Efficiency of recombinant human TNF in human cancer therapy

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Recombinant human tumour necrosis factor (TNF) has a selective effect on angiogenic vessels in tumours. Given that it induces vasoplegia, its clinical use has been limited to administration through isolated limb perfusion (ILP) for regionally advanced melanomas and soft tissue sarcomas of the limbs. When combined with the alkylating agent melphalan, a single ILP produces a very high objective response rate. In melanoma, the complete response (CR) rate is around 80% and the overall objective response rate greater than 90%. In soft tissue sarcomas that are inextirpable, ILP is a neoadjuvant treatment resulting in limb salvage in 80% of the cases. The CR rate averages 20% and the objective response rate is around 80%. The mode of action of TNF-based ILP involves two distinct and successive effects on the tumour-associated vasculature: first, an increase in endothelium permeability leading to improved chemotherapy penetration within the tumour tissue, and second, a selective killing of angiogenic endothelial cells resulting in tumour vessel destruction. The mechanism whereby these events occur involves rapid (of the order of minutes) perturbation of cell-cell adhesive junctions and inhibition of αvβ3 integrin signalling in tumour-associated vessels, followed by massive death of endothelial cells and tumour vascular collapse 24 hours later. New, promising approaches for the systemic use of TNF in cancer therapy include TNF targeting by means of single chain antibodies or endothelial cell ligands, or combined administration with drugs perturbing integrin-independent signalling and sensitizing angiogenic endothelial cells to TNF-induced death.

Keywords: human, neoplasms, TNF, melphalan, isolated limb perfusion, survival rate

Introduction

Classical anticancer strategies are based on the direct destruction of tumour cells, be it by surgery, radiotherapy or chemotherapy. It has recently become apparent that indirect attacks by means of agents destroying intratumoural vessels can also be efficient. Indeed, cancer growth depends on the formation of a vascular supply, a process also referred to as tumour angiogenesis, which is promoted by angiogenic factors secreted by tumour cells [reviewed in (1)].

Tumour necrosis factor (TNF) is a major player in both innate and specific acquired immunity, being secreted by activated macrophages and activated T lymphocytes. It has pleiotropic properties, among which the ability to cause apoptosis of tumour-associated endothelial cells that can result in the complete destruction of the tumour vasculature. It is precisely this property that Lloyd Old’s laboratory discovered over 24 years ago (2, 3, 4) when they injected tumour-bearing mice with a soluble factor produced by mice infected with bacille Calmette-Guerin (BCG) and acutely challenged with endotoxin. The tumours underwent massive haemorrhagic necrosis, hence the name tumour necrosis factor. The same authors found evidence that the tumour-associated vessels were the prime target of TNF and were selectively destroyed. A soluble factor capable of inducing cachexia, called cachectin, was discovered at about the same time, and subsequently turned out to be the same molecule (5).

TNF is a transmembrane protein, which upon cleavage by the metalloprotease TACE produces a soluble trimer of 157 amino acids (6) (Figure 1). Both isoforms of TNF (membrane and soluble TNF) bind two distinct receptors that are ubiquitous, p55 or TNF-R1, and p75 or TNF-R2 (7). Intracellular signalling involving TNF-R1 has partly been deciphered. Evidence for two opposing pathways were found in endothelial cells: (i) an apoptotic pathway initiated by the clustering of death domain-containing proteins (8, 9), leading to caspase activation (10) and (ii) a proliferation and survival pathway involving the activation of nuclear factor NF–κB (11, 12, 13). Activation of p55/TNFR-1 is essential for the apoptotic pathway (14). Although not studied on endothelial cells, a caspase- and cytochrome C-independent cell death pathway was reported in tumour cells (15, 16). It is characterized by the increased production of reactive oxygen species and involves the persistent phosphorylation of the microtubule regulator, oncoprotein 18.

Figure 1

Tri-dimensional computer modelling of soluble TNF. Left (courtesy of Dr V. Jongeneel): External aspect of the trimer. Right (courtesy of Dr J. Tschopp): Open structure showing the assembly of the trimer. One part is shown in front, with the beta sheets coloured.
The mouse (17) and human TNF genes were cloned and recombinant TNF was produced in *E. coli*. The crystal structure was then solved a few years later (18). The availability of recombinant TNF paved the way for extensive studies in animals which revealed that TNF not only has antitumour properties, but also strong haemodynamic effects. Indeed it was found that TNF is an important mediator of septic shock (19), although later on toll like receptor 4 (TLR4) was found to play a critical role in initiating septic shock (20). Clinical phase I and II studies confirmed that the dose limiting toxicity of TNF is due to vasoplegia, a pathological condition leading to multiorgan failure, the "septic shock like syndrome". Most phase I studies reported a maximum tolerable dose (MTD) of 30-200 µg/m² when injected daily for 5 days; protracted infusion for 24 hours allowed a MTD of 200-545 µg/m² to be reached (21, 22, 23, 24, 25, 26). In phase II studies, doses of 350-400 µg/m² produced rare and minimal tumour responses (27, 28). There were, however, some anecdotic reports of tumour regression after TNF (29). Most tumour models, including human xenografts in nude mice, have shown that TNF alone is not sufficient to effectively suppress tumour growth and that definitive cure in animals could only be obtained by combining TNF either with chemotherapeutic agents or with interferon-gamma (IFN-γ). It was then shown that TNF synergizes with IFN-γ (30, 31, 32, 33), with several chemotherapeutic agents and hyperthermia to induce antitumour responses (34, 35, 36, 37, 38, 39, 40). The mechanisms responsible for these synergisms are still not fully understood, and, in the eighties, only rare clinical studies evaluated these synergisms. In addition, clinical investigators who were performing the phase II studies were correcting vasoplegia only when it appeared during therapy. In other words, no protocol included active measures to prevent side effects, especially the "septic shock like syndrome", also known as "systemic inflammatory response". Furthermore, vasoplegia immediately leads to poor vascular exchanges and poor drug biodistribution (41, 42), including of TNF itself. It is therefore not surprising that, in 1986-87, projects for the further clinical evaluation of TNF were abandoned due to the excessive systemic toxicity and lack of interesting clinical antitumour effects.

