO RE-ENVISAGE SYSTEMIC TNFα ADMINISTRATION?

The experience with rTNFα ILP has been very rewarding not only because of its performance as a highly effective therapy for in-transit melanoma metastases, irresectable sarcomas and carcinomas, but also as a model for biochemotherapy. Indeed, the concept of associating cytokines with chemotherapy has been attempted in other settings, including systemic treatment, using, for example, interleukin-2 and chemotherapy, or rIFN-γ and chemotherapy (reviewed in ref. [51]).

Since we have demonstrated that, by means of appropriate measures, our patients tolerated nanogram levels of TNFα, it is hoped that there is a future for the systemic application of rTNFα. Circulating soluble receptors [32] may be extremely useful for buffering TNFα in the systemic blood. In fact, a construct made of Fc fragments of human immunoglobulins with two TNF receptors was shown to provide a 20-fold protection of animals receiving rTNFα, compared with TNF antibodies [52, 53]. What is unknown is the potential neutralisation of the antitumour effect of rTNFα, when such constructs are used, but it will be worthwhile designing pilot studies, based on the protection achieved in patients with septicaemia. Another approach, which has been successful in an experimental model, is the use of mutant TNFα where one or two amino acids were changed in the area of receptor binding, rendering the mutant unable to bind p75 and less toxic. This approach is based on the observation made by Fiers' group [54] that human TNFα is much less toxic in mice than is mouse TNFα, and that human TNFα does not bind to the mouse p75 receptors. This mutant was still able to destroy human tumour xenografts [55], but unfortunately showed toxicity in baboons [33]. However, the lack of binding p75 soluble receptors may increase the mutant TNFα half life in the plasma, resulting in more toxicity.

Our experience has been that the systemic side-effects of TNFα can only be counteracted by appropriate intensive care management. However, new drugs, such as lipo- [56] and cyclo-oxygenase inhibitors, NO synthetase inhibitors, or platelet aggregation inhibitors will be tried, but it will be important to verify that these inhibitors do not interfere with the antitumour effects of TNFα. Further research is needed to understand and predict the individual susceptibility to TNFα toxicity [57].

CONCLUSION

The results obtained in isolation perfusion with rTNFα are gratifying in terms of response and rates of limb salvage in melanoma and sarcoma. It is of interest to try, in this setting, other regional tumours, such as carcinomas, primary and metastatic bone tumours. Encouraged by our results, Boehringer Ingelheim has decided to apply for an ILP TNFα combination

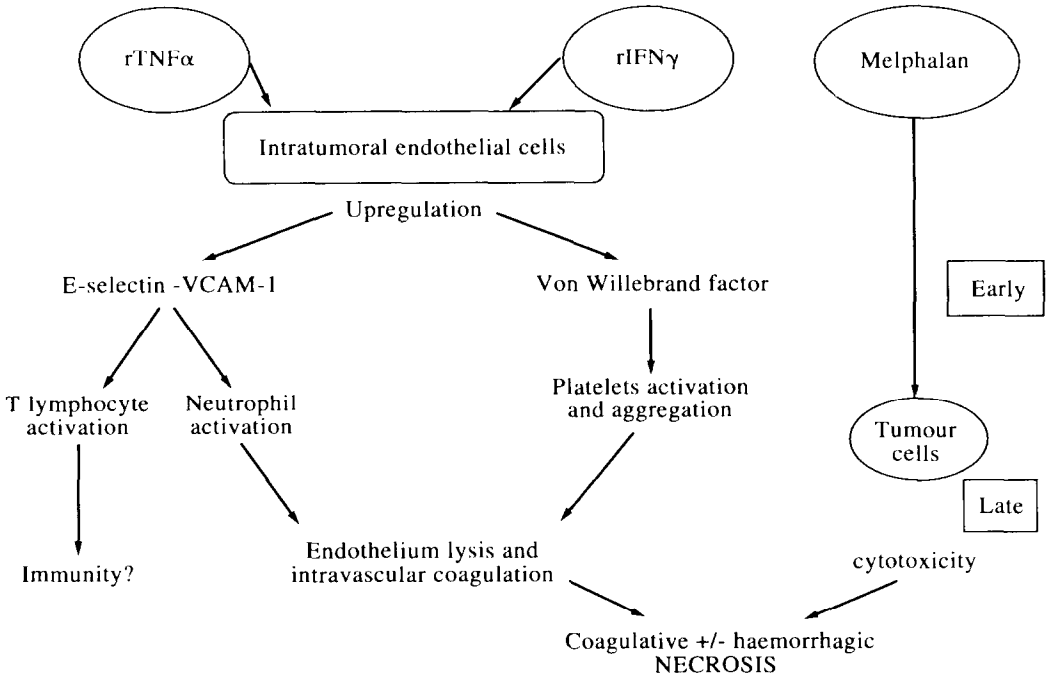

Figure 2. Dual targeting: rTNFα is effective on tumour associated endothelial cells; melphalan has a direct antitumour effect.

registration and its subsidiary, Bender (Vienna) has restarted rTNFα production for clinical trials.

Systemic release of nanogram levels of bioactive rTNFα was well tolerated when appropriate ILP techniques and intensive care were applied. Evidence of systemic inflammatory response syndrome and of lymphocyte activation suggest a beneficial systemic antitumour effect in some patients. A. Eggermont in Rotterdam and D. Fraker in Bethesda have recently launched pilot studies on isolation perfusion of the liver with rTNFα. Results are awaited.

It makes sense to try to administer rTNFα in organs which are not isolated, such as the pleura or in ascitis. In Lausanne, we have designed a protocol for intratumoral injection of rTNFα in bladder carcinoma.

As stated before, previous systemic phase I and II studies lacked appropriate measures for coping with TNFα side-effects. The experience of rTNFα ILP and other regional administrations should be helpful in the design of new protocols of administering rTNFα systemically.

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