SYSTEMIC SIDE EFFECTS
OF
ISOLATED LIMB PERFUSION
WITH
TUMOR NECROSIS FACTOR ALPHA

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De reis, niet de bestemming

Aan mijn ouders
Voor Mieke

Contents

Introduction

Tumor necrosis factor alpha (TNF-α) is a naturally occurring glycoprotein which displays an intriguing variety of biological functions. On the one hand it is cytotoxic for tumor cells, and as such has been used in the treatment of cancer since, with molecular cloning, it became available in sufficient quantities. On the other hand TNF-α is a central mediator in the metabolic changes observed during the systemic response to infection, be it bacterial, viral or protozoal. It is identical to 'cachectin', the lipoprotein lipase inhibitor that was isolated by Cerami and coworkers from the serum of severely wasted rabbits, infected with Trypanosoma brucei [1]. Administration of recombinant TNF-α (r-TNF-α) to experimental animals induces a clinical picture that is virtually identical to the septic shock syndrome [2].

In view of these potent pro-inflammatory actions of TNF-α, it is hardly surprising that systemic toxicity has been a notorious drawback of cancer treatment with r-TNF-α. Early clinical trials have been disappointing due to both a lack of objective tumor response and serious systemic side effects. Treatment related fatalities have been described. It was generally felt that higher concentrations of r-TNF-α were needed to achieve significant anti-tumor effect, but that toxicity would preclude the use of such high dosages. A breakthrough in the clinical use of r-TNF-α occurred in 1992 when the first report on isolated limb perfusion with high dose r-TNF-α was published [3]. With this technique, very high concentrations of r-TNF-α were achieved locally, while systemic toxicity seemed to be manageable. An impressive clinical response rate was obtained in patients with unresectable sarcomas and melanomas of a limb [4,5].

When isolated limb perfusion with r-TNF-α was introduced at the University Hospital in Groningen, it became readily apparent that toxicity, though indeed manageable in a modern Intensive Care Unit, was still considerable. During perfusion and in the immediate post-operative phase, most patients developed a sepsis syndrome characterized by high fever, systemic vasodilation and low bloodpressure. The studies presented in this thesis were conducted to explore the exact nature of this toxic response and, if possible, to clarify its mechanism.

Chapter I summarizes what is known of the side effects of treatment with r-TNF-α in general. It also offers some guidelines for the treatment of patients undergoing isolated limb perfusion with r-TNF-α, and it suggests a number of experimental strategies that may prove useful in the future. Chapter II defines the clinical response as it is seen in post-perfusion patients and relates the severity of symptoms to the amount of leakage of r-TNF-α from the perfusion circuit to the systemic circulation. In chapter III the effects of leakage of r-TNF-α on systemic coagulation variables are studied, in particular the added influence of pretreatment with interferon gamma (IFN-γ). Chapter IV is about the impact of isolated limb perfusion with r-TNF-α on the human fibrinolytic system. In chapter V renal effects of this type of treatment are studied in some detail. Finally, in chapter VI, the role of nitric oxide as a possible mediator of TNF-α-induced systemic vasodilation is investigated.

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Chapter I

Side effects of cancer treatment with recombinant human tumor necrosis factor alpha: A new challenge for the intensive care

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Side effects of cancer treatment with recombinant human tumor necrosis factor alpha: A new challenge for the intensive care
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Summary

The purpose of this chapter is to familiarize intensive care staff with the concept of isolated limb perfusion with recombinant human tumor necrosis factor alpha (r-TNF-α), a new modality in cancer treatment, that has attracted considerable attention. The systemic toxicity of this type of treatment, and of cancer therapy with r-TNF-α in general, is reviewed. This review is based on Phase I and Phase II trials published in the English language, along with supportive documentation and data on 64 patients treated with r-TNF-α in our own institution. Guidelines are offered for the successful management of this type of patient.

Treatment with r-TNF-α results in a characteristic clinical syndrome, which resembles the sepsis syndrome. Hypotension and respiratory failure are the main features of this syndrome. Toxicity is largely independent of the route of administration. Very high serum concentrations of TNF-α, if shortlived, can be less toxic than sustained low serum concentrations. Treatment of patients who have undergone isolated limb perfusion with high dose r-TNF-α is feasible and effective in a modern ICU setting, even if high serum concentrations of TNF-α , due to leakage from the perfusion circuit, cannot be avoided. Most patients can be discharged from the ICU within 24 hours.

Introduction

Cancer treatment with bacteria or bacteria-induced serum factors is not new. Late in the previous century, the surgeon William Coley reported considerable success in the treatment of advanced cancers with injections of live bacteria or bacterial extracts, although at the cost of serious morbidity [1-2]. Later, Shear and coworkers could show that endotoxin, a part of the cell wall of Gramnegative bacteria, was indeed capable of inducing hemorrhagic necrosis in laboratory animals with transplanted tumors [3]. Since endotoxin did not kill tumor cells in vitro, the possibility of an intermediate cytotoxic host factor released by the administration of endotoxin was suggested. In a series of elegant experiments Carswell and coworkers were able to demonstrate the presence of such a substance in the serum of BCG-infected mice treated with endotoxin [4]. When this serum was intravenously administered to animals bearing a subcutaneous transplant of a murine sarcoma, visible necrosis of the tumor was observed. Accordingly, the active substance was named tumor necrosis factor (TNF). In the mid-eighties the DNA for human tumor necrosis factor was cloned and, with recombinant techniques, large amounts of human TNF became available for research purposes [5-6]. Since TNF had shown in vitro anti-tumor activity against a number of different cell lines, while showing no effect on human fibroblasts, there was considerable interest to try this new drug in clinical trials in various types of neoplastic disease. Between 1987 and 1991 a series of Phase I and Phase II trials was carried out [7-43]. Unfortunately, none of them showed unequivocal clinical benefit. As toxicity turned out to be pronounced and lethal complications of systemic treatment with TNF were reported, interest in this new drug waned as quickly as it had been aroused. Recently a revival of TNF as antineoplastic drug was introduced by Lejeune and coworkers who combined high-dose human recombinant TNF-α (r-TNF-α) with an alkylating drug in a system of isolated regional perfusion [44]. A response rate of 87% was seen in patients with locally irresectable sarcoma of a limb [45]. Later, encouraging results with this technique were also described in patients with melanoma [46]. Toxicity was considered to be acceptable, although all patients needed intensive care after the procedure [47-48]. This technique of regional perfusion with r-TNF-α in combination with other antineoplastic drugs is rapidly gaining acceptance as a valuable alternative in the treatment of several types of cancer. Post-perfusion patients will find their way to the ICU in increasing numbers and intensivists should be thoroughly familiar with the serious side-effects of treatment with r-TNF-α to be able to provide proper care.

TNF-α toxicity in vivo

TNF-α is generally considered to be the key mediator in the mammalian response to bacterial infection. It is mainly produced by stimulated macrophages and monocytes but many other cells have been reported to be able to synthesize TNF-α. It is a strong pro-inflammatory agent that will affect the function of almost any organ system, either directly or by inducing the formation of other cytokines like IL-1, or prostaglandins. TNF-α, which naturally occurs in a trimeric form, exerts its influence on cells by binding to a specific receptor. Two types have been identified: the smaller 55 kilodalton TNF-R55 receptor, present on virtually all nucleated cells and the larger 75 kilodalton TNF-R75 receptor, mainly present on lymphocytes. The extracellular portions of these receptors can become separated from the cellmembrane by proteolysis, and can bind and inactivate TNF-α in the circulation. They are referred to as soluble receptors. Their shedding from cellmembranes is upregulated by TNF-α itself. If injected in small amounts, TNF-α is bound rapidly to tissue receptors and degraded in the cells. If administered

in larger amounts it is broken down and partially cleared by the kidneys, and, to a lesser extent, by the liver. The serum half-life of TNF-α is dose-dependent: at doses of 25 μg/m^2 a serum half-life of 13 min has been described , increasing to 26 min at higher doses [9].

If administered intravenously to laboratory animals it causes a clinical picture very similar to septic shock. Excessive vasodilation will cause hypotension, acidosis and oliguria with renal failure. Increased capillary permeability will result in edema which in the lung will interfere with gas exchange. Activation of the clotting system may lead to a disseminated intravascular coagulopathy. Liver cell necrosis may occur. Even small doses of TNF-α can cause death in susceptible animals. If administered in smaller quantities to human volunteers it causes fever, headache, anorexia, myalgia, hypotension, capillary leak syndrome, increased rates of lipolysis and skeletal muscle protein degradation [49].

Toxicity of TNF-α in the treatment of cancer: general remarks

Although the exact mechanism, leading to tumor cell necrosis in vivo, is still largely unknown, TNF-α has been tried for almost any kind of malignant disease. It has been administered subcutaneously, intramuscularly and intravenously in many different dose regimens. It has also been used locally, for instance in tumors of the bladder and in ovarian carcinoma. Intratumoral application of r-TNF-α has been studied extensively and has even been tried in the treatment of brain tumors [23, 24, 40, 41]. Recently, combined treatment of r-TNF-α with alkylating drugs in an isolated perfusion model has received considerable attention [44, 45, 46]. This technique can only be applied with tumors of a limb. After dissection and cannulation of femoral, popliteal or axillary vessels, a perfusion circuit is established using a roller pump and a membrane oxygenator (Fig 1). The system is primed with red blood cells, a colloid solution and heparin and kept at a temperature of 39^o C.

Fig.1 Set-up of isolated limb perfusion

After equilibration, up to 4 mg of r-TNF-α is infused into the perfusion circuit. r-TNF-α is usually combined with an alkylating agent such as melphalan to obtain maximal anti-tumor efficacy. Some of these patients are pretreated with recombinant interferon gamma (r-IFN-γ) to enhance the sensitivity of the tumor to r-TNF-α. Human r-IFN-γ increases the number of TNF-receptors on human tumor cells [50, 51]. Additionally, r-TNF-α and r-IFN-γ show synergy in antitumor effects on human tumor cells [52].

Subcutaneous and intramuscular routes of administration have been largely abandoned because of unacceptable local side effects such as soreness and ulceration [12]. Instillation of the drug into body cavities (intravesical, intraperitoneal) does not usually lead to detectable serum levels of TNF-α. Although systemic side effects of this type of treatment are reported to be mild by some [25, 43], others have observed severe systemic toxicity with fever, influenza-like symptoms and prolonged malaise [21]. Intralesional application of r-TNF-α will results in highly variable plasma levels of this cytokine [27], but most reports show plasma concentrations comparable to what is observed after intravenous dosing [23, 24, 27]. Again, systemic side effects may be severe, with intolerable rigors, fatigue and tachycardia [40].

Thus, it appears from the literature that all routes of administration of r-TNF-α can cause serious systemic side effects. Severe toxicity has been reported with low plasma concentrations of TNF-α [42], while very high concentrations of this cytokine are relatively well tolerated if its presence in the systemic circulation is short-lived. For example, toxicity in a study where r-TNF-α was infused over 24 hours, resulting in peak plasma concentrations of 8000 ng/L [33], was more severe than in a study where a 30 min. infusion of the drug led to peak levels of 300.000 ng/L [36]. Systemic toxicity seems to be determined mainly by the length of time that elevated concentrations of TNF-α can be found in the circulation. This is in keeping with the observation by Pinsky and coworkers that persistence of TNF-α and interleukin-6 concentrations, rather than peak concentrations of cytokines, predicts a poor outcome in septic shock [53]. On the other hand, the promising results of isolated limb perfusion with very high doses of r-TNF-α suggest that the effects of this drug on the tumor are dependent on high levels of TNF-α, and that, with such high levels, an exposure time of 90 min is sufficient to destroy virtually all malignant tissue. It follows that, generally speaking, short courses with high dose r-TNF-α hold most promise for the future, from the point of efficacy as well as from the point of toxicity.

Regional perfusion techniques were originally developed to avoid systemic toxicity. Ideally the drug would not spread beyond the limb that was being perfused. Indeed, perfusions with melphalan alone showed little systemic toxicity. However, it appeared that adding r-TNF-α to the perfusate invariably induced a sepsis syndrome, probably due to leakage of r-TNF-α into the systemic circulation [44, 47, 48, 54, 55]. We were able to demonstrate plasma levels of TNF-α in these patients of up to 267000 ng/L at the end of perfusion [56]. For comparison, in meningococcal disease and/or septicemia all patients with TNF-α levels over 100 ng/L died [57]. In other types of septic shock median TNF-α levels of 120 ng/L have been reported [58]. TNF-α levels in perfused patients were very high initially but fell rapidly: 12 hours postperfusion TNF-α levels had returned to baseline values [48].

It is still unclear whether the very high levels of systemic TNF-α, measured in perfusion patients, represent free TNF-α or neutralized TNF-α, bound to soluble receptors. The latter possibility would explain why clinical signs and symptoms in these patients are less severe than anticipated from the very high serum TNF-α levels. Thom and coworkers have reported a moderate increase in the soluble 55 kd

TNF receptor (TNF-R1) during perfusion with r-TNF-α, possibly representing an upregulation of receptor shedding [54]. With melphalan alone, the rise in serum TNF-R1 was less pronounced.
Interleukin 6 (IL-6) concentrations rise significantly during isolated limb perfusion with r-TNF-α and may contribute to toxicity [54]. Interleukin 1 (IL-1), which can act synergystically with TNF-α, seems to be less important in this setting. The role of anti-inflammatory cytokines like interleukin 4 (IL-4) and interleukin 10 (IL-10), which could mitigate the toxic effects of TNF-α, remains to be elucidated.

Toxicity of r-TNF-α treatment is variable and not always dose-dependent. Hepatic and cardiovascular toxicity have been generally found to increase with increasing dose, but constitutional symptoms like fever, chills, rigors and prostration are probably not dose-related. The maximum tolerated dose of r-TNF-α, administered intravenously over 30 min, is reported to be between 100 and 300 $\mu g/m^2$ [7-10, 12, 13, 14, 17, 29, 39]. Signs of late toxicity are notoriously absent in all reports.

Specific signs and symptoms of toxicity in patients treated with r-TNF-α

Constitutional
As was stated before, symptoms after treatment with r-TNF-α by any route are variable and individually determined. However, virtually all patients will experience constitutional symptoms. This influenza like syndrome is characterized by fever up to 40^0 C, chills, rigors, headache, back pain, fatigue, prostration and malaise. These symptoms are probably not dose-related [13, 17, 19, 30-31] and will disappear spontaneously even if treatment with r-TNF-α is continued without dose adjustment.

Cardiovascular
Cardiovascular toxicity is usually dose-limiting. A fall in blood pressure is observed in many patients [7-10, 12, 14-20, 22, 23, 27-28, 30-33, 37-38, 55], sometimes preceded by a short period of hypertension [33]. Hypotension can be severe with systolic blood pressures < 60 mm Hg. Volume resuscitation and inotropic support of the circulation are frequently necessary to maintain acceptable tissue perfusion. Sinus tachycardia is common but other rhythm disturbances are rare [37]. In one patient a transmural myocardial infarction soon after starting treatment with r-TNF-α has been described [7]. Excessive production of nitric oxide, a potent vasodilator, by cytokine-inducible nitric oxide synthase is believed by many to be the underlying mechanism. TNF-α can induce nitric oxide synthase in vascular smooth muscle cells from rat aorta in vitro [59]. In mice the administration of anti-TNF-α antibodies markedly reduces endotoxin-induced shock and nitric oxide synthesis in vivo [60]. Kilbourn and coworkers have induced hypotension in dogs by administering recombinant human TNF-α. N^G-monomethyl-L-arginine, a competitive inhibitor of nitric oxide formation from L-arginine, completely reversed this fall in blood pressure, which reappeared after the administration of excess L-arginine. The authors conclude that excessive nitric oxide production mediates the hypotensive effect of TNF-α [61]. Whether the same mechanism applies to humans remains to be seen. In a series of 8 patients treated with hyperthermic isolation perfusion with r-TNF-α we have measured nitric oxide metabolites in plasma. All patients developed vasodilation and hypotension, secondary to a pronounced leak of r-TNF-α from the perfusion system to the systemic circulation. However, we were unable to demonstrate any elevation in plasma nitrite or nitrate [56]. Other mechanisms, not involving the nitric oxide pathway, are therefore likely to play a role in the generation of hypotension and septic shock in the setting of r-TNF-α perfusion. The existence of a nitric oxide independent pathway to explain

cytokine-induced vasodilation in humans was already suggested by Beasley and coworkers [62]. Alternatively, cyclooxygenase products like prostacyclin could also play a role [63].

Respiratory

Respiratory compromise is common after treatment with r-TNF-α [2, 3, 7-10, 13, 18, 23, 30-33, 39, 64, 65] and may range from slight symptoms of tightness of the chest and tachypnea to severe respiratory distress requiring mechanical ventilation. Administration of r-TNF-α has been shown to affect pulmonary function parameters: it decreases vital capacity, capillary blood volume, diffusing capacity of the alveolo-capillary membrane and transfer capacity for carbon monoxide [64]. It is unclear whether these effects are dose-related [48, 64]. Significant reductions in these parameters are observed 1 week after isolated limb perfusion with r-TNF-α and melphalan. Eight weeks after the perfusion procedure, they have returned to pre-treatment values. Treatment with r-TNF-α also increases pulmonary permeability, which could explain the transient pulmonary infiltrates described in the original publication by Eggimann [47, 66]. A relationship between the Lung Injury Score (Murray) and TNF-α levels has been described [48].