Finding a new way to administer TNF: isolated limb perfusion

The efficient antitumour dose of TNF in mice is around 50 mg/kg. If extrapolated to humans, this dose is 10-fold higher than the dose that was found to be toxic in humans with only anecdotic tumour responses [reviewed in (43)]. In 1988 we designed a protocol for the application of TNF by isolated limb perfusion (ILP) (44, 45, 46). ILP is a method originally designed for administering high doses of chemotherapy in limbs affected by locally advanced tumours (Figure 2). This method consists in surgically isolating the vessels irrigating the limb affected by the tumour, to cannulate and connect them to a heart-lung machine to maintain perfusion and oxygenation. The resulting extracorporeal circulation, under tourniquet, receives high dose chemotherapy reaching up to 30-fold the levels obtained by systemic administration. Systemic toxicity will be abolished, depending upon the efficiency of the isolation (47) (Figure 3). ILP with the single chemotherapy agent melphalan at high dose was found to produce a CR rate of around 50% in unexcised, in-transit melanoma metastases (48, 49, 50), but had minimal effect on soft tissue sarcomas of the limbs (51). This treatment modality allows the administration of high drug doses with no or minimal systemic toxicity. It was therefore interesting to investigate the combination of TNF and chemotherapy with the aim to improve upon the results obtained after chemotherapy alone.

Rationale of protocols for administering TNF by ILP

As previously mentioned, preclinical data indicated that TNF cytotoxicity to tumours could be enhanced when combined with other treatment modalities, that is with: (i) alkylating agents, (ii) IFN-γ and (iii) hyperthermia. Based on these data, we set-up a series of feasibility studies to test TNF: First, as a single agent or in combination with IFN-γ, in ILP for melanoma in-transit metastases. In this pilot study of TNF alone or in combination with IFN-γ we observed only minimal or no tumour response (52). Next we designed a triple combination study with high dose TNF, low dose IFN-γ and high dose melphalan (44, 46, 53). This procedure is an intensive biochemotherapy: TNF dosage is 10-fold the MTD, with perfusate concentration reaching
2-7 µg/ml and melphalan peak concentrations of 20-60 µg/ml (45). Continuous radionuclide-based monitoring of the leakage allowed to dose perfusion pressure/volume to avoid leakage, and with it major side effects (54).

From mouse model, it is known that TNF is eliminated by binding to soluble p75 receptors (55), followed by renal secretion. In ILP this elimination does not take place and plateau levels of TNF can be maintained throughout the perfusion period (45). This ‘infinite’ half-life of TNF in the perfusate is in contrast with the shorter half-life of melphalan, which was measured at 10 minutes for the alpha curve and 40 minutes for the beta curve. This means that 10% or more leakage of TNF at any time point during ILP results in severe systemic toxicity because it reaches systemic MTD, whilst this is not the case for melphalan.

There are oncological conditions where tumour spreads extensively and exclusively for a period of time in a limb. This can be the case in melanoma, where in-transit metastases occur in 6 to 10% of the patients, and in soft tissue sarcomas of the limbs that can be inextirpable in 10% of the cases. Indeed, multiple and recurrent in-transit melanoma metastases and inextirpable soft tissue sarcomas are conditions were surgery is either inefficient or it produces severe functional sequelae. Some cases, especially sarcomas, are mere indications for limb amputation.

The results of the first single institution pilot study on melanoma and sarcoma were impressive (46): there was fast and intensive tumour necrosis, similar to the one reported in animal models, with virtually no severe systemic toxicity. The good efficacy and tolerability of TNF administration through ILP in contrast to the systemic application, was attributed to the absence of vasoplegia and to good drug biodistribution. An overall response rate of 100% was obtained, with a CR rate of 89% and a PR rate of 11%.

Results of phase II studies in melanoma patients

In order to confirm the above results, a multicentric phase II study with the triple combination TNF, IFN-γ and melphalan (TIM-ILP) was organised in melanoma patients only. The results of this study confirmed the previously obtained 100% objective response rate, with a 90% CR rate (56). Using the same regimen, Fraker et al. obtained a 92% objective response rate and a 76% CR rate (57). In the same study, the TNF dose was escalated from 4 mg to 6 mg. The CR rate dropped to 36% with more side effects being observed. These results suggest that 4 mg might be an optimal dose for TNF.

In Europe, several single institutions used TM-ILP, that is, TNF and melphalan without IFN-γ, with the classical dose of 3-4 mg TNF. The objective response rates ranged from 80 to 100%, with CR rates around 65 to 70% (58, 59, 60). The role of IFN-γ was then questioned. A randomized phase II study compared the triple combination (TIM-ILP) to the double combination (TM-ILP, i.e. TIM-ILP without IFN-γ). The results of this trial showed a 10% drop in the complete response rate when IFN-γ was omitted, but this drop was not statistically significant. However, no firm conclusion could be drawn because the study design was for a null hypothesis and it was therefore underpowered to establish a difference between the two groups (61). Although no control melphalan arm was designed, a comparison of the two TNF arms with matched cases from a databank confirmed that ILP with melphalan only resulted in a 52% complete response rate (Figure 4).

Phase III studies in melanoma patients

Two randomized studies aiming at comparing TNF-containing ILP (TIM-ILP) to melphalan-only ILP (M-ILP) were undertaken, one in the US and the other in Europe. An uncompleted American College of Surgeons trial compared TIM-ILP to M-ILP (62). Although a trend for a higher CR rate was found in the TIM arm, there was a much higher response in bulky disease (see next section). The European study sponsored by the company Boehringer Ingelheim compared TIM-ILP to M-ILP. A premature closure to patient entry was decided because of insufficient accrual. No difference could be seen in terms of response.

Bulky, in-transit melanoma metastases are highly sensitive to TNF and melphalan ILP

TIM-ILP is the treatment which gives the most dramatic response of in-transit melanoma metastases. Within 1 to 7 days, collapses and softening of skin tumours are apparent. This is similar to the first observations reported by Old et al. in experimental models. This potent effect is especially observed in bulky tumours, less so in small tumours (Figure 5). This is in opposition to the paradigm of cancer chemotherapy: the smaller the tumour, the better the response. It seems that vascularization of large tumours is more elaborate than in small tumours and results from a progressive process triggered by tumour growth. In addition, bulky tumours are often heterogeneous, with areas of necrosis. It can therefore be hypothesized that two phenomena could be responsible for the better response of bulky tumours: (i) the destruction of elaborate vascularization, and (ii) improved drug penetration into poorly vascularized regions of the tumour. Strong support for this hypothesis has come from in vivo animal studies (see the section ‘Evidence for two distinct effects of TNF on tumour associated vessels’). The US study demonstrated a high response rate in bulky tumours, with a 67% CR rate as compared to 17% in tumours which were not bulky (62).
Figure 5

Efficacy of TIM-ILP on bulky melanoma metastases. The patient, a 70-year-old woman with bulky in-transit melanoma metastases on a lower limb, had been considered for hip exarticulation in other hospitals. She received one TIM-ILP through the iliac vessels. From left to right: presentation on the day of TIM-ILP; on day 14, extensive necrosis, and, on day 44, complete response. The patient enjoyed CR for 9 months and thereafter developed extensive visceral metastases and died from disease.

Figure 6

Efficacy of TIM-ILP on proximal melanoma limb metastases. The patient, an 83-year-old woman, presented with numerous in-transit melanoma metastases extending to the proximal aspect of the left thigh. Upper left: before TIM-ILP. Lower left: day 1 after TIM-ILP; a general tumour collapse can be seen. Upper right: complete response was obtained on day 21. Lower right: 10 months after TIM-ILP. Black spots were tattoos from previously regressed metastases. Work-up demonstrated lung and liver metastases and the patient died from her disease 12 months after TIM-ILP.

ILP with TNF is effective on proximal metastases of the limbs

Most protocols reported on ILP with melphalan alone for melanoma excluded primary melanomas and melanoma metastases situated above the mid-level of the affected limb (63), because of the repeatedly observed lack of efficacy due to the lower distribution of chemotherapy in the proximal aspect of the limbs. This is reflected by reduced skin toxicity to melphalan, i.e. erythema followed by pigmentation, after perfusion in this area.

TNF-ILP allows a better distribution, including of the proximal aspects of the limbs. As exemplified in one representative case (Figure 6), combining TNF with melphalan can produce CRs in the proximal aspect of the limbs, up to the inguinal region. Indeed, this property might also be ascribed to an improved drug penetration due to TNF (see below).

Duration of the response to TNF ILP and survival in melanoma patients

Typically, ILP is a one-shot procedure that cannot easily be repeated because of difficult vascular access after previous ILP. In addition, tissue fibrosis can impair limb function and is a typical side effect which is mainly due to melphalan, and multiple ILPs can worsen it further (64). Repeated ILPs, however, were attempted and positive results were observed in certain patients (65). Moreover, patients with in-transit metastases are at high risk to develop distant metastases, a condition that is in principle a contra-indication for loco-regional treatment. Currently, only 10% of patients are reperfused. Time to recurrence was found to range from half a year to slightly more than 1 year.

Information on survival after ILP is scarce. Despite the very high response rate, this treatment is a regional treatment, and as such, was not expected to have an impact on survival. Indeed, survival curves from patients treated with TNF-containing ILP were identical to those treated with melphalan alone (M-ILP); in this multicentric study, the median survival was 2.5 years (61). The Lausanne series of 75 patients has a median survival of 5 years, in spite of a median time to recurrence of 6 months. In the Rotterdam series (60), 35% of patients survived 5 years and local progression occurred in 55% of patients after a median time of 16 months. These data show that survival was rather long, in spite of local progression. This suggests that in-transit melanoma metastases correspond indeed to a different disease where the tumour restricts its extension in an area of the body for several years before producing distant metastases. This is an argument that reinforces the indication of ILP for in-transit melanoma metastases.

ILP with TNF for inextirpable soft tissue sarcomas

Soft tissue sarcomas (STS) of great volume or which are recurrent are usually found to be invading several anatomical compartments, a clinical situation leading to the indication of amputation or disarticulation in 5 to 10% of all limb STS. However, the survival rate of patients with STS treated with limb salvage surgery seems to be similar to that obtained after mutilating surgery (66, 67). This observation suggested that any regional treatment that increases limb salvage would not be detrimental to the life expectancy of the patients. Attempts to increase the local operability of soft tissue sarcomas have involved using systemic neoadjuvant chemotherapy. The literature showed that the overall response rate is in the range of 38 to 47%, with few complete responses (68, 69). ILP with melphalan, with or without other drugs, gives a 5 to 10% complete response of short duration [reviewed in (51)]. The first unicentric study which included several soft tissue sarcomas (46) indicated that TNF-containing ILP could be used in a neoadjuvant setting, rendering inextirpable tumours removable without major mutilation.