Renal

Renal toxicity is surprisingly mild. In most series renal toxicity does not exceed moderate proteinuria and minimal elevation of serum creatinine [7, 9, 13, 17, 20, 31-33, 37-38, 55]. Others have reported more serious renal symptoms, such as a marked reduction in creatinine clearance, oliguria and elevated tubular enzyme excretion [12, 14, 23, 27, 28, 30, 67]. Whether renal toxicity in this setting is a consequence of inadequate perfusion pressures or a direct toxic effect of r-TNF-α, remains to be determined [23, 27, 67]. Even in patients who could be kept normotensive throughout the perfusion procedure and during their postoperative stay in the ICU, a temporary decrease in proximal tubular function was observed [67]. This may represent a direct toxic effect of TNF-α.

Hepatic

Many patients will develop a significant rise in either bilirubin or ASAT and ALAT or both [7-9, 14-16, 18-20, 22, 23, 27, 28, 30, 32-35, 37-38, 55]. Liver cell damage can be dose-limiting [8-9, 18, 20, 32], but is usually rapidly reversible on discontinuation of the drug. It does not cause clinically significant disturbances of coagulation, due to deficient synthesis of clotting factors.

Digestive tract

Upper as well as lower digestive tract symptoms are common in this type of treatment. Nausea and vomiting can be distressing [7, 23, 32, 33] and in some cases dose-limiting [35]. Watery diarrhea has been observed in a number of patients [15, 19, 23, 32, 33].

Blood

A dose-dependent decrease in platelets is common [20, 22, 23, 29-31, 55]. It can be very pronounced (< 25.000/mm^3) and dose-limiting [20, 22]. However, to our knowledge, petechiae and overt bleeding have not been reported.

A decrease in hemoglobin has also been reported, but its cause remains unclear [29]. Hemodynamic instability will often necessitate infusion of large quantities of fluids, which in turn will aggravate any existing low hemoglobin level.

Initially, granulocytes tend to decline in numbers in the peripheral blood, probably because of sequestration. After the infusion of r-TNF-α has been discontinued, a significant increase in numbers is observed [9-10, 15, 18, 22, 23, 28, 31, 39, 55]. Monocytes and lymphocytes also decrease dramatically in number in the early phase but their recovery seems to be less quick [9].

It has been shown by various authors that TNF-α has a procoagulant effect on the hemostatic mechanism in humans through expression of tissue factor and downregulation of thrombomodulin [68-70]. Signs of activated coagulation have been confirmed in a few clinical series [35, 39, 55, 71], but prolonged clotting parameters are rare [48, 55]. A normal clotting profile seems to be most common [17, 22, 23, 31-33]. Fibrinolysis is inhibited by a large increase of plasma activator inhibitor type 1 (PAI-1) and a decrease of tissue plasminogen activator (t-PA) [72, 73]. Inhibition of fibrinolysis has been shown in healthy subjects treated with r-TNF-α [72], in patients treated with intravenous r-TNF-α [73], and in patients treated with isolated limb perfusion with r-TNF-α and melphalan [74].

Nervous system

Neurological sequelae of treatment with r-TNF-α have been mentioned by a number of authors. Confusion and hallucinations can be severe but seem to be quickly reversible after discontinuation of the drug [23, 31, 33, 37]. Transient aphasia and diplopia have also been described [22, 30, 32, 33]. Three cases of blindness have been reported, one cortical [7], and two as a consequence of a retinal vein thrombosis [14]. Seizures have been reported in one series [31].

Miscellaneous

Wasting, considered to be due to prolonged administration of r-TNF-α has been described [25].

Authors who have studied the effect of r-TNF-α on lipid metabolism have reported a decrease in high-density lipoproteins, as well as increases in triglycerides and very-low-density lipoproteins [22].

In one series, bacteremia and sepsis were considered to be causally related to treatment with r-TNF-α [31].

To our knowledge at least six patients have died as a consequence of treatment with r-TNF-α: one after a cardiac arrest 90 minutes after receiving the first dose of r-TNF-α [8], two following septic episodes during a 5-day continuous infusion [31], one from pulmonary embolism [39], one from intracranial hemorrhage [39] and one from treatment-related pulmonary edema [39].

The most relevant side effects of hyperthermic isolated limb perfusion with r-TNF-α are summarized in Table 1.

Clinically relevant side effects of HILP with r-TNF-α
hypotension
respiratory failure
liver cell necrosis
thrombocytopenia
fever
rigors
malaise

Table 1. Side effects.

Treatment

Some symptoms of r-TNF-α toxicity can be prevented, others can be minimized by adequate treatment (Table 2). There is ample evidence that non-steroidal anti-inflammatory drugs can alleviate constitutional symptoms, probably because they are partially mediated by prostaglandins. Paracetamol [34, 35], indomethacin [28-30], ibuprofen [10] and ketoprofen [16, 30] have been used successfully for this indication. Routine administration of one or more of these drugs should be considered in all patients treated with r-TNF-α. Pethidine (meperidine) is possibly effective against chills and rigor [17, 30, 34, 35, 40]. Steroids are of unproven efficacy [10].

Prevention and treatment of r-TNF-a toxicity	
prevention	**invasive monitoring**
	volume loading
	leakage monitoring
	low perfusion flow rates
	extensive washout
treatment	**invasive monitoring**
	volume resuscitation
	dopamine
	norepinephrine
	positive pressure ventilation

Table 2. Prevention and treatment of toxicity

Adequate fluid resuscitation is mandatory to prevent r-TNF-α induced hypotension as much as possible. Patients on chronic diuretic therapy are at special risk for this complication. Volume loading with 500-1000 ml of saline is recommended if invasive measurement of filling pressures is not feasible.

In the case of isolated limb perfusion with r-TNF-α meticulous surgical technique can limit leakage to the systemic circulation to a minimum. Low perfusate flow rates (up to 500 ml/min for a lower limb and up to 300 ml/min for an upper limb) have been reported to reduce systemic leakage and attenuate side effects, probably by reducing vascular pressures in the isolated limb [55]. A thorough washout procedure at the end of perfusion with approximately 6-10 liters of washout fluid, may also contribute to a reduction of leakage and systemic side effects [47].

Leakage during the procedure should be monitored continuously with a radioactive marker, viz. I^{131}-albumin, as described previously [75]. In this way unacceptably large leaks can be discovered early and corrected before extensive damage is done.

Management in theatre can be facilitated by inserting a Swan-Ganz catheter after induction of anesthesia. Most patients will require extensive fluid administration to maintain adequate filling pressures. Since the main circulatory problem is excessive vasodilation, the majority will also require vasopressors. In our institution all patients who have undergone isolated limb perfusion with r-TNF-α are routinely admitted to the ICU. However, if the patient has been hemodynamically stable during perfusion and leakage is minimal (≤ 1%), the chances to develop severe toxicity are low [54] and postoperative treatment in a 24-hour recovery facility can be considered.

Patients with a substantial leak should definitely be managed in the ICU. In our experience hypotension and respiratory failure due to pulmonary edema are the only side-effects demanding immediate attention. As was pointed out above, other side effects are common, but they are usually selflimiting, do not need treatment and will not delay the patients discharge from the ICU. Hypotension

is treated with colloids and cristalloids to maintain filling pressures. A pulmonary artery wedge pressure of 12 mm Hg should be adequate. If hypotension persists, and it usually will, dopamine or norepinephrine is added to maintain perfusion pressures. Norepinephrine has the advantage of not being a positive chronotrope, as opposed to dopamine, and is a more potent vasopressor. Since most patients will be in sinustachycardia anyway this would favor the use of norepinephrine. In view of the hemodynamic profile of most of these patients, with a high cardiac output and low systemic vascular resistance, norepinephrine is probably the drug of choice. If blood pressure can be maintained renal failure is quite rare. In our own series of 64 patients only one needed continuous veno-venous hemofiltration because of renal failure. In this patient we were unsuccessful in our attempts to maintain adequate blood pressures.

Care should be taken that infusion of fluids does not aggravate non-cardiogenic pulmonary edema, which in a subclinical form is present in the majority of patients [48]. If this can be avoided most patients can be extubated within 24 hours after the perfusion procedure. Of the 64 patients treated in our institution the median duration of post-operative ventilation was between 24 and 48 hours; only 2 patients needed mechanical ventilation for longer than 48 hours. As soon as the patient has become hemodynamically stable, a diuretic can be useful to excrete the surplus of fluids that had to be administered in the acute stages of the sepsis syndrome. This will usually improve oxygenation by reducing extravascular lung water. Once symptoms in the acute phase have resolved, the patients can be safely sent to a general ward for further care; delayed toxicity is not a feature of treatment with r-TNF-α.

It would appear that a sepsis syndrome, that occurs as side effect of a medical intervention, is easier to treat than sepsis from an infectious source. In the treatment of naturally occurring septic shock, many attempts to stop the relentless propagation of the cascade of inflammatory mediators with blocking agents have been unsuccessful, simply because they could not be given early enough in the process. Obviously, timing of interventions could be much more favorable in iatrogenically-induced sepsis. The set-up of isolated limb perfusion with r-TNF-α would seem ideally suited for prophylactic treatment with a monoclonal antibody against TNF-α. Continuous systemic infusion of such an antibody during perfusion could theoretically neutralize all r-TNF-α that would leak from the perfusion circuit to the general circulation. If production of IL-1 could be shown to substantially increase the toxicity of r-TNF-α, continuous systemic infusion of an IL-1 receptor antagonist could also be of benefit. In theory, such an approach could prevent virtually all side effects of perfusion with r-TNF-α. However, before such treatment can be attempted, it is of crucial importance to make certain that the anti-tumor effect of r-TNF-α is in no way abrogated by anti-TNF-α antibodies, delivered systemically. A different approach is the development of mutant TNF's, with, ideally, anti-tumor activity similar to native TNF but reduced toxicity [76].

Finally, if nitric oxide does play a role in TNF-α induced hypotension in this setting, prophylactic treatment with competitive inhibitors of nitric oxide formation from L-arginine, like N^G-monomethyl-L-arginine, could block nitric oxide production and subsequently prevent excessive vasodilation. In naturally occurring septic shock this approach has had limited success [77], but, again, it could be more successful in a situation that permits early administration and timely discontinuation of the drug.

Concluding remarks

TNF-α has a remarkable capacity to kill tumor cells in vitro, while largely sparing normal cells. Apart from direct cytotoxic/cytostatic effects, multiple indirect processes induced by TNF-α as a "biological response modifier", are possibly involved in regression of in vivo tumor. These include potent immunomodulatory effects of TNF-α, in recruiting and activating immune cells, augmenting the expression of cell surface molecules, and inducing the production of intermediate cytokines. These characteristics make TNF potentially useful as an anti-cancer drug in vivo. Early clinical trials with recombinant human TNF have been disappointing: at best a small clinical response has been obtained at the cost of serious toxicity. Recently, encouraging results have been reported in patients with irresectable extremity sarcomas and melanomas with a combination of high dose r-TNF-α, IFN-γ and an alkylating drug, administered into a system of hyperthermic isolated regional perfusion [45, 46]. Due to leakage of r-TNF-α from the perfusion system to the general circulation, this type of treatment is also complicated by considerable clinical toxicity, mainly hypotension and respiratory failure. However, as we and others have shown, modern intensive care is able to cope with this, providing there is sufficient knowledge of the clinical syndrome of TNF toxicity. Our experience with this technique so far has led us to believe that even higher doses can be tolerated by the fully monitored, sedated and ventilated patient. In our series, serum concentrations of TNF-α with isolated limb perfusion are higher than the levels that have been documented with the intravenous administration of 300 $\mu g/m^2$ r-TNF-α, the dose traditionally considered to be maximal. The majority of our patients recovered within 24 hours and could leave the ICU on the day following perfusion. Accordingly we believe that there may be a place for new trials with intravenously administered r-TNF-α in dosages exceeding what has been considered the maximum tolerable dose for bolus iv. administration.

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Chapter II

High plasma levels of tumor necrosis factor alpha and shortlived sepsis syndrome in patients undergoing hyperthermic isolated limb perfusion with recombinant tumor necrosis factor alpha, interferon gamma and melphalan

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High plasma tumor necrosis factor (TNF)-α concentrations and a sepsis-like syndrome in patients undergoing hyperthermic isolated limb perfusion with recombinant TNF-α, interferon-γ and melphalan
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Summary

This chapter describes the post-operative course of 25 consecutive patients, who underwent hyperthermic isolated limb perfusion with recombinant tumor necrosis factor α (r-TNF-α) and melphalan, following pretreatment with recombinant interferon gamma (r-IFN-γ), as treatment for recurrent melanoma, primary nonresectable soft tissue tumors, planocellular carcinoma or metastatic carcinoma. It is a retrospective, descriptive study, relating systemic TNF-α levels with indices of disease severity.

All patients developed features of sepsis syndrome and required intensive care treatment. Most patients recovered quickly with a median ICU stay of 2 days (range 1-25). Maximum systemic TNF-α levels ranged from 2284 to 83000 ng/L (median 25409 ng/L) and returned to baseline values within 8 hours. Despite these high levels of TNF-α no patient died in the ICU, although the patient with the highest TNF-α level developed multiple organ failure and required continuous venovenous hemofiltration for 16 days.

Linear regression analysis showed a positive correlation between maximum TNF-α levels and systemic vascular resistance ($p<0.01$), cardiac index ($p<0.02$), lung injury score ($p<0.02$), prothrombin time ($p<0.02$) and activated partial thromboplastin time ($p<0.05$). It is concluded that hyperthermic isolated limb perfusion with r-TNF-α leads to high systemic levels of TNF-α, probably due to leakage of r-TNF-α from the perfusion circuit, mainly through collateral bloodflow. A sepsis like syndrome is seen in all patients. Despite high levels of systemic TNF-α, this sepsis syndrome is short-lived and recovery is rapid and complete in most patients.

Introduction

The cytokine TNF-α, originally defined by its antitumor activity in vivo, is now recognized to play a key role as a polypeptide mediator in the pathogenesis of septic shock [1-7]. TNF-α has been shown to cause myocardial depression, vasodilation, and diffuse lung injury in animal studies [8-10]. Levels of TNF-α in the serum of septic patients show a positive correlation with the severity of illness and mortality [11-14]. Cardiovascular, and fibrinolytic responses to the administration of endotoxin and TNF-α have also been described in volunteer studies, although the severity of symptoms has limited the dose used [15-18].

With hyperthermic isolated limb perfusion a very high dose of a cytostatic agent can be administered locally in a limb while minimizing systemic toxicity. A combination of r-TNF-α, r-IFN-γ and melphalan was used in 25 patients with, mostly, primary nonresectable soft tissue tumors and melanomas stage III A/AB of the limb, as an alternative to amputation. This triple combination with hyperthermia was chosen because of the reported synergistic antitumor effect of r-TNF-α with r-IFN-γ, hyperthermia, and alkylating agents [19]. We have studied the clinical course of these patients and its relationship to leakage of r-TNF-α to the systemic circulation during perfusion.

Subjects and methods

Subjects

Between January 1991 and June 1993, 25 patients received hyperthermic isolated limb perfusion at the Division of Surgical Oncology of the Groningen University Hospital, after approval of the Medical Ethics Committee and informed written consent had been obtained. Tumor histology is summarized in Table 1.

TUMOR HISTOLOGY	NUMBER OF PATIENTS
Malignant Melanoma	7
Liposarcoma	4
Malignant Fibrous Histiocytoma	3
Epithelioid Sarcoma	3
Leiomyosarcoma	2
Myxoid Chondrosarcoma	1
Malignant Schwannoma	1
Neurofibrosarcoma	1
Embryonal Rhabdomyosarcoma	1
Planocellular Carcinoma	1
Metastatic Renal Cell Carcinoma	1

Table 1 Tumor histology.

Four additional patients underwent hyperthermic isolated limb perfusion without pretreatment with r-IFN-γ and without the addition of r-TNF-α to the perfusate.

Anesthesia and intensive care

Anesthesia was induced with thiopental, after which the patients were paralyzed with vecuronium and the trachea intubated. Anesthesia was maintained with midazolam, sufentanyl, nitrous oxide and isoflurane. After induction arterial and pulmonary artery catheters were inserted. Blood pressure, ECG, urine output, venous and pulmonary pressures, as well as pulmonary wedge pressures and blood gas values were checked at standard intervals. All patients were admitted to the intensive care unit following surgery. Patients received mechanical ventilation until hemodynamically stable. Fluid resuscitation with crystalloid and colloid solutions was given to maintain pulmonary wedge pressures above 11 mmHg and a dopamine infusion was added if systolic arterial bloodpressure fell to 90 mmHg or decreased by > 30 mmHg from preoperative values despite adequate fluid replacement.