The main difference with the melanoma indication is that the majority of STSs eligible for TNF-ILP were not metastatic.
Therefore, ILP was to be considered as a neoadjuvant treatment, prior to resection of tumour remnants. The surgical procedure aims at removing the tumour without mutilation. In other words, when classical wide surgery could create serious limb function impairment, narrow margins are taken, considering the tumour necrosis expected from magnetic resonance imaging (MRI) and clinical evaluation. An interesting feature is the development of an intense peritumoral inflammation, followed by the build-up of a thick capsule that facilitates the surgical procedure (Figure 7).

Figure 7

Thick capsule formation after TIM-ILP. Shown are two cases of tumour remnants (surgical samples). (A) Malignant fibrohistiocytoma of the left calf (refer to the legends of Figures 8 and 9) removed 3 months after TIM-ILP. (B) Extrasosseous osteosarcoma of the right thigh (refer to the legend of Figure 12) removed two months after TM-ILP. In both cases, there was slight tumour shrinkage, but more than 60% histological necrosis. Tumour remnants were surrounded by a thick capsule (arrow). Histological appearance of a tumour capsule after TIM-ILP: (C) 30 months after TM-ILP. In both cases, there was slight tumour shrinkage, but more than 60% histological necrosis. Tumour remnants were surrounded by a thick capsule and oedema.

A series of prospective, multicentric phase II studies were launched in Europe. The triple combination, TNF, IFN-γ and melphalan (TIM-ILP), gave the highest complete response rate ever obtained in STS treatment, 36% (70). ILP without IFN-γ (i.e. TM-ILP) yielded complete response rates of 28 to 70% (71, 72, 73, 74). For all studies combined, the objective response rate (CR + PR) was between 81 and 91%, resulting in limb salvage in 81 to 90% of the cases.

Enlarging the number of European centres and increasing the number of cases to 260 resulted in the demonstration that treatment with TNF plus melphalan in soft tissue sarcoma is a limb salvage neoadjuvant therapy, provided the tumour remnants are removed several months later (70, 71, 75).

The European TNF/ILP assessment group evaluated 260 patients with irresectable STSs enrolled over 10 years in 4 studies involving treatment with TIM-ILP and TM-ILP (76). In order to determine whether TNF and melphalan (TM) or TNF, IFN-γ and melphalan (TIM) ILP offers durable limb salvage by effective local control of the tumour, with or without subsequent surgery, patients were reviewed by an independent review committee and compared with conventionally-treated patients (often by amputation) of a population-based Scandinavian STS database. The proportion of patients who had a durable limb salvage ranged from 74 to 87% in the 4 studies, and the objective response rate (CR + PR) was 56.5 to 82.6%. The independent review committee considered that 80% of all enrolled patients met the criteria for irresectability. As proved by matched comparison, the ILP-treated patients survived as long as conventionally-treated patients from the Scandinavian STS database (51, 71).

In Lausanne, as a single centre performing TM- or TIM-ILP, the experience was reviewed in order to determine the final outcome with respect to disease-free survival, time to distant metastases, and survival (77). Twenty-four ILPs were performed in 22 patients: 18% of them experienced a complete response and 64% a partial response. Seventy-seven per cent underwent limb sparing resection after a median time of 3.4 months: 10 resections were intracompartmental and 7 extracompartmental (Figures 8-11). Adjuvant chemotherapy was given to 8 patients and radiotherapy to 6. Post-resection radiotherapy was optional, although it was reported that it can be very efficient in this situation (78). One patient had to be amputated after a second ILP. After a median follow-up of 28 months, 10 recurrences were recorded: 7 were both local and systemic, whereas 3 were local only. The overall survival has been 18.7 months, which is similar to the outcome after primary amputation in similar cases.

Melphalan is not a drug of choice for systemic chemotherapy of sarcoma, whilst doxorubicin as a single agent can produce a response in up to 40% of patients. For this reason an Italian group has tried doxorubicin alone with some promising results (79, 80). This was followed by studies with the combination of doxorubicin with TNF, which gave rather similar response rates to those obtained with the combination of melphalan and TNF, but apparently with slightly lower limb salvage rates (75%) (79, 81). ILP with recombinant human TNFα-1A (tasonermin) and melphalan was registered in Europe (1999) and in Switzerland (2001) for both sarcoma and melanoma.

Figure 8

Neo-adjuvant TIM-ILP for soft tissue sarcoma (I). The woman, 42, had a grade 3, stage III, malignant fibrohistiocytoma of the left calf which was in contact with neurovascular elements. She had progressed on two courses of systemic ifosfamide/doxorubicine. Left: MRI before TIM-ILP. Right: MRI 3 months after TIM-ILP. Massive necrosis except for a 5% area (arrow) that was still containing live sarcoma cells in the surgical specimen.
Neo-adjuvant TIM-ILP for soft tissue sarcoma (II). Left: Clinical aspect of the calf before surgical resection of tumour remnants, 3 months after ILP. Right: complete recovery of leg mobility 10 months after surgical resection. Two years later, the patient developed groin metastases. She underwent radical groin dissection and postoperative radiotherapy. The patient is alive and well 6.5 years after ILP.

Figure 10

Neo-adjuvant TIM-ILP for soft tissue sarcoma (III). The woman, 62 years old, had a gigantic, stage IV malignant fibrohistiocytoma of the left thigh that had progressed on 4 courses of systemic ifosfamide/doxorubicine. The patient had been offered hindquarter exarticulation in another town. Left: clinical aspect before TIM-ILP. The scar of the open biopsy is visible. Right: clinical aspect on day 30 after perfusion. The tumour has shrunk and there is necrosis of the biopsy scar (refer to the text).

Is a TNF dose close to the systemic MTD efficient in ILP?
Pharmacokinetics of TNF in ILP perfusate shows a plateau that suggests a saturation of the perfused tissues (45). The lowest dose of TNF active in ILP for soft tissue sarcoma is not known. A relevant question is whether doses lower than the registered ones might be as efficient and less toxic. Lowering TNF dosage could improve safety and reduce the cost. There are indeed some small series in the literature which suggest that lower doses of TNF can be efficient, but no comparative study was available (82, 83).

A multicentric randomized phase II study was initiated to compare four dosages of TNF in terms of complete response as determined by MRI (84). The secondary objectives were to evaluate toxicity, histological responses and the percentage of amputations avoided. Overall response rates were 68%, 56%, 72%, 64% for the 0.5 mg, 1 mg, 2 mg and 3/4 mg groups, respectively. No statistically significant differences were observed as the study was designed to detect a difference of 10% or more. Systemic toxicity was significantly related to high doses of TNF. The 2-year overall and disease-free survival rates are 82% (73%-89%, 95% confidence interval) and 49% (39%-59%, 95% confidence interval) respectively. Although the overall results are similar to the ones obtained with high dose TNF, it cannot be definitively concluded that lower doses are equal to high doses because this study was not powered to prove it. Today, many ILP centres have adopted the 1 mg dose of TNF, with the conviction that it is as effective as the effective, classical 3-4 mg dose. Indeed, a phase III study would have no chance to be launched in sarcoma and melanoma because the number of patients required to confirm or reject this hypothesis with enough statistical power would be too large for such a non-inferiority trial to be conducted in patients with these rare conditions (85).

ILP for other tumour types
Squamous cell carcinomas and Merkel carcinomas, when locally advanced, are not amenable to surgery. Isolation perfusion with TNF and melphalan resulted in 60% of patients showing a complete response with histopathological confirmation and 27% showing a partial response (86). For unresectable bone sarcoma of the lower extremity, limb-sparing surgery can also be envisaged according to a preliminary report.
An example of limb salvage in case of recurrent extraosseous osteosarcoma is shown (Figure 12).

Small series have addressed the question of the efficacy of cisplatin administered by ILP for STS and osteosarcoma with documented tumour responses (88, 89). It could be envisaged that cisplatin might be combined with TNF in osteosarcoma in future studies.

Figure 12

Recurrent osteosarcoma treated by neo-adjuvant TM-ILP. The patient is a 75-year-old male with extraosseous osteosarcoma of the external aspect of the left thigh that had been widely resected 1 year before, with marginal osteotomy and osteosynthesis. Recurrent sarcoma developed in the internal aspect of the thigh. TM-ILP produced tumour shrinkage and marginal resection showed 65% histological necrosis.

Toxicity of ILP with TNF and chemotherapy

Continuous monitoring of leakage from perfusate to systemic circulation is of paramount importance. It is well established that it permits to take action at any moment, mainly by reducing the pump flow rate which directly correlates to perfusion pressure, and hence to an imbalance between the two compartments (90, 91, 92, 93). Indeed, a 10% leakage means that the systemic MTD is reached. In spite of this monitoring, to suppress leakage in some patients is sometimes an unmet goal for peculiar anatomical/vascular reasons. Table 1 reports severe systemic toxicity after TNF-based ILP published in the literature. It is worth mentioning that only one randomised phase II study (61) allows TIM-ILP to be compared with TM-ILP. Toxicity was surprisingly lower when IFN-γ was added. Indeed, toxicity is more due to leakage from perfusate to systemic circulation, than to the addition of IFN-γ. Although not reported, one can speculate that there had been more leakage in the patient population treated by TM-ILP. The same factor can explain the important variations in the percentage of toxicity reported by different authors. Table 2 reports all toxicity grades observed in the Lausanne series (unpublished data), where most patients have been treated with TIM-ILP. It seems that most side effects were mainly due to TNF. They were all reversible with no sequelae. An important finding was that peak levels of bioactive TNF measured in the plasma varied considerably, with a minimum in the picogram range and a maximum of the order of hundreds of nanograms. This very high TNF concentration was not life-threatening in our patients. A tentative explanation comes from studies on septic shock where not only TNF, but also TLR-4 triggering by endotoxin, is necessary to elicit a septic shock (18). Patients treated with TNF-based ILP had no infection and therefore were not exposed to endotoxin. Interestingly, the haemodynamic changes that occurred, especially the drop of peripheral vascular resistance, rarely correlated with the level of TNF in the systemic circulation (94) (Figure 13). This observation clearly shows that haemodynamic sensitivity to TNF varies tremendously among individual patients. As this is an unpredictable variable, careful monitoring of perfused patients is mandatory during and after ILP.

Isolated liver perfusion with TNF

Ocular melanoma has a predilection to metastasize selectively in the liver, which can be the sole organ affected for up to several years. Systemic treatments are poorly effective and a majority of patients die from melanoma progression in the liver. This is also the case for a fraction of patients with colorectal cancer. Therefore, intrahepatic delivery of high dose anti-cancer agents might be beneficial. To this aim, Alexander et al. performed phase I and II studies on isolated hepatic perfusion (IHP) with TNF. A total of 147 patients underwent IHP on various approved institutional protocols using TNF alone, melphalan alone, or a combination of melphalan and TNF. All patients had histologically proven, unresectable metastatic or primary cancers confined to the liver. In a phase II study with diverse histologies, 75% objective responses were obtained after a single IHP with 1 mg TNF and 1.5 mg/m² melphalan (95).