Isolated Limb Perfusion

For two days prior to the perfusion patients received 0.2 mg r-IFN-γ subcutaneously. The perfusion technique employed at the Groningen University Hospital is based on the technique developed by Creech and Krementz [20]. Briefly, after ligation of all collateral vessels and heparinization of the patient with 3.3 mg heparin/kg bodyweight (Thromboliquine, Organon BV, Oss, The Netherlands) the axillary, iliac, femoral or popliteal vessels were dissected, cannulated and connected to the extracorporeal circuit. The perfused limb was wrapped in a thermal blanket to reduce heat loss and four thermistor probes were inserted subcutaneously and intramuscularly for continuous monitoring of the temperature during perfusion. A tourniquet was applied to the proximal limb in an attempt to minimize leakage of the perfusate into the systemic circulation through skin collaterals. Perfusion was performed during 90 minutes under mild hyperthermic conditions (39-40^{o}C). The perfusate consisted of 350 ml 5% dextran 40 in glucose 5% (Isodex, Pharmacia AB, Uppsala, Sweden), 500 ml blood (250 ml red blood cells, 250 ml plasma), 30 ml 8.4% $NaHCO_3$ and 0.5 ml 5000 IU/ml heparin (Thromboliquine). The perfusate was oxygenated with a bubble oxygenator and driven by a roller pump. At the start of perfusion r-TNF-α (Boehringer, Ingelheim, Germany, 4 mg for leg perfusions and 3 mg for arm perfusions) and 0.2 mg r-IFN-γ (Boehringer, Ingelheim, Germany) were injected as a bolus into the arterial line of the perfusion circuit. Melphalan (Burroughs Wellcome, London, England, 10 mg/L volume of an affected leg and 13 mg/L volume of an affected arm) was administered 30 minutes later. During perfusion potential leakage to the systemic circulation was monitored with I^{131} -labeled albumin [23]. After 90 minutes of perfusion, the limb was flushed with 2 L dextran 40 in glucose 5% (Isodex) and 500 ml blood (250 ml red blood cells, 250 ml plasma), catheters were removed, the circulation restored and the heparin antagonized with protamine chloride. A lateral fasciotomy of the anterior compartment of the lower leg was performed in leg perfusions or a fasciotomy of the forearm in arm perfusions to prevent a compartment syndrome.

Four additional patients underwent hyperthermic isolated limb perfusion with melphalan in exactly the same way but without the addition of r-TNF-α to the perfusate and without pretreatment with r-IFN-γ.

Hemodynamic measurements

Hemodynamic variables were measured immediately after the patient had arrived in the intensive care unit and then at hourly intervals. Measured variables included the heart rate, mean arterial pressure, central venous pressure, mean pulmonary artery pressure, pulmonary capillary wedge pressure. Cardiac output, cardiac index, systemic vascular resistance and pulmonary vascular resistance were determined at two hourly intervals. Pressure transducers were set to zero at the level of the midaxillary line. Cardiac output was measured in triplicate by the thermodilution method, with the use of a cardiac output computer and cold saline.

Oxygen consumption, oxygen delivery, oxygen extraction ratio and alveolar arterial oxygen difference were calculated according to standard formulas. Lung Injury Score as a measure of Adult Respiratory Distress Syndrome was calculated from the chest roentgenogram, hypoxemia and positive end-expiratory pressure scores as described by Murray et al [21]. The APACHE II score was calculated for each patient on the basis of the worst results in the first 24 hours after admission to the intensive care unit. The Simplified Sepsis Score for each patient was calculated as described by Baumgartner et al [22].

Assay for tumor necrosis factor

TNF-α levels were determined by specific immunoradiometric assay (Medgenix Diagnostics, Soesterberg, the Netherlands). Samples were processed according to the guidelines of the manufacturer. Blood samples (3 ml) from an indwelling radial artery cannula were collected in EDTA vacutainer tubes, and kept on melting ice during transport to a centrifuge. Samples were centrifuged for 10 min at 3000 rpm at 0^0C and the separated plasma kept at -20^0C until analysis. A baseline sample was taken for TNF-α assay after the insertion of the arterial line, then at 5, 30, 60, and 89 minutes after the start of the perfusion. Samples from the extracorporeal circuit were also taken at the same sampling times. After restoration of circulation to the perfused limb, systemic samples were taken at 1, 5, 10, 30 and 60 minutes after removal of arterial clamps, hourly thereafter for at least eight hours and finally the next morning.

Statistical analysis

Data were analyzed using SPSS for MS WINDOWS (release 5.0). Results are tabulated to show preoperative values, mean values, standard error of the mean, and range. Correlations were sought between variables and maximum TNF-α levels. A p-value < 0.05 was considered significant.

Results

Demographic Data

Eleven male and fourteen female patients were studied (mean age 49.2 years, range 18-74 yrs). Twenty three perfusions of the lower limb were performed (iliac vessels cannulated in 15 patients, femoral or popliteal vessels cannulated in 8 patients). Two perfusions of the upper limb were performed. Histology of the tumors is summarized in Table 1. All perfusions were performed without technical complications. Leakage from the perfused limb circuit ranged from 0 to 8% (median 2%).

Clinical Course

All patients developed clinical sepsis syndrome with fever, rise in cardiac output, fall in systemic vascular resistance and the need for fluid resuscitation and inotropes. Maximum temperature, APACHE II scores, Simplified Sepsis Scores, Lung Injury scores, fluid balance, maximum dopamine requirements in the first 24 hrs of Intensive Care Unit (ICU) stay, and length of ICU stay are summarized in Table 2 and 3. One patient developed multiple organ failure and required continuous venovenous hemofiltration for 16 days.

All 4 patients who received hyperthermic isolated limb perfusion with melphalan only (i.e. without r-TNF-α) did well without invasive hemodynamic monitoring. They did not need large infusions of fluid or continued treatment with dopamine and were not admitted to the ICU.

PARAMETER	MEAN	SEM	RANGE
Length of stay (days)	3*		1-25
Maximum temperature (°C)	40.0	0.12	39.0-41.8
APACHE II Score	15.6	0.66	11-22
Simplified Sepsis Score	4.9	0.4	1-9
Fluid Balance 1ST 24HRS (L)	+10.15	0.87	+2.43 - +18.93
Time to Extubation (days)	2*		0-21

SEM, standard error of the mean.

*nonparametric distributed variable, therefore mean value should be read as median and no standard error of the mean is given.

Table 2 Clinical course data.

TNF-α *levels*

Systemic TNF-α levels were measured in 13 patients. Maximum TNF-α levels ranged from 2284 to 83000 ng/L (median 25409 ng/L). TNF-α levels peaked towards the end of the isolated limb perfusion and immediately after reperfusion of the limb. Levels then rapidly declined over the next hours of measurement (Figure 1).

TNF-α was detected in plasma up to 8 days after perfusion, but at very low levels. In the 4 patients who were treated with hyperthermic isolated limb perfusion without pretreatment with r-IFN-γ and without the addition of r-TNF-α to the perfusate, maximum postperfusion TNF-α levels ranged form 1-53 ng/L (mean 42 ng/L, standard deviation 22 ng/L, standard error of the mean 11 ng/L).

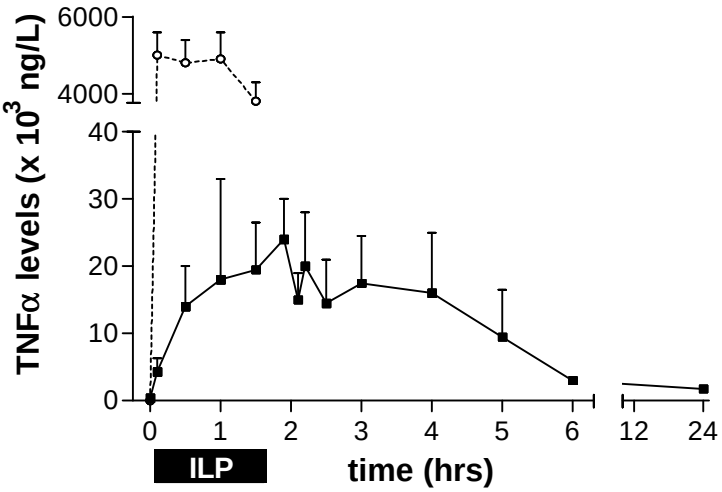

Fig. 1 Median systemic TNF-α levels (straight line and black squares) with SEM and median TNF-α levels and SEM in the perfusion circuit (dotted line and open circles).

Hemodynamic Variables

A full set of values for cardiac output, cardiac index, systemic vascular resistance and pulmonary vascular resistance was available for 22 patients. Cardiovascular variables on admission to the ICU are summarized in Table 3.

PARAMETER	BEFORE	MAXIMUM	SEM	RANGE
Cardiac Index (L/min/m ^{2})	3.06	6.3	0.4	3.6-9.9
SVR dyne.sec/cm ^{5}	1055	537	27	317-757
PVR dyne.sec/cm ^{5}	n.a.	79	6	21-133
DO_2 (ml/min)	n.a.	759	49	420-1341
VO_2 (ml/min)	n.a.	183	25	73-401
Extraction ratio	n.a.	0.21	0.03	0.1-0.4
Max. Dopamine conc. (mg/kg/min)	0	5.1	0.6	0-10.2
AaGradient	n.a.	20.6	2.0	10-59.3
Lung Injury Score	0	0.88	0.2	0-3
Thrombocytes (x10 ^{9}/L)(normal 150-400)	286	108	11	22-228
Fibrinogen (g/L)(normal 1.7-3.5)	4.3	2.2	0.3	0.9-5.2
PT (sec)(normal 11-16)	13.7	22.7	1.4	14.9-34.5
APTT (sec)(normal 26-36)	35.6	46.2	3.1	25.5-64.9
ATIII (%)(normal 80-120)	85	47.6	5.0	27-77

SEM, standard error of the mean; SVR, systemic vascular resistance; PVR, pulmonary vascular resistance; DO_2, oxygen delivery; VO_2, oxygen consumption; Max, maximum; AaGradient, alveolair arterial oxygen difference; PT, prothrombin time; APTT, activated partial thromboplastin time; ATIII, antithrombin III.

Table 3 Hemodynamic, respiratory and coagulation variables.

Linear regression analysis showed significant correlations between maximum TNF-α levels and systemic vascular resistance ($p < 0.02$, $r=0.70$), cardiac index ($p < 0.02$, $r=0.72$), volume of fluid

infused in the operating room ($p < 0.02$, $r=0.69$) and in the ICU ($p<0.02$, $r=0.80$) and maximum infusion rate of dopamine ($p < 0.02$, $r=0.69$).

Respiratory System
Twenty patients remained intubated for less than 24 hrs, 3 for 24-48 hrs and 2 patients required prolonged ventilatory support (14 and 21 days). Respiratory variables are summarized in Table 3. There was a significant correlation between maximum TNF-α level and the Lung Injury Score ($p < 0.02$, $r=0.64$).

Renal Function
One patient developed multiple organ failure and required continuous venovenous hemodialysis for 16 days. The remaining 24 patients showed no significant change in creatinine clearance from preoperative values. The patient who developed multiple organ failure had the highest top level of TNF-α recorded in this study: 83.000 ng/L.

Coagulation Parameters
Coagulation parameters are summarized in Table 3. Values for thrombocyte count were available for 24 patients, fibrinogen levels for 16 patients, prothrombin time and activated partial thromboplastin times for 15 patients, and antithrombin III levels in 14 patients. There were significant correlations between maximum TNF-α level and prothrombin time ($p < 0.02$, $r=0.79$) and activated partial thromboplastin time ($p < 0.05$, $r=0.72$) but not with fibrinogen and antithrombin III levels.

Discussion

Isolated limb perfusion with r-TNF-α, melphalan and r-IFN-γ produced a severe but short-lived systemic reaction characterized by high fever, high cardiac output, low peripheral resistance, activated clotting and an increased A-a gradient for oxygen. All patients required either fluid resuscitation or dopamine infusion or both to maintain adequate bloodpressure. The clinical picture resembled septic shock and adult respiratory distress syndrome, lasting for about 24 hours. Since, in 4 other patients, perfusion with only melphalan (without r-TNF-α and r-IFN-γ) did not trigger a rise in systemic TNF-α levels, and extremely high levels of TNF-α were measured in the systemic circulation of the patients described here, both during and shortly after perfusion, leakage of r-TNF-α from the perfusion system into the systemic circulation is likely to be the explanation for the sepsis-like state that was observed. Washout of remnant r-TNF-α once the circulation was restored may have contributed to the high systemic TNF-α levels. The observed leakage of I^{131} labeled albumin from the perfusion system, which ranged between 0 and 8% (median 2%) supports the role of TNF-α leakage as the primary cause of the postoperative events described earlier. The extent of leakage is similar to that reported in the literature [23]. Moreover, it is known from animal experiments, that administration of r-TNF-α leads to tissue injury and metabolic derangements similar to those seen in septic shock [24]. In theory, systemic TNF-α levels in these patients may also have been raised by the systemic response to extracorporeal circulation, hyperthermia, and the presence of indwelling plastic cannulae [25 -27]. Tissue damage induced by the combined effects of heat, melphalan and r-TNF-α was also considerable in all cases and this in itself could have contributed to an increase in TNF-α levels and signs of systemic inflammatory response. However, we feel that the TNF-α levels we measured were too high to be explained by

endogenous production of this cytokine. Maximum systemic TNF-α levels (median 25409 ng/L, range 2284-83000) were several orders of magnitude higher than TNF-α levels previously reported in other forms of shock. In a series of 79 patients with meningococcal disease and/or septicemia all patients with TNF-α levels over 100 ng/L died [28]. In septic shock median TNF-α levels of 120 ng/L have been reported [11]. In our patients TNF-α levels were very high initially but fell rapidly: 12 hours postperfusion TNF-α levels had returned to baseline values. In view of their high TNF-α levels recovery in our patients was remarkably rapid: 80% could be transferred from the ICU the day following perfusion. How can this discrepancy between TNF-α levels and clinical course be explained?

Theoretically the measured systemic TNF-α levels could represent an inactive form of the polypeptide. Since the preparation was tested in a bioassay by the manufacturer it is unlikely that r-TNF-α was administered to the patients in an inactive form. It has recently been shown that TNF-α binds to two different types of receptors, one of them 55 kilodalton in size, the other 75 kilodalton. The extracellular portions of these receptors, TNF-α binding protein type 1 and 2 respectively, can become separated from the cellmembrane by proteolysis, and can bind and inactivate TNF-α in the circulation. In theory, rapid and extensive binding of r-TNF-α to these soluble receptors could therefore explain the relatively mild clinical course in our patient group. In our study only total TNF-α was measured. Because of these limitations the question whether the high TNF-α levels represent unbound, active TNF-α cannot be answered. Future studies should determine both unbound TNF-α and TNF-α soluble receptor complexes. TNF-α in our series was not cleared fast; the mean half-life of 80 minutes in our patients is considerably longer than the 10 - 15 minutes reported in the literature. It has been suggested that patients with cancer are chronically exposed to increased levels of endogenous TNF-α and so may have increased tolerance to the effects of TNF-α [29]. The patients in this study received r-IFN-γ subcutaneously for 2 days preoperatively to sensitize them to the effects of TNF-α. In animals this is highly effective [30]. It is not unreasonable to assume that any downregulation of receptors in our patients was probably negated by the effects of r-IFN-γ pretreatment. Moreover, baseline values of plasma TNF-α were several orders of magnitude lower than maximum TNF-α levels so any pre-existing tolerance probably had insignificant effects on the subsequent clinical course. Pinsky and coworkers have shown that the persistence of TNF-α and IL-6 levels rather than peak levels of cytokines predicts a poor outcome in patients with septic shock [31]. Accordingly, it is possible that the rapid decline in TNF-α levels and the lack of a repetitive stimulus for TNF-α release was the reason that our patients recovered so quickly with no deaths in this series.

Finally it should be borne in mind that much of the injury induced by TNF-α results from local production of the protein and its subsequent action at short range. Systemic levels of this cytokine only weakly reflect what is going on at the local level. Low levels in the circulation do not exclude high levels of TNF-α in the tissues and, inversely, high systemic levels do not necessarily reflect high concentrations in the tissues of the patient.

As far as management of these patients in the ICU is concerned, hypotension was the key problem and the presence of a Swan-Ganz catheter proved to be very valuable in its treatment. Colloids and cristalloids were infused to a pulmonary wedge pressure of 12 mmHg. However, large infusions of volume (mean of 10 L within the first 24 h) did not correct the hypotension in the majority of patients. Accordingly 24 out of 25 patients were treated with intermediate dose dopamine infusions (mean 5 μg/kg/min). The patient with the highest TNF-α level also needed norepinephrine to maintain adequate bloodpressure but this could not prevent the development of renal failure. Dopamine could usually be stopped the day following admission to the ICU and the Swan-Ganz catheter was removed a few hours

later. All patients but one retained good renal function. A large spontaneous diuresis followed the resolve of the sepsis syndrome on day 2. Patients were ventilated in a pressure support mode as soon as their ventilatory drive was restored following anesthesia. Signs of Adult Respiratory Distress Syndrome were usually discrete and only two patients needed high levels of positive end-expiratory pressure or inspiratory oxygen concentration to maintain adequate saturation. These two patients required mechanical ventilation for almost three weeks. Successful extubation was performed in 21 out of 25 patients within 24 hours. Clinically relevant coagulopathy was not observed in any patient.
There is little doubt that patients undergoing isolated limb perfusion with r-TNF-α will benefit from postoperative care in an ICU. Data derived from a Swan-Ganz pulmonary artery catheter are helpful to direct fluid administration and treatment with inotropes. Preferably a pulmonary artery catheter is introduced before the perfusion is started.

In conclusion this study shows that isolated limb perfusion with r-TNF-α leads to very high systemic TNF-α levels and transient serious signs of sepsis, pulmonary dysfunction and activated clotting, probably due to leakage of r-TNF-α from the perfusate. Despite high systemic TNF-α levels recovery in most cases is rapid and complete.

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Chapter III

Augmented procoagulant activity in cancer patients, treated with recombinant interferon gamma in addition to recombinant tumor necrosis factor alpha and melphalan

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Augmented procoagulant activity in cancer patients, treated with recombinant interferon-γ in addition to recombinant tumor necrosis factor-α and melphalan
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Summary

Several investigators have reported that interferon gamma can alter tumor necrosis factor alpha induced effects in vitro. We assessed in vivo effects of recombinant interferon gamma (r-IFN-γ) on recombinant tumor necrosis factor alpha (r-TNF-α) induced activation of systemic blood coagulation in a non-randomized study in 20 consecutive cancer patients. Eight patients were treated with r-IFN-γ prior to and during hyperthermic isolated limb perfusion with r-TNF-α and melphalan (IFN-γ group). They were compared with twelve patients who did not additionally receive r-IFN-γ (non-IFN-γ group).