Table 1

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<th>Reference</th>
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*Abbreviations: TIM, TNF/IFN-γ/melphalan; TM, TNF/melphalan.

*Randomised phase II study in melanoma where a comparison of TIM with TM was made.
Table 2
Postoperative systemic side effects recorded in 104 patients in Lausanne after TNF-based ILP

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<td>45 (43)</td>
<td>59 (56.7)</td>
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*Intravascular coagulation disease.
Values in brackets represent the percentage (%) of patients.

Evidence for two distinct effects of TNF on tumour-associated vessels

TNF has two distinct effects occurring at different time points: (i) an early effect (within 30 minutes) - the improved penetration of anti-cancer agents, and (ii) a late effect (24 hours to a few days later) - the selective destruction of the tumour-associated angiogenic vessels.

Improved penetration of anticancer agents

In vitro data on human umbilical vein endothelial cells (HUVECs) and microvascular endothelial cells can explain why penetration of drugs into tumours is enhanced by TNF and why tumour angiogenic vessels are selectively destroyed by TNF-based ILP. TNF alone, or in combination with IFN-γ at a concentration of 10 ng/ml, strongly alters the endothelial cell monolayer integrity (99). From a confluent cobblestone monolayer, treated cultures show spindle endothelial cell morphology with interrupted confluence. An increased permeability of endothelial cells and pericytes was seen in vitro upon treatment with TNF (100, 101). This effect seems to be mainly mediated by TNFR-1 (102). The molecular mechanisms leading to enhanced permeability involve remodelling of the cytoskeleton (103, 104), as well as the redistribution of the cell-cell adhesion molecules PECAM-1 (105) and VE-cadherin (106).

In vivo, these gaps in the endothelial cell monolayer translate into increased permeability of perfused microvessels. In a xenogenic colon carcinoma, intratumoural or systemic application of TNF produced an increased vascular permeability which resulted in higher uptake of monoclonal antibodies (107), whilst there was no modification of blood flow. In rat limb sarcoma models, ILP with TNF produced a selective increased penetration of melphalan (108) and doxorubicin (109) in the tumours, whilst there was no modification of uptake in normal muscle and skin.

Selective destruction of angiogenic vessels

The initial work of Old et al. (2, 4) indicated that the rapid necrosis of mouse tumours was the result of the selective destruction of tumour-associated vessels. Moreover, this same group showed that this effect was important when the tumours were grafted in well-vascularized skin and negligible when they were grafted in the poorly vascularized peritoneum cavity. This phenomenon seems to be due to a direct effect of TNF on the endothelium, as it was demonstrated that TNF induces apoptosis in normal endothelial cells (110).

Indeed, in patients treated by TNF-ILP, angiograms performed on sarcoma-bearing limbs reproduced the early disappearance of tumour-associated angiogenic vessels, whilst bystander normal tissue vessels were spared (46, 111) (Figures 14 and 15). New methods can provide an early evaluation of the effect of TNF-ILP on tumour vasculature, namely MRI and Doppler ultrasonography. A prospective comparison study was undertaken on soft tissue sarcomas. According to MRI and histological analysis, 51% of patients were good responders, with tumour necrosis exceeding 90%, while 49% were poor responders. In contrast, as of 1 day post-ILP the accuracy of DUPC (Doppler ultrasonography with perfusion software and contrast agent injection) in predicting tumour response was...
Selective tumour-associated vessel destruction by TIM-ILP (I). The patient, a 50 year old male, had a large recurrent high-grade neurofibrosarcoma of the anterior aspect of the leg. Angiograms performed before and 7 days after TIM-ILP reveal that tumour-associated hypervascularisation disappeared whereas the peritumoural and normal vessels were spared.

Selective tumour-associated vessel destruction by TIM-ILP (II). Histology of surgical specimen of sarcoma remnants 3 months after TMI-ILP. Necrotic tumour tissues contain some damaged vessels whilst normal vessels surrounding thick tumour capsules appear intact.

82% [72% in good responders and 92% (22/24) in poor responders] and increased to 91% at 7 days post-ILP, 95% at 15 days post-ILP and 96% at 30 days post-ILP. At 15 days post-ILP, DUPC was predictive of a good response in 100% of the cases. DUPC is a simple technique, allowing early prediction of tumour response after ILP (112). A direct way of assessing tumour viability is positron emission tomography (PET) scan. It is especially useful for the follow-up of patients with multiple in-transit melanoma metastases (Figure 16).

The histological study of tumour biopsies taken from TNF perfused patients demonstrated the swelling of intratumoural endothelial cells and early destruction of the endothelium (113). A change in the distribution of von Willenbrand (vWF) staining occurred, from a discrete endothelial pattern in the untreated lesions to a diffuse, perivascular and intratumoural pattern in the treated lesions. Within 24 hours, this was accompanied by intravascular thrombocyte aggregation and erythrocytosis, in the absence of tissue factor and fibrin deposits. We concluded that the thrombocyte aggregation observed is not caused by local procoagulant activity, but rather results from therapy-associated vascular damage (114).

We have demonstrated that TNF-induced endothelial cell death is associated with the selective inhibition of integrin αvβ3, an adhesion receptor highly expressed on angiogenesis endothelial cells, but not, or to a lower extent, on quiescent endothelial cells (115, 116). Indeed, integrins promote physical cell adhesion to the extracellular matrix and deliver survival signals to the cell (117). Subsequently we have observed that activation of protein kinase B (PKB, or Akt), together with NF-κB, is essential for endothelial cell survival in response to TNF. Importantly, PKB activation required integrin ligation to ECM, while NF-κB does not (118). Based on these data, we initiated the search for drugs that may interfere with PKB signalling and sensitize endothelial cells to TNF-induced death. We then discovered that zoledronate, a nitrogen-containing bisphosphonate, suppressed endothelial cell migration, disrupted established focal adhesions and actin stress fibers, as well as endothelial cell adhesion mediated by integrin αvβ3 without modifying cell surface integrin expression level or affinity. These effects were associated with the dephosphorylation of focal adhesion kinase (FAK), an upstream activator of PKB. Consistent with these data, zoledronate strongly enhanced TNF-induced cell death. Strikingly, addition of zoledronate inhibited sustained phosphorylation of PKB in the presence of TNF. These effects were specific to zoledronate, as clodronate, a non-nitrogen containing bisphosphonate, had no effect on HUVEC adhesion, migration and survival, nor did it enhance TNF cytotoxicity (119). Current experiments in our laboratory are aimed at testing a possible synergy between TNF and zoledronate in vivo.

Taken together, the results, especially those obtained in bulky tumours of different histologies (i.e. melanoma, soft tissue sarcoma, skin carcinomas), suggest that the synergism of the combination of TNF and melphalan is not limited to a direct interaction, but rather to a double and distinct targeting: endothelial cells and tumour cells. TNF rapidly enhances tumour vessel permeability, resulting in increased accumulation of melphalan in the tumour, while later on it causes vascular...
Inflammatory response after TNF-based ILP

After ILP, several soluble factors involved in inflammation are secreted in the plasma and seem to result from the systemic effect of TNF. There is a peak of IL-6 (120, 121) and of IL-8 (120, 122), an increase of C-reactive protein, and the release of tenascin-C (123) and of phospholipase A2 (124). Release in the plasma of soluble TNFR-1 and TNFR-2 occurs within 30 minutes and it does not correlate with the amount of TNF leakage (117). We found that this amount of receptors was only able to neutralize picograms of TNF. At the tumour tissue level, biopsies taken early after TIM-ILP and up to 60 days later revealed that an intense inflammatory response takes place (113). There is an upregulation of the adhesion molecules E-selectin and V-CAM on endothelial cells, with perivascular recruitment of polymorphonuclear leukocytes (PMNs). This is followed by PMN colonization of tumours a few days later and infiltration by lymphocytes and macrophages after two weeks (113). These results suggested that tumour destruction with tumour antigen shedding in the context of an inflammatory reaction, with recruitment of antigen presenting cells and lymphocytes, could have elicited some sort of immune response. Previous unpublished data (125) on circulating CD8+ lymphocytes in HLA-A2 melanoma patients suggested that CD8+ T cell activation had occurred after TIM-ILP. An ongoing study at the Ludwig Institute for Cancer Research, in Lausanne, aims at evaluating the CD8+ T cell response in melanoma patients after TNF-based ILP, using HLA-A2 multimers against Melan-A/Mart-1 peptides.

Events occurring during and after TNF-based ILP. Events in the tumour compartment (bottom) are listed in parallel to those taking place in peripheral tissues (top) over time. During the 90 minutes ILP, TNF activates angiogenic endothelial cells in the tumour tissue, resulting in increased permeability and improved melphalan penetration within the tumour tissue. After ILP, suppression of αvβ3 integrin occurs and endothelial cells undergo progressive apoptosis that parallels the apoptosis of tumour cells induced by melphalan. After ILP, a systemic inflammatory response develops in the peripheral tissues, with the secretion of inflammatory cytokines, soluble TNF receptors and an extracellular matrix degradation product that appear in the plasma.

Is there a prospect for the systemic application of TNF?

Several attempts were made in order to improve the local delivery of TNF in tumours and to decrease the systemic exposure to the cytokine. Repeated intravenous injection of stealth liposomes with TNF and doxorubicin in rats bearing soft tissue sarcoma resulted in objective tumour responses without significant systemic side effects (126). However, half-life of stealth liposomes can exceed several days, a condition giving rise to a long-lasting, low TNF concentration. Therefore the risk of tumour metastasis enhancement is to be considered, since a low dose of TNF can be angiogenic (127), although no such effect has been recorded in this setting. To the best of our knowledge, no clinical trial with this combination in liposomes has been performed. For the systemic delivery of TNF, targeting TNF to the tumour is an appealing approach.

Targeting the extra domain B+ (ED-B) isoform of fibronectin

Different isoforms of fibronectin are generated by alternative splicing of pre-mRNA. The isoform containing the ED-B domain (B-FN) is highly expressed in tumours and foetal tissues, and in tissues undergoing physiological remodelling, such as the endometrium, ovary and wound healing tissue, but cannot be detected in normal adult tissues. It was demonstrated that B-FN is especially expressed in intratumoural angiogenic vessels, where the endothelial cells migrate and proliferate in the tumour along an extracellular matrix rich in B-FN. With the aim of targeting TNF in the intratumoural angiogenic endothelial cells, Zardi et al. generated murine (128) and human (129) single chain (scFv) recombinant antibodies specific for the B-FN isoform. The human scFv L19 was fused with monomeric TNF. Interestingly, the molecules reassemble as trimers which can bind to both the ED-B motif of B-FN and the TNF receptors. Using an aggressive mouse teratocarcinoma with intense angiogenesis, they could demonstrate that the murine construct specifically accumulates in the tumour tissue and that a strong synergism with free melphalan can be obtained (130). It resulted in impressive tumour growth retardation, while the same dose of construct or melphalan alone is poorly efficient.