Before start of perfusion, higher levels of TNF-α, prothrombin fragment 1 and 2 (F_{1+2})and thrombin-antithrombin-complexes (TAT) were found in the IFN-γ group. Fibrinogen and antithrombin III (ATIII) levels tended to be lower in this group. High TNF-α levels, due to leakage during perfusion, were associated with activation of coagulation in all patients, that became obvious after the end of perfusion, when heparin treatment had been antagonized. Activation, measured by increased F_{1+2} and TAT levels, was significantly stronger in the IFN-γ group. Monocytic tissue factor (TF) remained low, possibly due to shedding of TF positive vesicles and/or sequestration of TF positive activated monocytes against the vessel wall. In both groups F_{1+2} and TAT levels declined 24 hours after the perfusion, whereas monocytic TF increased to levels that were higher in the IFN-γ group.

In conclusion, our data confirm a strong activation of coagulation induced by r-TNF-α in cancer patients. They suggest that r-IFN-γ may lead to a slight activation of coagulation and augments TNF-α induced procoagulant activity. These effects may be due to r-IFN-γ induced sustained monocytic TF activity.

Introduction

Tumor necrosis factor alpha (TNF-α), an inflammatory mediator, has been demonstrated to play an important role in several pathological and experimental conditions [1-5]. It also has been associated with disturbances of the hemostatic balance, particularly changes of coagulation and fibrinolysis [6,7]. Administration of recombinant TNF-α (r-TNF-α) to both cancer patients [6] and healthy volunteers [7] resulted in activation of coagulation. Since the intrinsic pathway of coagulation was not activated [7], it seems likely that TNF-α acts by activation of the extrinsic route. Tissue factor (TF) is assumed to be the main in vivo initiator of this pathway [8-10]. Under normal conditions TF is not found on cells in direct contact with blood [11,12]. However, TF expression can be induced in vitro by TNF-α both in monocytes [13,14] and endothelial cells [15,16].

In vivo, several cytokines are present concomitantly or consecutively so that they may contribute to amplification or inhibition of their respective activities. It has been demonstrated that one of these cytokines, interferon gamma (IFN-γ), augments macrophage procoagulant activity induced by TNF-α in vitro [17]. Reports on in vivo and in vitro induction of TF expression by IFN-γ itself are inconsistent [18-21]. Recent in vitro findings suggest that adhesion of lymphocytes to IFN-γ stimulated endothelium results in TNF-α production and subsequent induction of endothelial TF [22]. In the present study, we assessed the effects on blood coagulation of recombinant IFN-γ (r-IFN-γ), administered to cancer patients in addition to r-TNF-α and melphalan.

Materials and Methods

Patients

Twenty consecutive patients with either advanced melanomas or nonresectable soft tissue tumors of a limb were treated by hyperthermic isolated limb perfusion [23]. Two different therapeutic regimes were applied (Fig. 1).

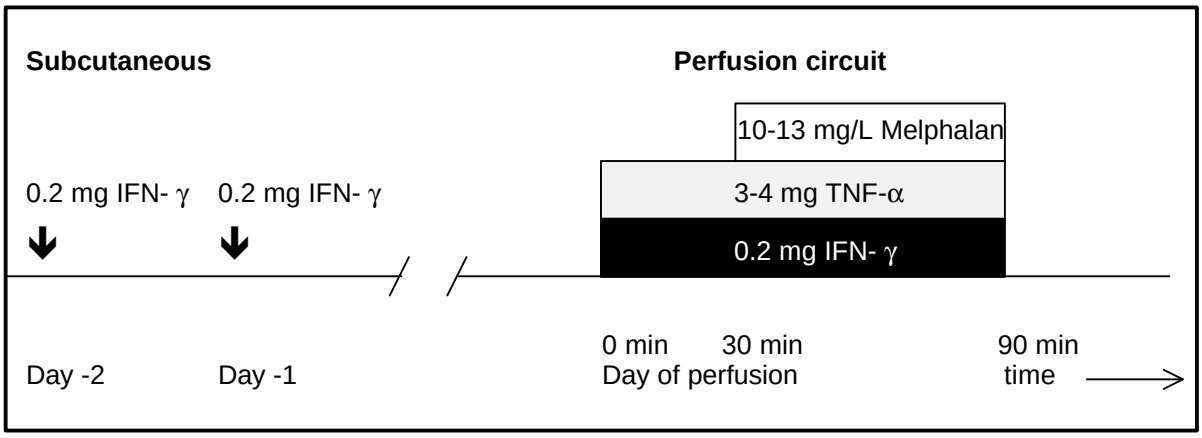

Fig. 1 **Treatment scheme. Eight patients received IFN-γ, TNF-α and melphalan (IFN-γ group), 12 patients only TNF-α and melphalan (non IFN-γ group).**

The first eight patients received a combination of r-IFN-γ, r-TNF-α and melphalan (IFN-γ group). The remaining twelve patients received only r-TNF-α and melphalan (non-IFN-γ group). Patients with abnormal hepatic and/or renal function were excluded. The study protocol was approved by the medical ethical committee of our hospital.

Hyperthermic isolated limb perfusion
The perfusion technique employed is based on a technique developed by Creech and Krementz [24]. Briefly, after ligation of all collateral vessels and heparinization of the patient with 3,3 mg heparin/kg body weight iv, either the axillary, iliac, femoral or popliteal vessels were dissected, cannulated and connected to an extracorporeal circuit. A tourniquet was applied to the proximal limb in an attempt to minimize leakage of the perfusate into the systemic circulation. Perfusion was performed during 90 minutes under mild hyperthermic conditions (39-40°C). The perfusate consisted of 350 ml 5% dextran 40 in glucose 5%, 500 ml blood products (250 ml red blood cells, 250 ml plasma), 30 ml 8.4% $NaHCO_3$ and 0.5 ml 5000 IU/ml heparin (Thromboliquine^R). At the start of the perfusion r-TNF-α (Boehringer Ingelheim, Ingelheim, Germany; 4 mg for leg perfusions and 3 mg for arm perfusions) was injected as a bolus into the arterial line of the perfusion circuit. Melphalan (Burroughs Wellcome, London, England, 10 mg/L volume of an affected leg and 13 mg/L volume of an affected arm) was administered 30 minutes later. Treatment with r-IFN-γ consisted of a daily subcutaneous injection with 0.2 mg r-IFN-γ on the two days preceding perfusion and a bolus injection of 0.2 mg r-IFN-γ into the perfusion circuit. After 90 minutes of perfusion, the limb was flushed with 2 L dextran 40 in glucose 5% and 500 ml blood products (250 ml red blood cells, 250 ml plasma), catheters were removed, the circulation restored and heparin antagonized with protamine chloride. A lateral fasciotomy of the anterior compartment of the lower leg was performed in leg perfusions or a fasciotomy of the forearm in arm perfusions to prevent a compartment syndrome.

Sample collection
Blood samples were collected from an indwelling radial artery cannula and anticoagulated with either EDTA (TNF-α measurements) or 1/10th volume of 0.109 mol/L trisodium citrate, pH 6.0 (all other measurements). Samples were taken before start of the perfusion, 5 minutes after starting perfusion, 1 min before ending perfusion, and 5 min, and 2 and 24 hours after restoration of the circulation. Collected blood samples were kept on melting ice during transport to the laboratory. Plasmas were prepared by centrifugation at 2000 x g and subsequently at 14,000 x g to remove residual platelets and stored at -80 °C until analysis. Mononuclear cell (MNC) suspensions were obtained by density-gradient centrifugation on Ficoll-Hypaque.

Assays
TNF-α concentrations were measured using a specific immunoradiometric assay (Medgenix Diagnostics, Soesterberg, The Netherlands). Prothrombin fragment 1+2 (F_{1+2}) and thrombin-antithrombin III complex (TAT) levels were measured using enzyme linked immunosorbent assays (ELISA) provided by Baxter, Miami, Florida (USA) and Behringwerke, Marburg, (Germany), respectively. Normal values for F_{1+2} ranged (geometric mean ± 2SD) from 0.08 to 0.51 nmol/L and for TAT from 1.0 to 4.1 mg/L. Levels of antithrombin III (ATIII) and fibrinogen (Fg) were measured by standard laboratory methods. Normal values ranged from 74 to 113% and 1.7 to 3.5 g/L, respectively. Monocytic procoagulant activity was measured in cell lysates, prepared by resuspending the MNCs in assay buffer (10 mmol/L HEPES, 137 mmol/L NaCL, 4 mmol/L KCL, 11 mmol/L D-glucose, 5 mg/ml of ovalbumin and 2.5 mmol/L $CaCL_2$, pH 7.45) and subsequent exposure to three freeze-thaw cycles (-80°C/37°C). TF activity was determined by a two-stage amidolytic assay [25]. Since TF activity is exclusively generated by monocytes in this system, monocytes

were not purified from the mixed mononuclear cell population before estimating monocytic procoagulant activity and the data were expressed as TF activity/monocyte. The estimated numbers of monocytes were calculated from its proportion in the MNC fraction of whole blood multiplied by the number of isolated MNCs. Normal values for TF, estimated in a group of 12 healthy individuals, comparable in age and sex with the treatment groups, ranged from 0 to 227 fmol Xa/min/10^6 cells.

Statistical analysis.
TF, F_{1+2} and TAT data were LOGe-transformed, because they were skewed. Differences between the medians or means of both groups were analyzed using the program CIA (confidence interval analysis) [26]. When the 95% or 99% confidence intervals for the mean levels at separate time points did not overlap the normal range, the values were considered significantly different from normal. When the 95% or 99% confidence intervals for the difference between medians (TNF-α measurements) or means (all other parameters) of both groups did not contain zero, the difference was considered statistically significant. The course of monocytic TF activity was analyzed with the random coefficient model.

Results

Before perfusion, the IFN-γ group as compared with the non-IFN-γ group showed a higher median level of TNF-α (25 vs 10 ng/L, p<0.01), and higher mean levels of F_{1+2} (0.51 vs 0.23 nmol/L, p<0.01, Fig. 2) and TAT (12.1 vs 4.6 mg/L, p<0.01, Fig. 3).

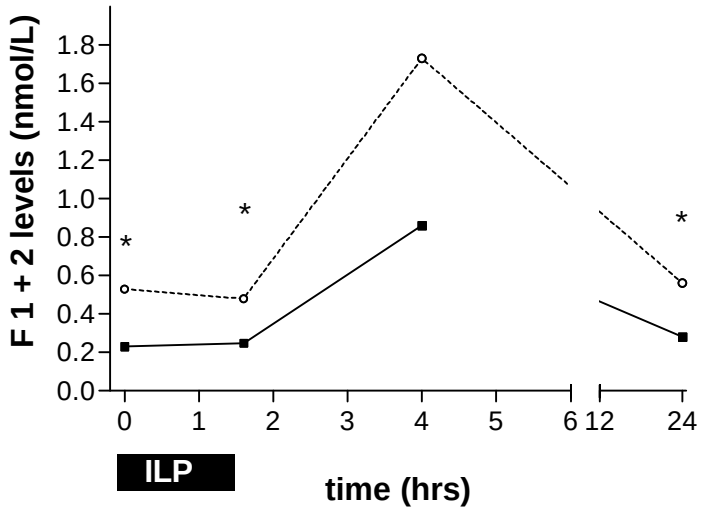

Fig. 2 **Geometric means of F_{1+2} (nmol/L) in patients who were treated by hyperthermic isolated limb perfusion (ILP) with TNF-α and melphalan, with (interrupted line) or without (solid line) IFN-γ. * : p<0.01**

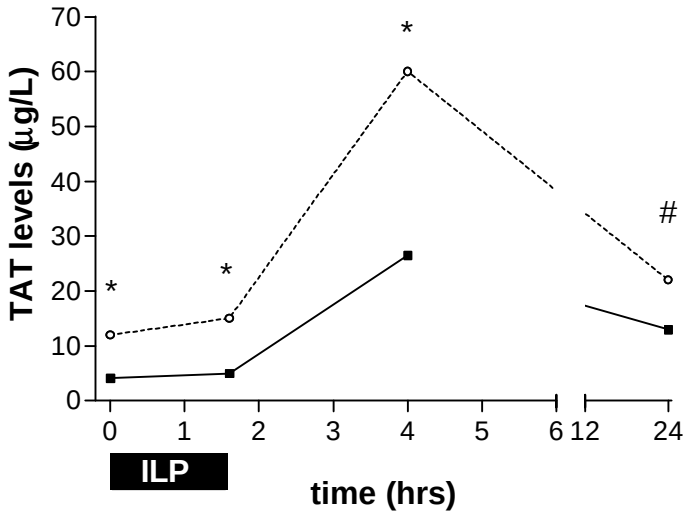

Fig. 3 **Geometric means of TAT (mg/L) levels in patients who were treated by hyperthermic isolated limb perfusion (ILP) with TNF-α and melphalan, with (interrupted line) or without (solid line) IFN-γ. * : p<0.01. #: p<0.05.**

Mean levels of TF (184 vs 145 fmol Xa/min/10^6cells, Fig. 4), ATIII (63 vs 73%) and Fg (2.72 vs 3.31 g/L) were not significantly different in both groups.

TNF-α concentrations increased during perfusion (IFN-γ group, 24180 ng/L and non-IFN-γ group, 18159 ng/L), followed by a decline after perfusion. At 24 hours, TNF-α concentrations remained elevated as compared with baseline values (123 and 122 ng/L, respectively). There were no statistically significant differences between the two groups.

F_{1+2} and TAT levels did not change during perfusion (Fig. 2 and 3). They increased afterwards with maximum levels measured two hours after perfusion. The differences present at baseline were maintained over time. At 24 hours, F_{1+2} levels approximated baseline values (IFN-γ group, 0.54 nmol/L and non-IFN-γ group, 0.28 nmol/L; $p<0.01$), while TAT levels, although declined, were still elevated (21.2 vs 13.1 mg/L, $p<0.05$).

AT-III and Fg levels in both groups decreased slightly during perfusion. Fg levels returned to pre-perfusion levels at 24 hours, while ATIII levels remained lowered. There were no statistically significant differences between the two groups (data not shown).

Although the difference in monocytic procoagulant activity between the two treatment groups was not statistically significant at the end of the perfusion, monocytic TF activity showed a different course in both groups, as was demonstrated by the random coefficient model (Fig. 4).

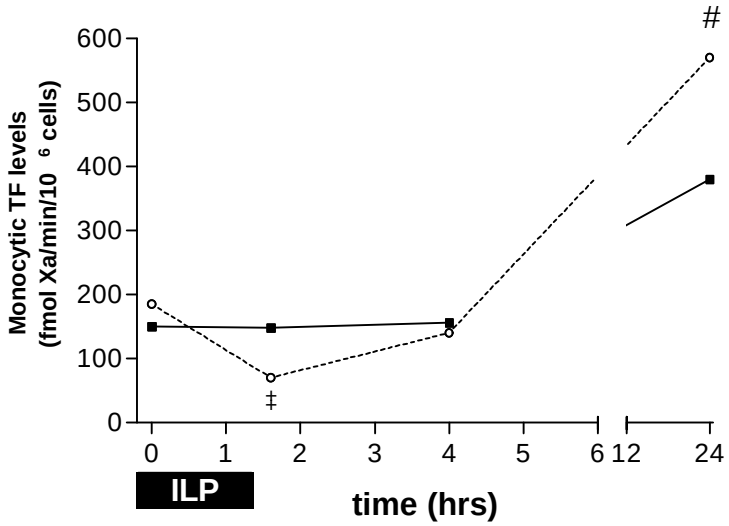

Fig. 4 **Geometric means of monocytic TF activity (fmol Xa/min/10^6 cells) in patients who were treated by hyperthermic isolated limb perfusion (ILP) with TNF-α and melphalan, with (interrupted line) or without (solid line) IFN-γ. # : $p<0.05$ (comparison between the two groups), ‡ : $p<0.05$ (compared with baseline in the IFN-γ group).**

A quadratic equation resembled the course in the IFN-γ group (p=0.0019), while a linear equation was found in the non-IFN-γ group (p=0.02). A significant, more than two-fold decrease of monocytic TF activity was found in the IFN-γ group during perfusion, while the levels in the non-IFN-γ group did not change significantly. Two hours after the end of perfusion, monocytic TF activity had returned to its approximate pre-perfusion level in the IFN-γ group, while TF levels in the non-IFN-γ group had remained unchanged. At 24 hours, TF levels in both groups showed a clear increase to levels that were higher in the IFN-γ group than in the non-IFN-γ group (560 vs 380 fmol Xa/min/10^6 cells, $p<0.05$).

Discussion

We studied the effects of r-IFN-γ treatment on r-TNF-α induced activation of coagulation in patients with a malignancy of a limb. Twelve of these patients, who were treated by hyperthermic isolated limb perfusion with r-TNF-α and melphalan, were compared with 8 patients, who additionally received r-IFN-γ prior to and during perfusion (Fig. 1).

Patients who had received r-IFN-γ for two days showed higher levels of TNF-α, F_{1+2} and TAT prior to perfusion, as compared with controls. Levels of ATIII and fibrinogen tended to be lower. These differences might be due to r-IFN-γ induced monocytic TF activity. In vitro studies have provided conflicting data on the potency of IFN-γ to induce TF [18-21]. Nevertheless, it has been demonstrated that IFN-γ increases macrophage TNF-α production [27] and more recently, Schmid et al. showed that adhesion of lymphocytes to IFN-γ stimulated cultured endothelium resulted in TNF-α production with subsequent induction of endothelial TF [22]. These observations are consistent with our findings, showing higher F_{1+2} and TAT levels that coincided with an elevated endogenous TNF-α level in the IFN-γ group. IFN-γ possibly acts indirectly on the coagulation system by increasing TNF-α activity.