Targeting an isoform of aminopeptidase N

An isoform of aminopeptidase N (CD13), which is extensively expressed by angiogenic tumour vessels, binds peptides containing the CNGRC motif. This prompted Corti et al. (131) to design a fusion protein where the N-terminus of TNF is coupled to the C-terminus of the peptide. This construct was called NGR-TNF. This molecule used as a single agent was found to be is 10- to 15-fold more active than wild type TNF in a murine lymphoma model. Evidence was obtained that NGR-TNF was improving doxorubicin penetration in tumours. In the B16 melanoma model, a strong synergism with doxorubicin was
observed, using picogram doses of NGR-TNF (132). In addition to angiogenic endothelial cells, many normal tissues express CD13: most cells of the myeloid lineage, epithelial cells of kidney proximal tubules, of small intestine, of the prostate, of bile ducts, and less frequently in fibroblasts and smooth muscle cells. Fortunately, direct binding assays and competition assays by immunohistology on myeloid cells and on kidneys showed that NGR-TNF failed to bind to this isoform of CD13. Biodistribution studies on normal organs confirmed these results. Considering the proposed mechanism of synergy between NGR-TNF and chemotherapy, studies in several tumour models demonstrated that the time interval between NGR-TNF and chemotherapy was critical. A two-hour interval was better than either a one-hour or a three-hour interval. This was found in several experiments with different drugs and is consistent with a permeability-inducing activity of TNF (133). A clinical phase I study in solid tumours has been activated at the EORTC.

**Targeting a melanoma antigen**

TNF was coupled to the murine antibody ZME-018, which recognizes a 240 kDa glycoprotein present on over 80% of melanoma cells (134). This was found to increase TNF cytotoxicity and to reduce cell resistance. The same group decided to prepare a recombinant fusion construct, scFvMEL/TNF, composed of the human TNF and a scFv recognizing gp240 (135). The scFvMEL/TNF fusion protein was more cytotoxic to antigen-positive A375 melanoma cells than TNF alone, and furthermore was active against AAB-527 melanoma cells completely resistant to TNF. Co-administration of scFvMEL/TNF and chemotherapeutic agents (vincristine, etoposide, cisplatin and doxorubicin) to A375 cells for 72 hours demonstrated synergistic antitumour activity with doxorubicin or 5-FU, and additive effects in combination with vincristine, etoposide or cisplatin (136).

Taken together, these three TNF targeting preclinical studies indicate that it is possible to efficiently and safely administer TNF systemically if it is targeted to angiogenic vessels or to tumour cells. The efficiency clearly resides essentially in the improved efficacy of chemotherapy because of improved intratumoural penetration. However, it can also be hypothesized that the intratumoural delivery of TNF might produce other biological effects, such as to improve the immunological response to the tumour or to inhibit angiogenesis in case of repeated administration.

**II. Gene transfer of TNF and radiation-induced expression**

The radiation-inducible promoter region of the Egr-1 gene was linked to the gene encoding TNF and a replication-deficient adenovirus was used to deliver this Egr-TNF construct to human tumours growing in nude mice. Combined treatment with Ad5.Egr-TNF and 5,000 cGy (rad) resulted in increased intratumoural TNF-α production and increased anti-tumour effect compared with control treatments. The increase in tumour control was achieved without an increase in normal tissue damage, as compared to tissue injury from radiation alone (137, 138). The same combination resulted in tumour suppression in a murine glioma xenograft model mediated by the destruction of tumour microvasculature (139). Others have demonstrated that the TNF gene under the control of the stress-inducible promoter, gadd 153, did not result in cytotoxicity when introduced into the human glioma cell line. When the transfected cells were irradiated with 10 or 20 gray (Gy), the gadd 153 promoter was highly induced and the expression level of TNF increased, resulting in higher cytotoxicity than with radiation alone (140).

In the same way, “TNF-erade” is a second-generation (E1-, E3-, and E4-deleted) replication-deficient adenovector carrying the transgene encoding for human TNF, regulated by the radiation-sensitive promoter Egr-1. Radiation increased intratumoural levels of TNF by a factor of 12 (141). This approach was translated in the clinic. Patients with large soft tissue sarcomas of the extremities received repeated intratumoural injections of TNF-erade and fractionated irradiations. The treatment was well tolerated. This phase I study showed 85% objective clinical or pathological responses, including two complete responses (142). Although the antitumour mechanism is not fully understood, it seems that this approach allows the local production of an effective concentration of TNF.

**Conclusion**

TNF was the first agent registered for the treatment of cancer that improve drug penetration in tumours and selectively destroy angiogenic vessels. ILP with TNF and melphalan provided the proof of concept that an antivascular strategy combined to chemotherapy may produce a strong anti-tumour effect. The registered application of ILP is as a regional therapy for tumours that have spread regionally (143). It has a great regional efficiency but it is unlikely that it has any impact on survival. High TNF dosage induces endothelial cell apoptosis, leading to vascular destruction. However, lower TNF dosage produces a very efficient effect, which is to increase drug penetration into the tumour, presumably by decreasing the intratumoural hypertension. It is worth mentioning that melphalan is not a drug that is active in soft tissue sarcoma when given alone systemically or by ILP. Its efficacy when given with TNF strongly suggests that it is due to better tumour uptake, and not to an abolition of the so-called resistance to this drug. TNF-ILP allowed uncovering the role of αvβ3 integrin deactivation as an important mechanism of antiangiogenesis. Several recent studies showed that TNF targeting is possible, paving the way to a new opportunity to administer TNF systemically to improve cancer drug penetration.

**Abbreviations**

CR, complete response; ILP, isolated limb perfusion; M-ILP, ILP with melphalan alone; MTD, maximum tolerable dose; STS, soft tissue sarcomas; TIM-ILP, triple combination TNF, IFN-γ and melphalan given by ILP; TM-ILP, combination of TNF and melphalan given by ILP

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