Five minutes after start of limb perfusion, systemic TNF-α levels strongly increased in all patients. Apparently, significant leakage of r-TNF-α occurred in spite of isolated limb perfusion. As expected, considering that TNF-α-induced monocytic TF expression peaks after six hours [18] and a high dose of heparin was administered, there were no signs of activation of coagulation during perfusion. Accordingly, F_{1+2} and TAT levels did not change. A simultaneous decrease of ATIII and Fg was probably due to dilution by massive fluid infusion, rather than consumption of these proteins secondary to activated coagulation.

Consistently, systemic monocytic TF activity did not change markedly during perfusion in the non-IFN-γ group. However, a statistically significant and more than two-fold decrease was observed in the IFN-γ group. Several mechanisms could account for this apparently paradoxical decrease in TF activity, including neutralization by an inhibitor, shedding of TF containing membrane vesicles, or loss of peripheral circulating TF positive monocytes. Increased neutralizing activity by inhibitors, like tissue factor pathway inhibitor or ATIII, possibly potentiated by heparin [28,29], is unlikely. This would have resulted in a reduction of TF activity to the same extent in both groups. Moreover, IFN-γ has not, to our knowledge, been reported to stimulate expression of one of these inhibitors [30]. Shedding of TF-rich vesicles has been observed from the surface of tumor cells [31], fibroblasts [32], and monocytes [33]. Accordingly, recent findings have demonstrated that TNF-α only causes shedding of L-selectin [34] or leukocytic activation [35,36] in the presence of secondary stimuli. Thus, loss of monocytic TF by shedding of TF-rich vesicles induced by TNF-α in the presence of IFN-γ, might be a more valid explanation.

Alternatively, we speculate that migration of TF positive monocytes from the systemic circulation could have contributed to the decrease in measured monocytic TF activity. The latter view is supported by reports showing that IFN-γ can promote upregulation of specific adhesion molecules for adhesion of monocytes to endothelium [37,38]. In this study, we found, in line with previous findings [39], that MNC counts decreased rapidly in all patients after start of the perfusion (data not shown), suggesting peripheral consumption rather than bone marrow suppression. Because we did not purify monocytes from the mixed MNC population before estimating monocytic procoagulant activity, we can not provide direct evidence for loss of TF positive monocytes from the systemic circulation.

After heparin had been antagonized at the end of perfusion, a strong increase in F_{1+2} and TAT levels was observed in both treatment groups, in agreement with previous reports on activation of coagulation by TNF-α administered to cancer patients and healthy humans [6,7]. It should be noticed that actual F_{1+2} and TAT levels were higher if corrected for dilution. Moreover, our data suggest that the effects of r-TNF-α on coagulation are potentiated by r-IFN-γ, as F_{1+2} and TAT levels remained higher in the IFN-γ group.

However, monocytic TF activity showed only a limited increase in the IFN-γ group, while in the non-IFN-γ group the levels even remained unchanged, despite pronounced activation of coagulation in both treatment groups. Perhaps mechanisms like induction of endothelial TF or concentration of monocytes at vessel wall sites thus supporting TF-independent, factor VIIa-mediated activation of factor X [40] might have been the cause of this apparent discrepancy. Alternatively, activation of coagulation might have been due to TF, expressed on shed vesicles and/or on the surface of adherent, activated monocytes. Monocytic TF activity, as measured in the systemic circulation under our experimental conditions, probably depends on the balance between induced expression of monocytic TF and loss of it through shedding of TF positive vesicles and/or sequestration of TF positive activated monocytes. Because we measured only a limited increase in monocytic TF activity, despite pronounced activation of coagulation, we hypothesize that most of the monocytic TF was shed and/or most of the TF positive monocytes participated in vessel wall associated coagulation.

Consistent with the latter hypothesis, we found 24 hours after perfusion in both treatment groups low F_{1+2} and TAT levels concomitantly with increased monocytic TF, possibly residual from maximal induction

by TNF-α earlier after perfusion, considering that TNF-α-induced monocytic TF expression peaks after six hours [18]. The higher level of monocytic TF in the IFN-γ group at that time might be attributed to a late effect of r-IFN-γ, as IFN-γ induced TF activity is maximal at 24 hours [18].

Because of the non-randomized design of this study and the small number of patients, the observed differences between the two groups might be attributed to selection bias. However, consecutive patients were enrolled and there were no differences in clinical baseline characteristics between the two groups. Another more important limitation is the absence of measurements two days before perfusion, to assure comparability of the groups with regard to the reported parameters prior to r-IFN-γ treatment.

In conclusion, the results of this study in cancer patients confirm previous reports on a strong activation of coagulation induced by r-TNF-α administration. Furthermore, our data suggest that r-IFN-γ may lead to a slight activation of coagulation, due to increased endogenous production of TNF-α, and augments TNF-α induced procoagulant activity by sustained induction of monocytic TF.

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Chapter IV

Effects of hyperthermic isolated limb perfusion with recombinant tumor necrosis factor alpha and melphalan on the human fibrinolytic system

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Effects of hyperthermic isolated limb perfusion with recombinant tumor necrosis factor alpha and melphalan on the human fibrinolytic system
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Summary

This study was undertaken to determine the effects on systemic fibrinolysis of hyperthermic isolated limb perfusion with recombinant tumor necrosis factor alpha (r-TNF-α) and melphalan, with or without pretreatment with recombinant interferon gamma (r-IFN-γ). Twenty patients were treated with r-TNF-α and melphalan; four patients, treated with melphalan only, served as controls. Of the twenty patients treated with both r-TNF-α and melphalan, eight received r-IFN-γ for two days before the perfusion and as a bolus into the perfusion circuit. A significant leak of r-TNF-α from the perfusion circuit to the systemic circulation was observed in all r-TNF-α treated patients (mean maximum TNF-α 87227 ng/liter *versus* 31 ng/liter in controls, $p<0.002$). In these patients, but not in controls, there was an almost instantaneous rise in systemic tissue plasminogen activator (t-PA) activity (from 0.26 IU/ml to 5.28 IU/ml in 90 min), causing activation of fibrinolysis. After a delay of 90 minutes, plasminogen activator inhibitor-1 (PAI-1) antigen rose to high levels in the r-TNF-α treated group (mean maximum PAI-1 1652 ng/ml *versus* 211 ng/ml in controls, $p<0.02$), associated with a sharp decrease of tPA-activity and a slower decrease of plasminogen-antiplasminogen complexes (from 5.28 IU/ml to 0.02 IU/ml in 2 h, and from 1573 μg/L to 347 μg/L in 22 h respectively). No additional effect of IFN-γ pretreatment on fibrinolysis could be demonstrated. These results suggest that in isolated limb perfusion with r-TNF-α and melphalan an initial activation of systemic fibrinolysis, induced by leakage of r-TNF-α from the perfusion circuit, is set off by a subsequent inhibition of the fibrinolytic system by PAI-1. This large increase in PAI-1 could place the patient at risk for deposition of microthrombi in the systemic circulation.

Introduction

Isolated limb perfusion with cytotoxic drugs is used in patients with nonresectable soft tissue tumors and locally advanced melanomas of a limb, as an alternative to amputation [1,2]. It allows the administration of high doses of cytostatic agents locally while minimizing systemic toxicity. Traditionally an alkylating agent like melphalan has been added to a mildly hyperthermic perfusate. Recently melphalan has been combined with recombinant tumor necrosis factor alpha (r-TNF-α) in an attempt to maximize the anti-tumor effect of the perfusion [3-5]. Some of these patients have been pre-treated with recombinant interferon gamma (r-IFN-γ) to enhance the sensitivity of the tumor to r-TNF-α [3-5]. Human r-IFN-γ increases the number of TNF-receptors on human tumor cells [6, 7]. Additionally, r-TNF-α and r-IFN-γ show synergy in antitumor effects on human tumor cells and on human melanoma xenografts in nude mice [8-10].

It has been recognized by us, as well as by others, that isolated limb perfusion with r-TNF-α induces a sepsis like state in all patients, characterized by fever, tachycardia and a low blood pressure due to systemic vasodilation [11, 12]. Vigorous fluid resuscitation and vasopressor therapy are usually required to maintain adequate tissue perfusion. The sepsis response can be quite severe but is remarkably short-lived: most patients can be discharged from the intensive care unit on the day after perfusion. The occurrence of this syndrome is explained by leakage of r-TNF-α from the perfused limb into the systemic circulation; very high levels of TNF-α have been documented in peripheral blood of these patients during and directly after perfusion. Leakage has been confirmed by adding radiolabeled albumin to the perfusate, which can be traced to the systemic circulation during the procedure [13]. Lower perfusate flow rates have been reported to reduce systemic leakage and attenuate side effects, probably by reducing vascular pressures in the isolated limb [14]. A thorough washout procedure at the end of perfusion may also contribute to a reduction of leakage and systemic side effects [12].

In vitro and *in vivo* experiments have shown the effects of TNF-α on blood coagulation and fibrinolysis to be profound, although the mechanism is still incompletely understood [15]. Because patients treated with isolated limb perfusion with r-TNF-α show high systemic TNF-α levels, it was hypothesized that blood coagulation and fibrinolysis in these patients may be profoundly disturbed, especially during and directly after perfusion. In theory, either a bleeding diathesis or, conversely, a prothrombotic state could well occur as a consequence of the treatment and expose the patient to an additional risk.

The aim of this study was to investigate the effects of isolated limb perfusion with r-TNF-α and melphalan on systemic fibrinolysis. Because part of the study population was additionally treated with r-IFN-γ before and during perfusion the added effects of r-IFN-γ on fibrinolysis were also studied.

Patients and Methods

Outline of Experiments.
Patients treated with r-TNF-α (with or without additional treatment with r-IFN-γ) were compared with control patients who received melphalan only. Subsequently, patients additionally treated with r-IFN-γ were compared with patients who had no such additional treatment, in order to evaluate the effects of r-IFN-γ in addition to r-TNF-α.
Blood samples (before, during and after perfusion) were taken at regular intervals to determine TNF-α levels and parameters of fibrinolysis. Activation of the fibrinolytic system was monitored by measuring t-PA antigen and activity. Inhibition of the fibrinolytic system was measured by determining levels of PAI-1. The balance between activation and inhibition was assessed by determining FbDP (fibrin degradation products) and PAP (plasminogen - anti-plasminogen complexes).

Subjects.
Between April 1993 and June 1994, 24 patients received hyperthermic isolated limb perfusion at the division of surgical oncology of the Groningen University Hospital after approval of the medical ethical committee and informed consent had been obtained. Of these 24 patients, 12 received melphalan and r-TNF-α without additional treatment with r-IFN-γ, 8 received melphalan and r-TNF-α with additional r-IFN-γ treatment and the remaining 4 were treated with melphalan only.

Anesthesia and Intensive Care.
Anesthesia was induced with thiopental, after which the patients were paralyzed with vecuronium and the trachea was intubated. Anesthesia was maintained with midazolam, sufentanyl, nitrous oxide and isoflurane. All patients were monitored invasively and admitted to the intensive care unit after surgery.

Isolated Limb Perfusion.
The perfusion technique used at the Groningen University Hospital is based on the technique developed by Creech and Krementz [16]. Briefly, after ligation of all collateral vessels and heparinization of the patient with 3.3 mg heparin/kg (Thromboliquine^{R}, Organon BV, Oss, the Netherlands) the axillary, iliac, femoral or popliteal vessels were dissected, cannulated and connected to the extracorporeal circuit. The perfused limb was wrapped in a thermal blanket to reduce heat loss and four thermistor probes were inserted subcutaneously and intramuscularly for continuous monitoring of the temperature during perfusion. A tourniquet was applied to the proximal limb in an attempt to minimize leakage of the perfusate into the systemic circulation through skin collaterals. Perfusion was performed for 90 minutes under mildly hyperthermic conditions (39-40 ^{0}C). The perfusate consisted of 350 ml 5% dextran 40 in glucose 5% (Isodex^{R}, Pharmacia AB, Uppsala, Sweden), 500 ml blood (250 ml red blood cells, 250 ml plasma), 30 ml 8.4% $NaHCO_3$ and 0.5 ml 5000 IU/ml heparin (Thromboliquine^{R}). The perfusate was oxygenated with a bubble oxygenator and driven by a roller pump. At the start of perfusion r-TNF-α (Boehringer Ingelheim, Germany, 4 mg for leg perfusions and 3 mg for arm perfusions) was injected as a bolus into the arterial line of the perfusion circuit. Melphalan (Burroughs Wellcome, London, England, 10 mg/L volume of an affected leg and 13 mg/L volume of an affected arm) was administered 30 minutes later. Pretreatment with r-IFN-γ consisted of a daily subcutaneous injection with 0.2 mg r-IFN-γ (Boehringer, Germany) on the two days preceding the perfusion and a bolus injection of 0.2 mg r-IFN-γ into the perfusion circuit. During perfusion potential leakage to the systemic circulation was monitored with I^{131} -labeled albumin [6]. After 90 minutes of perfusion, the

limb was flushed with 2 L dextran 40 in glucose 5% (Isodex^{R}) and 500 ml blood (250 ml red blood cells, 250 ml plasma), catheters were removed, the circulation was restored and the heparin was antagonized with protamine chloride. A lateral fasciotomy of the anterior compartment of the lower leg (in leg perfusions) or a fasciotomy of the forearm (in arm perfusions) was performed to prevent a compartment syndrome.

Blood samples.
Blood samples were drawn from an indwelling radial artery line before cannulation (t=0), 5 min after starting perfusion (t=1), 1 min before ending perfusion (t=2), 5 min after normal circulation was restored (t=3), 2 hours thereafter (t=4) and finally after 24 hours (t=5). Samples were collected in either EDTA Stabylite Vacutainer^{R} tubes or in citrate-containing tubes, and kept on melting ice during transport to the laboratory. Samples were centrifuged for 10 min at 3000 g at 0^{o}C. Plasma was stored at -80^{o}C until analysis.

Immunochemical Analyses.
TNF-α levels were determined by specific immunoradiometric assay (Medgenix Diagnostics, Soesterberg, the Netherlands). Samples were processed according to the guidelines of the manufacturer. FbDP were measured with an ELISA (Fibrinostika FbDP, Organon Teknika, Turnhout, Belgium) and PAP with an ELISA (EIA APP micro, Behringwerke AG, Marburg, Germany). t-PA antigen was measured with an ELISA (Asserachrom tPA, Stago, Boehringer, Mannheim, Germany). t-PA activity was determined in a bioassay (Chromolize-t-PA, Biopool, Umeå, Sweden). PAI-1 antigen was measured with an ELISA (Innotest-PAI-1, Innogenetics, Antwerp, Belgium).
Normal values for FbDP and PAP ranged from 90 - 500 ng/ml and from 80 - 470 μg/L respectively. Normal ranges, as indicated by the manufacturer, for t-PA antigen, t-PA activity and PAI-1 antigen ranged from 0 - 5 ng/ml, from 0.0 - 1.0 IU/ml and from 0 - 40 ng/ml respectively.

Statistical Analysis.
Data were analyzed using SPSS for MS WINDOWS (release 5.0). The overall effect of perfusion with TNF-α on each separate parameter of fibrinolysis was assessed by comparing differences from baseline values (Δ FbDP, Δ PAP, Δ t-PA, Δ PAI-1) between TNF-α treated patients and controls, using a Kolmogorov-Smirnov test for nonparametrically distributed values. Differences in each separate parameter at different time points between TNF-α treated patients and controls were assessed with a Mann Whitney U rank sum test for nonparametrically distributed values. A p-value <0.05 was considered significant.

Results

All patients who received r-TNF-α showed an increase in systemic TNF-α-levels (Figure 1).

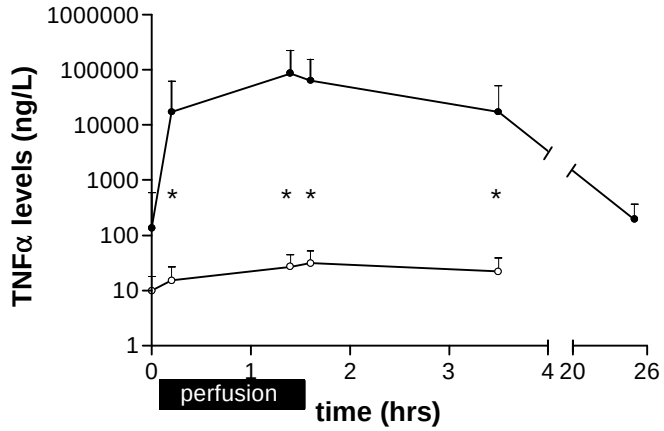

Fig. 1. Mean systemic TNF-α-levels in r-TNF-α treated patients (closed circles) and in controls (open circles) over time. Hyperthermic isolated limb perfusion is indicated by the black box. Statistically significant differences are marked with an asterisk (*). Error bars represent standard deviation.

Systemic levels of TNF-α varied over a wide range, but mean values were significantly higher in the r-TNF-α-treated group (mean maximum TNF-α-levels 87227 ng/L versus 31 ng/L in controls, $p<0.002$). Peak levels were reached just before the end of perfusion or 5 min after recirculation. Overall levels of mean t-PA activity were higher in the r-TNF-α group ($p < 0.02$ Figure 2).

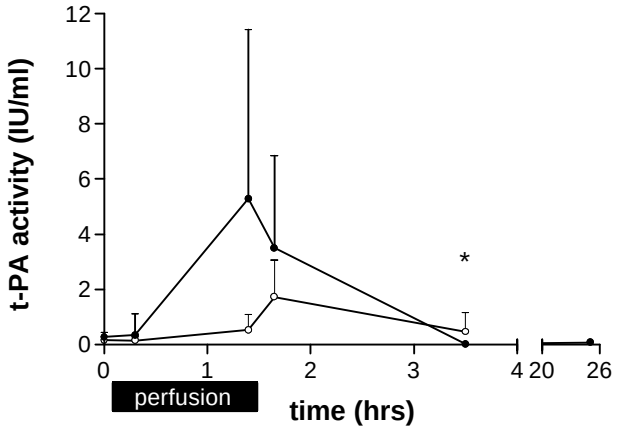

Fig. 2 Mean t-PA activity in r-TNF-α treated patients (closed circles) and in controls (open circles) over time. Hyperthermic isolated limb perfusion is indicated by the black box. Statistically significant differences are marked with an asterisk (*). Error bars represent standard deviation.

Mean levels ranged from 0 to 6 IU/ml. t-PA activity in the r-TNF-α-treated group peaked during perfusion; 2 hours later no t-PA activity could be demonstrated.

t-PA antigen levels were also higher in r-TNF-α-treated patients than in controls ($p < 0.01$) and remained elevated for 2 hours after perfusion (Figure 3).

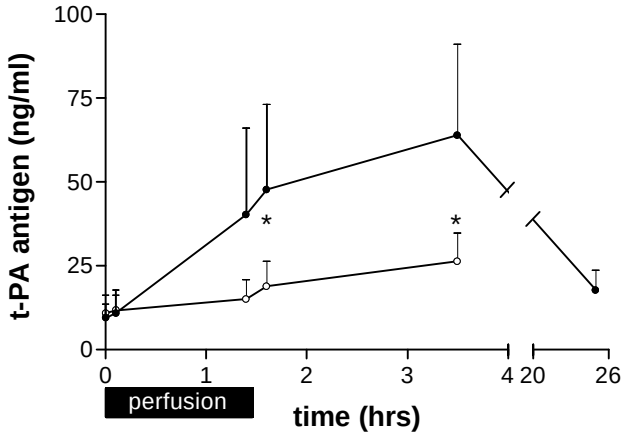

Fig. 3 Mean t-PA antigen levels in r-TNF-α treated patients (closed circles) and in controls (open circles) over time. Hyperthermic isolated limb perfusion is indicated by the black box. Statistically significant differences are marked with an asterisk (*). Error bars represent standard deviation.

Individual time point differences were significant at t=3 (5 min after normal circulation was restored, $p < 0.01$) and at t=4 (2 hours after ending the perfusion, $p < 0.03$).
After activation of the fibrinolytic system in the r-TNF-α-perfused group, a sharp rise in PAI-1 antigen was observed (Figure 4).

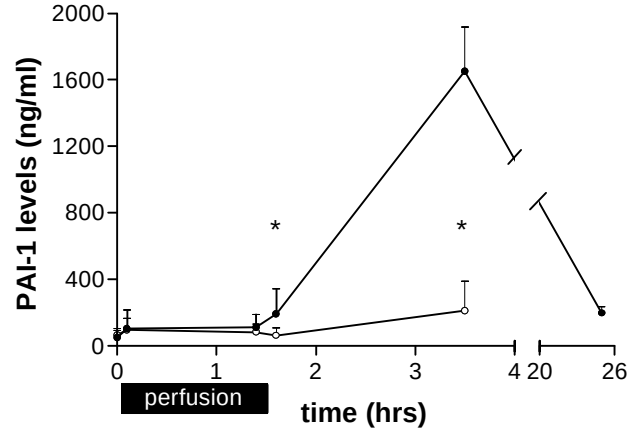

Fig. 4 Mean PAI-1 levels in r-TNF-α treated patients (closed circles) and in controls (open circles) over time. Hyperthermic isolated limb perfusion is indicated by the black box. Statistically significant differences are marked with an asterisk (*). Error bars represent standard deviation.

Mean levels ranged between 50 and 1652 ng/ml. Overall levels were higher in the r-TNF-α-treated group ($p < 0.005$). The peak level was observed 2 hours after the end of perfusion. For individual time

points differences were significant at t=3 (5 min after normal circulation was restored, p< 0.05) and at t=4 (2 hours after ending the perfusion, p < 0.02).

There were definite signs of activation of the fibrinolytic system in the group perfused with r-TNF-α.

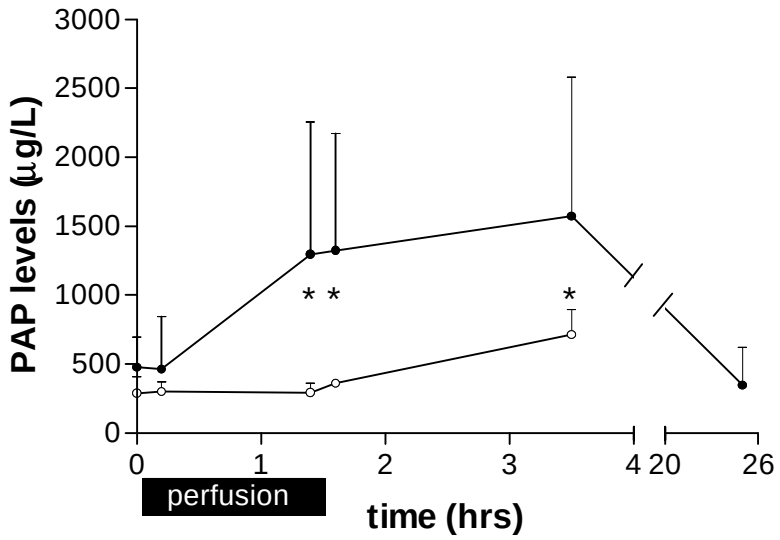

Fig. 5 Mean PAP-levels in r-TNF-α treated patients (closed circles) and in controls (open circles) over time. Hyperthermic isolated limb perfusion is indicated by the black box. Statistically significant differences are marked with an asterisk (*). Error bars represent standard deviation.

Mean PAP levels ranged from 200 to 1500 mg/l. Overall, PAP levels were higher in the r-TNF-α-perfused group (p < 0.03, Figure 5).
For individual time points differences were significant at t=2 (end of perfusion, p < 0,01), t=3 (5 min after normal circulation was restored, p < 0.005) and t=4 (2 hours after ending the perfusion, p<0.05). PAP levels returned to baseline at 24.hours.

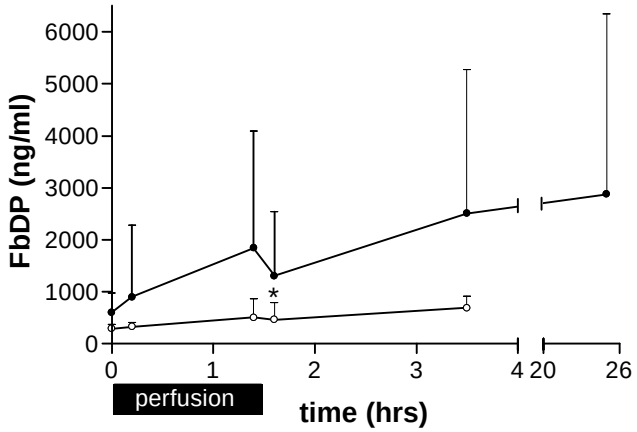

Fig. 6 Mean FbDP levels in r-TNF-α treated patients (closed circles) and in controls (open circles) over time. Hyperthermic isolated limb perfusion is indicated by the black box. Statistically significant differences are marked with an asterisk (*). Error bars represent standard deviation.

FbDP levels in r-TNF-α-treated patients and in controls showed a similar course (Figure 6). Median levels ranged from 250 ng/ml to 3000 ng/ml. Overall levels were higher in the r-TNF-α-treated group ($p < 0.03$). Differences for individual time points were significant at t=3 (5 min after normal circulation was restored, $p < 0.05$). Due to large differences in FbDP levels between individual patients in the r-TNF-α-treated group, no significant changes in mean FbDP level within this group could be demonstrated once normal circulation was restored.

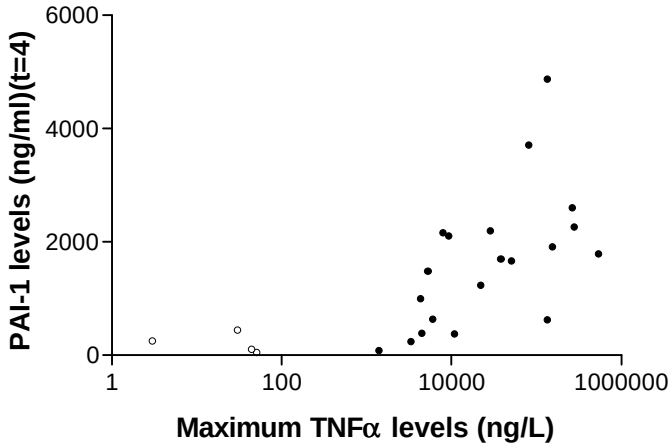

Fig. 7 Correlation between maximum systemic TNF-α levels (log scale) and maximum systemic PAI-1 levels (2 hours post-perfusion) in r-TNF-α treated patients (closed circles) and controls (open circles).

Figure 7 shows the relationship between maximum TNF-α-levels, measured from arterial blood, and PAI-1 antigen levels at t=4 (2 hours post-perfusion), which were invariable the highest PAI-1 levels recorded in the study. There was a weak but statistically significant relationship ($p < 0.05$, r=0,4).

Individual data on maximum systemic TNF-α-levels, FbDP at t=3 and t-PA antigen, PAP and PAI-1 antigen at t=4 are shown in Table 1.

Patient nr	TNF- α ng/L (normal: 0-15)	FbDP ng/mL (normal: 90-500)	tPA antigen ng/mL (normal: 0-5)	PAP µg/mL (normal: 80-470)	PAI ng/ml (normal: 0-40)
1	4,328	537	149	537	995
2	135,242	3,298	46	1,806	4,870
3	5,325	2,997	83	1,812	1,489
4	9,400	1,945	53	1,750	2,107
5	283,500	465	35	881	2,264
6	82,000	3,494	100	2,426	3,707
7	29,000	1,935	60	1,978	2,198
8	51,100	2,649	89	3,906	1,665
9	6,096	522	75	471	635
10	546,000	796	38	946	1,793
11	157,000	9,978	79	2,486	1,917
12	22,300	511	52	466	1,239
13	1,393	428	37	176	88
14	3,850	682	66	948	1,703
15	10,878	295	55	202	371
16	134,000	1,059	50	1,388	626
17	3,347	467	44	253	236
18	8,039	484	63	1,039	2,155
19	4,491	678	66	580	391
20	267,000	3,712	38	1,867	2,604
Control 1	30	522	34	301	443
Control 2	51	198	15	293	47
Control 3	3	335	32	208	250
Control 4	44	999	24	371	103

Table 1 Individual values of maximum systemic TNF-α, FbDP (t=3), t-PA antigen (t=4), PAP (t=4) and PAI-1 antigen (t=4) in patients and in controls.

Finally, patients additionally treated with r-IFN-γ had levels of TNF-α, t-PA activity and antigen, PAP and PAI-1 antigen that did not differ significantly from values recorded in patients who received r-TNF-α without additional treatment with r-IFN-γ.

Discussion

TNF-α, originally defined by its anti tumor activity in vivo, is now recognized to play a key role as a polypeptide mediator in the pathogenesis of septic shock [17-23]. It has also been reported to profoundly influence the dynamic balance between procoagulant and fibrinolytic factors in the blood.

In the study presented here we have measured parameters of fibrinolysis in patients undergoing isolated limb perfusion with r-TNF-α. Perfusion with r-TNF-α, in combination with r-IFN-γ and melphalan, has recently been shown to yield high remission rates in patients with irresectable extremity soft tissue sarcomas and in patients with melanoma in-transit metastases. In a multicenter study of 55 patients with irresectable soft tissue sarcoma, a major tumor response was seen in 87% of the patients, rendering the tumor resectable in most cases [4]. Fraker and coworkers have recently reported a series of 38 patients with extremity melanoma with a complete response rate of 76% and an overall objective response rate of 92% [5]. Unfortunately, this type of treatment is not without systemic effects: due to leakage from the perfusion circuit a high, but short-lived peak in systemic TNF-α is observed during

and immediately after perfusion [11, 12]. We have found evidence of an initial enhancement of fibrinolytic activity as documented by a modest increase of PAP-levels and FbDP with a peak towards the end of perfusion and immediately following recirculation. The five-fold increase in t-PA activity, preceding the increase in PAP and FbDP, was of the same order of magnitude as has been described in prior studies [24, 25]. The most striking finding of our study was a sharp rise in PAI-1 antigen, that followed the increase in t-PA antigen. Its peak was reached at 2 hours after perfusion. At that time levels of t-PA activity fell dramatically, while t-PA antigen could be detected for many hours. The highest PAI-1 antigen levels were found in patients with the highest maximum systemic TNF-α levels. PAI-1 antigen levels in our perfusion model were 10 to 20 times higher than levels described in earlier studies with a different design [24, 25].

These data show that isolated limb perfusion with r-TNF-α results in high levels of TNF-α in systemic blood during and immediately after perfusion, which cause initial activation of fibrinolysis due to increase of t-PA antigen and activity. Subsequently, fibrinolysis is inhibited by a more pronounced increase in PAI-1 antigen with a simultaneous fall in t-PA activity, probably due to binding of t-PA to PAI-1. The increase in PAI-1 antigen is proportional to the maximum level of TNF-α, measured in the systemic arterial circulation of the patient. A similar two-stage response has been described in experimental and clinical sepsis, where TNF-α is also of pivotal importance [26-30]. The overall inhibitory effect on fibrinolysis in the septic patient is hypothesized to contribute to end-organ damage by disseminated intravascular coagulation, which is a frequent and severe complication of sepsis [15, 30, 31]. Although t-PA activity was not detectable at 2 hours after the start of perfusion, PAP levels were still elevated at that time, suggestive of ongoing formation of plasmin. This could be due either to a delayed clearance of PAP or to release from the tumor which was visibly necrotic at this stage. Pretreatment with r-IFN-γ did not influence any of the measured parameters of fibrinolysis in a statistically significant way.

The precise mechanism of the early increase in fibrinolysis remains unclarified by this study. A direct effect of TNF-α on endothelial cells to produce t-PA has been proposed, although in vitro effects are variable and dose dependent [25]. Alternatively, van Hinsbergh and coworkers have suggested that thrombin, generated by activation of the coagulation cascade, rather than TNF-α, is the actual trigger for the increased level of t-PA during treatment with r-TNF-α [32]. Our experiments have shown an increase in t-PA during the perfusion phase of the study, when the patients were adequately heparinized. This effectively rules out the presence of relevant amounts of circulating thrombin. Although it cannot be excluded that TNF-α induces generation of thrombin bound to endothelial cells, a direct effect of TNF-α on vascular endothelial cells would seem a more probable explanation. This conclusion is supported by experiments in chimpanzees, where the effects of TNF-α on fibrinolysis were not influenced by the administration of a monoclonal antibody against tissue factor, suggesting that the triggering of the fibrinolytic response was not dependent on the generation of thrombin, but a direct effect of TNF-α [33].

The large increase in PAI-1 antigen levels is probably also due to a direct effect of TNF-α on vascular endothelium. Increased production of PAI-1 antigen following incubation with TNF-α has been shown in human umbilical vein endothelial cells, in human umbilical artery endothelial cells and in human foreskin vascular endothelial cells [34, 35]. Rats treated intraperitoneally with human r-TNF-α showed a dose-dependent increase in PAI-1 activity [35, 36].

Silverman et al. have reported on cancer patients treated in various regimens with intravenously administered r-TNF-α, who reacted with a significant rise in t-PA activity, followed by a corresponding increase in PAI-1 activity [24]. After a 2 hour infusion with r-TNF-α all fibrinolytic

parameters returned to pretreatment values within 24 hours. Van Hinsbergh et al. have measured several indexes of fibrinolysis at 3 and 24 hours after a 24 hour continuous infusion of r-TNF-α in cancer patients. Fibrin- and fibrinogen degradation products as well as PAP complexes were increased after 24 hours [32]. Baars et al. could show that injection of interleukin-2 in cancer patients induced changes in fibrinolysis similar to those induced by r-TNF-α [37]. Van der Poll et all [25] described a series of six healthy human volunteers, treated with a single intravenous injection of 50 $\mu g/m^2$ r-TNF-α. A sharp rise in t-PA activity was observed reaching its maximum at 1 hour. Plasma levels of PAI-1 antigen did not change in the first hour following r-TNF-α administration, but peaked sharply thereafter, with a maximum PAI-1 level attained at 3 hours. D-dimer levels were also increased, reaching a summit after 1 hour and PAP-levels increased transiently with a peak at 45 min. The authors concluded that injection of r-TNF-α induces a rapid activation and a subsequent inhibition of the fibrinolytic system in human volunteers [25].

In the study presented here the effects of TNF-α on fibrinolysis were analyzed in an entirely different model. It also differs from earlier studies by Silverman [24] and van Hinsbergh [32] in that its design includes a control group treated in exactly the same way but without the use of r-TNF-α. Although TNF-α levels were not reported in the study of van der Poll on fibrinolysis [25], their study on coagulation [38], performed in the same small group of healthy volunteers, yielded mean peak TNF-α levels of 4261 ± 785 pg/ml. Peak TNF-α levels recorded in our patients were twice as high (mean maximum TNF-α 87227 ng/L. Moreover, high TNF-α levels persisted for much longer in our study.

Our study has several limitations. The control group of patients treated with perfusion with melphalan but without r-TNF-α was small and patients were not randomly assigned to either treatment arm. Due to its dramatic effects on tumor regression, perfusions without r-TNF-α came to be considered ethically unjustified. Obviously, this made any form of randomization impossible. Another source of variation is the variable extent to which leakage of r-TNF-α from the perfusion circuit to the systemic circulation occurred. This is reflected in widely varying levels of peak systemic TNF-α (range 1393 to 546000 ng/L). Such variation is inherent in the perfusion / leakage model used in these experiments. The response of parameters of fibrinolysis however was remarkably uniform in all patients.

It may well be that, in the treatment of cancer with r-TNF-α, the effects of this cytokine on coagulation and fibrinolysis are important for its antitumor potential. Tumor vasculature seems to be disproportionally sensitive to TNF-α, and vascular destruction precedes regression of the tumor in many cases [39]. However, as our experiments have shown, even with the technique of isolated limb perfusion an inhibition of the *systemic* fibrinolytic system, by a large increase of PAI-1 and decrease of t-PA activity, cannot be prevented, which might prove to be detrimental. As in sepsis, it may place the patient at danger of extensive deposition of thrombi in the systemic microvasculature and subsequent damage to multiple organ systems, especially if activation of the coagulation system occurs at the same time.

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Chapter V

Renal function in cancer patients treated with hyperthermic isolated limb perfusion with recombinant tumor necrosis factor alpha and melphalan

In press as:
Renal function in cancer patients treated with hyperthermic isolated limb perfusion with recombinant tumor necrosis factor alpha and melphalan
Nephron
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Summary

Hyperthermic isolated limb perfusion (HILP) with recombinant TNF-α (r-TNF-α) and melphalan has been shown to result in a sepsis like syndrome due to leakage of r-TNF-α from the perfusion system to the systemic circulation. We have studied renal function parameters in 11 cancer patients, who underwent 12 perfusions. Three patients, perfused with melphalan only, served as controls. All patients treated with r-TNF-α developed a sepsis syndrome and needed volume replacement and inotropes to remain normotensive; controls had an uneventful postoperative course. Creatinine clearance decreased transiently on the day of perfusion in both groups (mean preperfusion clearance 118 ml/min, mean postperfusion clearance 68 ml/min, $p<0.02$, $n=15$). Follow-up measurements of renal plasma flow and glomerular filtration rate in 9 r-TNF-α treated patients did not suggest permanent damage. One patient became hypotensive and developed transient multiple organ dysfunction with renal failure needing hemofiltration. In r-TNF-α treated patients, but not in controls, a transient increase in clearance of β_2 microglobulin (49 vs. 8171 ml/min, $p<0.001$) and urinary excretion of phosphate (12 vs. 48 mmol/L, $p<0.05$) was seen, compatible with proximal tubular dysfunction. These data suggest that HILP with melphalan decreases glomerular function, whether or not r-TNF-α is added to the perfusion circuit. Extension of the treatment regimen with r-TNF-α may result in additional proximal tubular dysfunction. If hypotension can be avoided this deterioration in renal function seems to be transient, with full recovery within weeks.

Introduction

Impairment of renal function is a frequent complication of septic shock, with a major impact on outcome. Its occurrence is at least partly explained by a decrease in renal blood flow secondary to a drop in arterial blood pressure. Additionally, cytokines like tumor necrosis factor alpha (TNF-α), which are released systemically and locally during bacterial sepsis, may directly compromise renal function.

Recently, hyperthermic isolated limb perfusion with melphalan and human recombinant TNF-α (r-TNF-α) has been studied in patients with locally advanced soft tissue tumors and advanced melanomas of a limb, as an alternative to amputation. The technique of isolated limb perfusion allows the administration of high doses of these agents locally while minimizing systemic toxicity. However, it has been recognized by us as well as by others that isolated limb perfusion with r-TNF-α induces a sepsis like state in all patients, characterized by fever, tachycardia and a low blood pressure due to systemic vasodilation [1,2]. The occurrence of this syndrome is explained by leakage of r- TNF-α from the perfused limb into the systemic circulation; very high levels of TNF-α have been documented in peripheral blood of these patients during and directly following perfusion. Leakage has been confirmed by adding radiolabeled albumin to the perfusate; radioactivity can be traced to the systemic circulation during perfusion treatment [3].

In view of the similarity of this clinical syndrome to bacterial sepsis and the unexpectedly high systemic levels of TNF-α, a detrimental effect on kidney function could be anticipated. This study was undertaken to assess the impact of isolated limb perfusion with r-TNF-α and melphalan on creatinine clearance, renal plasma flow (ERPF) and glomerular filtration rate (GFR). Fractional excretion of sodium (FE_{Na}) and urinary excretion of β_2 microglobulin and phosphate were determined to assess proximal tubular function.

Patients and methods

Outline of Experiments

Creatinine clearance and excretion of β_2 microglobulin and phosphate were determined in patients undergoing hyperthermic isolated limb perfusion with r-TNF-α and melphalan. The results were compared with data obtained in control patients who underwent a similar perfusion procedure but without the addition of r-TNF-α. In a subgroup of r-TNF-α treated patients ERPF and GFR were determined with radiopharmaceuticals prior to the perfusion. These experiments were repeated after the perfusion. Due to background radioactivity from radio-labeled albumin administered during the perfusion, postoperative renal function measurement had to be postponed with an average of 22 days (range 10-31).

Anesthesia and Intensive Care

Anesthesia was induced with thiopental, after which the patients were paralyzed with vecuronium and the trachea intubated. Anesthesia was maintained with midazolam, sufentanyl, nitrous oxide and isoflurane. After induction arterial and pulmonary artery catheters were inserted. Blood pressure, ECG, urine output, venous and pulmonary pressures, as well as pulmonary wedge pressures and blood gas values were checked at standard intervals. Fluid resuscitation with crystalloid and colloid solutions was given to maintain pulmonary wedge pressures above 12 mmHg and a dopamine infusion was added if

systolic arterial blood pressure fell to 90 mmHg or decreased by > 30 mmHg from preoperative values despite adequate fluid replacement. All patients were admitted to the intensive care unit following surgery, where the same algorithm was followed to prevent hypotension. Patients received mechanical ventilation until hemodynamically stable.

Hyperthermic Isolated Limb Perfusion
The perfusion technique employed at the Groningen University Hospital is based on the technique developed by Creech and Krementz [4]. Briefly, after ligation of all collateral vessels and heparinization of the patient with 3.3 mg heparin/kg bodyweight (Thromboliquine, Organon BV, Oss, the Netherlands) the axillary, iliac, femoral or popliteal vessels were dissected, cannulated and connected to the extracorporeal circuit. A tourniquet was applied to the proximal limb in an attempt to minimize leakage of the perfusate into the systemic circulation through skin collaterals. Perfusion was performed under mild hyperthermic conditions (39-40^{0}C). The perfusate consisted of 350 ml 5% dextran 40 in glucose 5% (Isodex, Pharmacia AB, Uppsala, Sweden), 500 ml blood (250 ml red blood cells, 250 ml plasma), 30 ml 8.8% $NaHCO_3$ and 0.5 ml 5000 IU/ml heparin (Thromboliquine). The perfusate was oxygenated with a bubble oxygenator and driven by a roller pump. At the start of perfusion recombinant human TNF-α (Boehringer, Ingelheim, Germany, 4 mg for leg perfusions and 3 mg for arm perfusions) was injected as a bolus into the arterial line of the perfusion circuit. Melphalan (Burroughs Wellcome, London, England, 10 mg/L volume of an affected leg and 13 mg/L volume of an affected arm) was administered 30 minutes later. After 90 minutes of perfusion, the limb was flushed with 2 L dextran 40 in glucose 5% (Isodex) and 500 ml blood (250 ml red blood cells, 250 ml plasma), catheters were removed, the circulation was restored and the heparin antagonized with protamine chloride.

Measurement of TNF-α
TNF-α was measured in blood drawn from the radial artery by specific immunoradiometric assay (Medgenix Diagnostics, Soesterberg, the Netherlands). Samples were processed according to the guidelines of the manufacturer.

Measurement of renal function parameters
GFR and ERPF were measured simultaneously using ^{125}I-iothalamaat and ^{131}I-hippurate, respectively, according to the method described by Donker et al. [5]. The radiopharmaceuticals were infused at a constant rate after a priming dose was given. After an equilibration period of one and a half hour, clearances were determined over a two hour period. Post-operative testing was performed at an average of 22 days (SD 6 days) after perfusion. Creatinine clearances were determined from 24 hour urine collections.

Statistical Analysis
Data were analyzed using SPSS for MS WINDOWS (release 5.0). Differences in mean values within groups were studied with either a t-test for paired differences (sytemic vascular resistance, creatinine clearance) or a Wilcoxon Matched Pairs Signed-Ranks Test (ERPF, GFR, urinary excretion of β_2 microglobulin, serum levels of β_2 microglobulin, clearance of β_2 microglobulin, urinary excretion of phosphate), depending on the assumption of distribution. Differences between groups were studied with either an independent samples t-test (creatinine clearance) or a Mann-Whitney U-Wilcoxon Rank Sum W Test (serum levels of β_2 microglobulin, urinary excretion of β_2 microglobulin and FE_{Na}).

Bonferroni's method was used to correct for the effect of multiple comparisons. For single testing a p-value <0.05 was considered significant.

Results

Between September 1992 and May 1993 twelve consecutive hyperthermic isolated limb perfusions with r-TNF-α and melphalan were studied in eleven patients. Three patients, perfused with melphalan but without r-TNF-α served as controls. Paired data on renal blood flow and glomerular filtration rate were available in seven and nine perfusions respectively.

Peak systemic TNF-α concentrations in patients treated with r-TNF-α ranged from 4328 ng/L to 267000 ng/L with a median of 16969 ng/L and a mean of 67693 ng/L. Maximum TNF-α concentrations were reached at the end of the perfusion procedure.

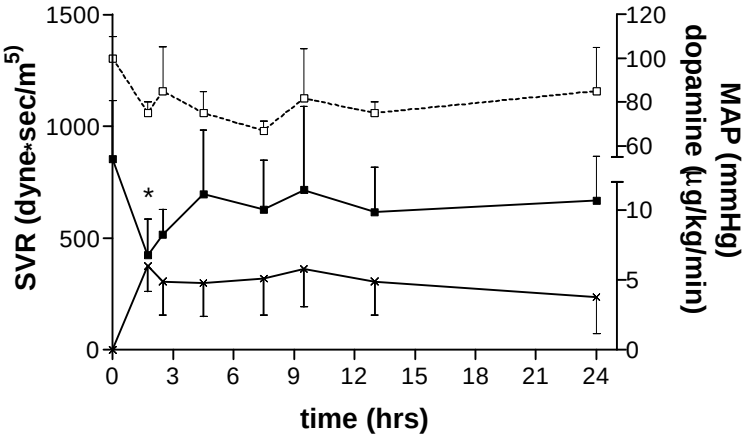

Fig. 1 Mean arterial pressure (MAP, open rectangles), systemic vascular resistance (SVR, closed rectangles) and dopamine requirement (crosses) in patients treated with r-TNF-α perfusion. The asterisk (*) illustrates a significant decrease in SVR compared to baseline values (p<0.005). Error bars represent standard deviations.

Figure 1 shows the effects of r-TNF-α perfusion on systemic vascular resistance, mean arterial pressure and the average need for dopamine. In the r-TNF-α-treated group mean systemic vascular resistance decreased from 854 dyne/sec/cm^{-6} preperfusion to a nadir of 423 dyne/sec/cm^{-6} postperfusion ($p<0.005$). Mean arterial pressure was not significantly decreased at any moment; the lowest mean arterial pressure recorded was 60 mm Hg. All but one r-TNF-α treated patient needed treatment with dopamine to maintain bloodpressure after adequate volume resuscitation (maximum dose 12 mg/kg/min). This patient, and 3 others, were treated with norepinephrine. None of the control patients needed postoperative care in the ICU and none of them were treated with dopamine and/or norepinephrine postoperatively.

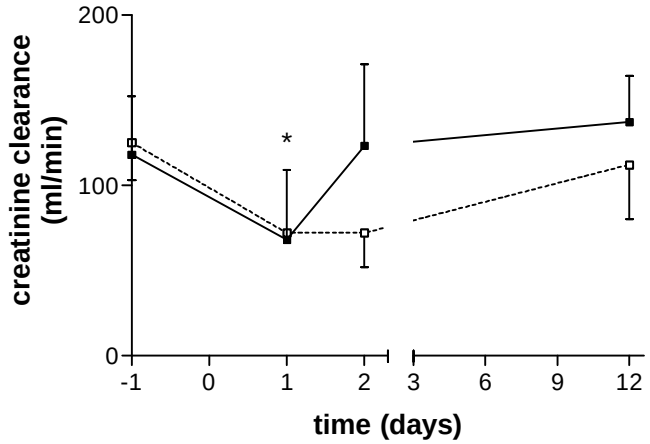

Fig. 2 Mean creatinine clearance in patients treated with r-TNF-α perfusion (solid line) and in controls (dotted line). The asterisk (*) illustrates a significant decrease in creatinine clearance in both groups compared to baseline values ($p<0.02$). Error bars represent standard deviations.

Preperfusion serum creatinine levels ranged from 67-90 μmol/L (mean 80 μmol/L). All patients, including controls, showed a transient drop in creatinine clearance within 48 hours following perfusion (Figure 2). Mean creatinine clearance decreased from a preperfusion level of 118 ml/min to a nadir of 68 ml/min on the second day after the perfusion ($p<0.02$). There was no significant difference in creatinine clearance between r-TNF-α treated patients and controls. Creatinine clearance had returned to normal in all patients on day 4, except in one patient who maintained a septic hemodynamic pattern and developed multiple organ failure.

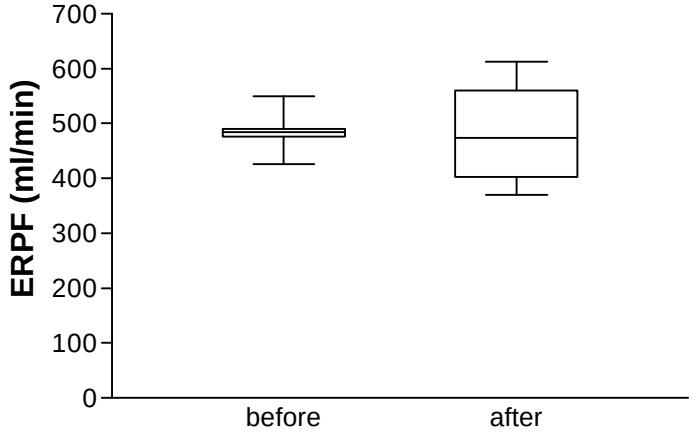

Fig. 3 Box and whiskers plot showing effective renal plasma flow (ERPF), standardized for body surface area, before and after perfusion with r-TNF-α.

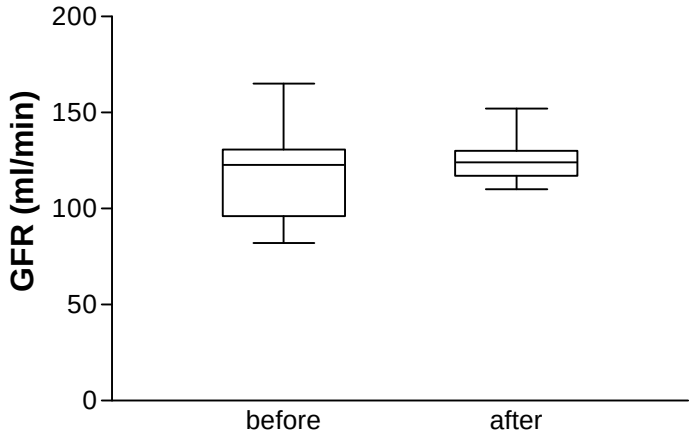

Fig. 4 Box and whiskers plot showing glomerular filtration rate (GFR), standardized for body surface area, before and after perfusion with r-TNF-α.

Figure 3 and 4 show pre- and postperfusion values in r-TNF-α treated patients for ERPF (n=7) and GFR (n=9) respectively; no significant differences were observed in any of these paired parameters.

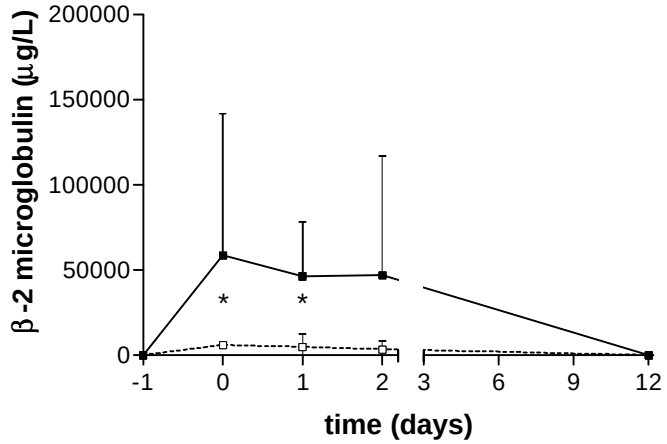

Fig. 5 Urinary excretion of β_2 microglobulin in patients treated with r-TNF-α perfusion (solid line) and in controls (dotted line). The asterisks (*) illustrate a significant increase in β_2 microglobulin excretion in the r-TNF-α -treated group compared to baseline values ($p<0.01$). Numbers represent median values.

In the r-TNF-α treated group there was an increase in the excretion of β_2-microglobulin in the urine from a median value of 41 mg/24 h preoperatively to a median value of 36727 mg/24 h one day postperfusion ($p<0.01$, Figure 5). In serum, β_2-microglobulin concentrations in this group rose from a mean preperfusion value of 1416 mg/L to a mean one day postperfusion value of 3060 mg/L ($p<0.01$, Figure 6).

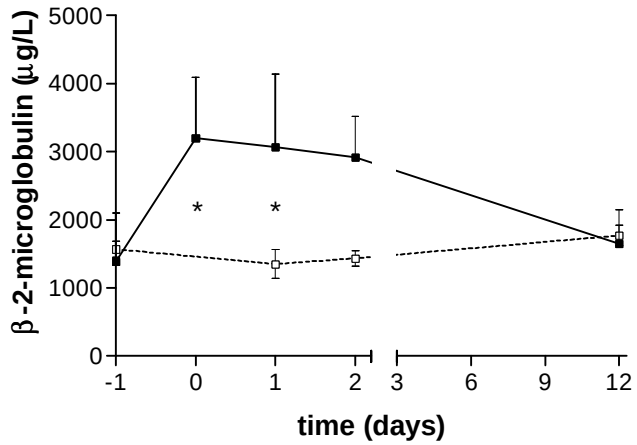

Fig. 6 Mean serum levels of β_2 microglobulin in patients treated with r-TNF-α perfusion (solid line) and in controls (dotted line). The asterisks (*) illustrate a significant increase in serum β_2 microglobulin concentrations in the r-TNF-α-treated group compared to baseline values ($p<0.01$). Error bars represent standard deviations.

Clearance of β_2-microglobulin in patients perfused with r-TNF-α increased from 49 ml/min pre-operatively to 8171 ml/min one day postperfusion ($p<0.001$). In the control group neither urinary excretion of β_2-microglobulin, nor serum concentrations of β_2-microglobulin changed significantly over time (Fig 5 and 6). On the first day after perfusion, levels of β_2-microglobulin in serum, as well as urinary excretion of β_2-microglobulin, were significantly higher in the r-TNF-α treated patients than in controls ($p<0.05$). Preoperative values were not significantly different.

In r-TNF-α perfused patients urinary excretion of phosphate increased from a mean pre-operative value of 12 mmol/24 h to a mean value of 48 mmol/24 h on day 2 after perfusion ($p<0.05$). If corrected for sodium excretion this difference was no longer significant.

Fe_{Na} increased from a preperfusion level of 0.4% to 1.1% on day 1 after perfusion with r-TNF-α, but this increase failed to reach statistical significance ($p=0.07$).

Discussion

This study describes a group of cancer patients who were inadvertently exposed to high systemic concentrations of TNF-α and developed signs of sepsis syndrome as a result. Exposure to TNF-α occurred as a side effect of hyperthermic isolated limb perfusion with r-TNF-α, through leakage of this antitumor cytokine from the perfusion circuit to the systemic circulation. The mean maximum systemic TNF-α concentration in r-TNF-α treated patients was 67693 ng/L (range 4328 to 267000 ng/L). This is several orders of magnitude higher than TNF-α concentrations previously reported in different forms of shock. In a series of 79 patients with meningococcal disease and/or septicemia all patients with TNF-α levels over 100 ng/L died [6]. In septic shock median TNF-α concentrations of 120 ng/L have been reported [7]. Despite much higher concentrations of TNF-α all our patients survived their ICU stay and could be discharged to the ward after a median stay in the ICU of two days.

The effects of TNF-α on the kidney have not yet been well characterized. TNF-α is an early mediator of endotoxemic shock and, if administered to laboratory animals, causes a clinical syndrome

which is very similar to bacterial sepsis [8]. In this setting, part of the effect of TNF-α on the kidney will be through decreased mean arterial pressure and decreased renal perfusion. However, there may also be a direct effect of TNF-α on kidney function. Rabbits given recombinant human TNF-α showed a decrease in renal blood flow, but also a decrease in the renal fraction of aortic blood flow, caused by a 17% increase in renal resistance [9]. Autoregulation was preserved as indicated by a compensatory increase of the filtration fraction with 17%. Hemodynamic changes were abolished by both thromboxane inhibitors and indomethacin, implying a role for the arachidonate cascade system. In another rabbit model TNF-α induced swelling of glomerular endothelial cells and accumulation of leukocytes in the glomerular capillary lumen [10]. In a plethora of animals models, local TNF-α production, mainly by mesangial cells, has been shown to play a role in the pathophysiology of inflammatory kidney disease [11- 13]. TNF-α is also involved in kidney transplant rejection [14]. Experiments in humans have been limited by the toxicity of TNF-α. Early trials with intravenous TNF-α have reported renal toxicity in sporadic cases [15], but normal kidney function is the rule [16]. In patients who underwent isolation perfusion with r-TNF-α Eggimann and coworkers reported a fall in creatinine clearance in 22% of their patients [1]. In a group of 9 patients reported by Sorkin and coworkers, 2 patients showed a transient rise in serum creatinine [17].

In the present study all r-TNF-α treated patients showed a decrease in creatinine clearance on the day of perfusion. There was a similar decrease in control patients, who were treated with melphalan only. In both groups creatinine clearance returned to normal levels within two days. Subsequent radiopharmaceutical studies in the r-TNF-α treated group did not show significant late changes in GFR and ERPF. It should be noted that in all patients but one hypotension could be prevented with early expansion of volume and judicious use of vasocactive drugs, guided by invasive haemodynamic monitoring in the operating room as well as in the intensive care unit. One patient went on to develop transient multiple organ failure. This patient remained inotrope-dependent with a septic hemodynamic profile and developed acute respiratory failure and non-oliguric renal failure for which he had to be treated with continuous veno-venous hemofiltration.

It appears from these data that hyperthermic isolated limb perfusion with melphalan *as such* transiently decreases creatinine clearance. The role of r-TNF-α in this setting remains to be determined. We were unable to show that the addition of r-TNF-α to the perfusion circuit resulted in any additional loss of glomerular function. Peri- and postoperative hypotension, due to TNF-α-induced systemic vasodilation, can be lifethreatening, and its management requires skill and experience. Aggressive support of perfusion pressures with cristalloid and colloid infusions with vasopressors in an ICU setting is mandatory to prevent the development of multiple organ dysfunction. In the series described here, no permanent renal damage occurred if peri- and postoperative hypotension could be avoided. In theory, this could be attributed to a specific protective effect of dopamine on the kidney. However, proof that dopamine influences the course of acute renal failure is lacking. Furthermore, in additional experiments in mechanically ventilated patients after aortic surgery, we were unable to show a specific renal hemodynamic effect of this drug; the observed increase in RBF and GFR could be fully ascribed to the increase in cardiac output [18].

On the day of perfusion, excretion of β_2 microglobulin increased greatly in r-TNF-α treated patients. There was a simultaneous rise in plasma β_2 microglobulin, which has been recognized as a TNF-α effect by others [19], but this was insufficient to explain the 150-fold increase in clearance of β_2 microglobulin. Like the creatinine clearance, clearance of β_2 microglobulin returned to normal levels within two days. Since β_2 microglobulin is almost completely reabsorbed in the proximal tubule these data suggest a temporary dysfunction of this proximal part of the nephron. The observed increase in

phosphate excretion is compatible with such a temporary proximal dysfunction, although it could also be explained by the observed increase in sodium excretion. An increase in sodium excretion in turn, could be attributed to sodium loading, which was carried out to correct hypovolemia and to prevent hypotension. Although FE_{Na} was >1% in the r-TNF-α treated group on day 1 postperfusion, a value compatible with a diagnosis of acute tubular necrosis, it did not differ significantly from FE_{Na} in the control group. Thus, the precise effect of r-TNF-α on proximal tubular function remains to be determined.

The present study has several limitations. The control group of patients treated with perfusion with melphalan but without r-TNF-α was small and patients were not randomly assigned to either treatment arm. Due to its dramatic effects on tumor regression, perfusions without r-TNF-α became to be considered ethically unjustified by the responsible clinicians. Obviously, this made any form of randomization impossible. A second drawback of the study design is the interference of GFR and ERPF measurements with standard I^{131}-albumin measurement of albumin leak during perfusion (as an indicator of r-TNF-α leak to the systemic circulation), forcing us to postpone GFR and ERPF measurements to well after the perfusion procedure. In theory early changes in renal function may thus have been masked. However, to our knowledge this is the first study to examine the renal effects of isolated limb perfusion with r-TNF-α in humans in some detail.

We conclude that, in hyperthermic isolated limb perfusion with r-TNF-α and melphalan, renal toxicity is acceptable, even if considerable leakage of r-TNF-α to the systemic circulation of the patient cannot be avoided. Although a transient decrease in creatinine clearance is observed, no lasting damage to the kidney will occur if hemodynamic stability can be maintained. This requires early treatment with volume expansion and pressor drugs, guided by invasive haemodynamic monitoring. If r-TNF-α is added to the treatment a transient decrease of proximal tubular function is seen, in addition to the effect on glomerular filtration. This may represent a direct nephrotoxic effect of TNF-α.

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Chapter VI

Role of nitric oxide in recombinant tumor necrosis factor alpha-induced circulatory shock: A study in patients treated for cancer with isolated limb perfusion

In press as:
Role of nitric oxide in recombinant tumor necrosis factor-α-induced circulatory shock: A study in patients treated for cancer with isolated limb perfusion
Crit Care Med
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Summary

This chapter aims to analyze the mechanism of vasodilation and circulatory shock in patients who were treated with isolated limb perfusion with melphalan and recombinant tumor necrosis factor α (r-TNF-α) for locally advanced malignant tumors. The role of nitric oxide, if any, was determined by measuring plasma nitrite and nitrate levels.

Eight consecutive patients developed sepsis syndrome due to leakage of r-TNF-α from the perfusion circuit to the systemic circulation. Despite the presence of very high systemic TNF- α levels during and immediately after perfusion and definite signs of hyperdynamic circulatory shock (increased heart rate, increased cardiac index, decreased systemic vascular resistance) nitrite and nitrate levels, measured in plasma at several time points, were not elevated.

The hypothesis that, in humans, TNF-α induces vasodilation and shock through activation of inducible nitric-oxide synthase and subsequent formation of excessive quantities of nitric oxide is not substantiated by our results. Normal nitric oxide metabolite levels were found in the presence of high TNF-α levels and shock. Other mechanisms that do not involve the nitric oxide pathway are likely to play a role in the generation of hypotension and septic shock in this setting.

Introduction

Isolated limb perfusion with melphalan and r-TNF-α, used in the treatment of locally advanced soft tissue sarcomas or melanomas of a limb, has been shown to result in a severe, but short-lived sepsis syndrome, characterized by fever, low blood pressure and the need for fluid resuscitation and inotropic support [1,2]. The occurrence of this syndrome is explained by leakage of r-TNF-α from the perfusion circuit into the systemic circulation; high levels of TNF-α are found in arterial blood during and directly after the procedure. Since TNF-α can induce nitric oxide synthase leading to a sustained release of nitric oxide and catecholamine-refractory vasodilation in animal models [3], we speculated nitric oxide to be an important mediator of the circulatory shock observed in this setting. To test this hypothesis we have measured metabolites of nitric oxide in eight patients during and after perfusion.

Subjects and Methods

Subjects
Eight patients received hyperthermic isolated limb perfusion at the division of surgical oncology of the Groningen University Hospital after approval of the medical ethical committee and informed consent had been obtained. Tumor histology is summarized in the table.
Normal values for nitrate and nitrite were obtained from studies in 26 healthy volunteers.

Anesthesia and intensive care
Anesthesia was induced with thiopental, after which the patients were paralyzed with vecuronium and the trachea intubated. Anesthesia was maintained with midazolam, sufentanyl, nitrous oxide and isoflurane. After induction a radial artery catheter and a pulmonary artery catheter were inserted. Blood pressure, ECG, urine output, venous and pulmonary pressures, as well as pulmonary artery wedge pressures and arterial blood gas values were checked at standard intervals. All patients were admitted to the intensive care unit following surgery. Patients received mechanical ventilation until hemodynamically stable. Fluid resuscitation with crystalloid and colloid solutions was given to maintain pulmonary artery wedge pressures above 12 mm Hg, and a dopamine infusion was added if systolic arterial blood pressure fell to 90 mm Hg or decreased by > 30 mm Hg from preoperative values despite adequate fluid replacement.

Isolated Limb Perfusion
The perfusion technique employed at the Groningen University Hospital is based on the technique developed by Creech and Krementz [4]. Briefly, after ligation of all collateral vessels and heparinization of the patient with 3.3 mg heparin/kg bodyweight (Thromboliquine, Organon BV, Oss, the Netherlands) the axillary, iliac, femoral or popliteal vessels were dissected, cannulated and connected to the extracorporeal circuit. The perfused limb was wrapped in a thermal blanket to reduce heat loss and four thermistor probes were inserted subcutaneously and intramuscularly for continuous monitoring of the temperature during perfusion. A tourniquet was applied to the proximal limb in an attempt to minimize leakage of the perfusate into the systemic circulation through skin collaterals. Perfusion was performed during 90 minutes under mild hyperthermic conditions (39-40^{o}C). The perfusate consisted of 350 ml 5% dextran 40 in glucose 5% (Isodex, Pharmacia AB, Uppsala, Sweden), 500 ml blood (250 ml red blood cells, 250 ml plasma), 30 ml 8.4% $NaHCO_3$ and 0.5 ml

5000 IU/ml heparin (Thromboliquine). The perfusate was oxygenated with a bubble oxygenator and driven by a roller pump. At the start of perfusion r-TNF-α (Boehringer, Ingelheim, Germany, 4 mg for leg perfusions and 3 mg for arm perfusions) was injected as a bolus into the arterial line of the perfusion circuit. Melphalan (Burroughs Wellcome, London, England, 10 mg/L volume of an affected leg and 13 mg/L volume of an affected arm) was administered 30 minutes later. During perfusion potential leakage to the systemic circulation was monitored with I^{131} labeled albumin [5]. After 90 minutes of perfusion, the limb was flushed with 2 L dextran 40 in glucose 5% (Isodex) and 500 ml blood (250 ml red blood cells, 250 ml plasma), catheters were removed, the circulation restored and the heparin antagonized with protamine chloride. A lateral fasciotomy of the anterior compartment of the lower leg was performed in leg perfusions or a fasciotomy of the forearm in arm perfusions to prevent a compartment syndrome.

Hemodynamic measurements
Hemodynamic variables were measured during perfusion and after the patient had arrived in the intensive care unit. Measured variables included heart rate and mean arterial pressure. Cardiac output, cardiac index and systemic vascular resistance were determined at two hourly intervals. Pressure transducers were set to zero at the level of the midaxillary line. Cardiac output was measured in triplicate by the thermodilution method, with the use of a cardiac output computer and cold saline.

Assay for TNF-a
TNF-α levels were determined by specific immunoradiometric assay (Medgenix Diagnostics, Soesterberg, the Netherlands). Samples were processed according to the guidelines of the manufacturer. Arterial blood samples (3 ml) were collected in EDTA Vacutainer tubes, and kept on melting ice during transport to a centrifuge. Samples were centrifuged for 10 min at 3000 rpm at 0^0C and the separated plasma kept at -20^0C until analysis. A baseline sample was taken for TNF-α assay after the insertion of the arterial line, then at 5, 30, 60, and 89 minutes after the start of the perfusion. After restoration of the circulation to the perfused limb, systemic samples were taken at 1, 5, 10, 30 and 60 minutes after removal of arterial clamps, hourly thereafter for at least eight hours and finally the next morning.

Assay for nitric oxide metabolites [6]
Nitrite was measured using the Griess reaction [7]. Briefly, plasma samples were diluted fourfold with distilled water and deproteinized by adding zinc sulfate (300 g/L) to give a final concentration of 15 g/L. After centrifugation at 10.000 *g* for 5 min at room temperature (or 1000 *g* for 15 min), 100 ml of supernate was applied to a microtiter plate well, followed by 100 ml of Griess reagent (1 g/L sulfanilamide, 25 g/L phosphoric acid, and 0.1 g/L *N*-1-naphthylethylenediamine). After 10 min of color development at room temperature, the absorbance was measured on a microplate reader (Titertek Multiskan MCC/340; Flow Lab, McLean, VA) at wavelength of 540 nm. Each sample was assayed in duplicate wells.
Nitrate was measured as nitrite after enzymatic conversion by nitrate reductase. Briefly, 100 ml of plasma was diluted fourfold with distilled water, nicotine adenine dinucleotide phosphate (reduced), FAD, and nitrate reductase from *Aspergillus* spp. (Boehringer Mannheim, Mannheim, Germany) were added to yield final concentrations of 50 mmol/L, 5 mmol/L, and 200 U/L, respectively. Samples were subsequently incubated for 20 min at 37°C, and then mixed with lactate dehydrogenase from rabbit muscle (Boehringer Mannheim) at a final concentration of 10 mg/L and sodium pyruvate at a final

concentration of 10 mmol/L. Samples were further incubated for 5 min at 37°C to oxidize nicotine adenine dinucleotide phosphate (reduced), deproteinized, and assayed with Griess reagent as described above. Values obtained by this procedure represent the sum of nitrite and nitrate. Nitrate concentrations were obtained by subtracting nitrite concentration from the total nitrate + nitrite concentrations.
Plasma samples for nitrate and nitrite measurements were taken at the following time points: 1 hour before perfusion, during perfusion at 5, 30, 60 and 89 minutes and after perfusion at 5, 30, 60 minutes and after 2, 3, 4, 5, 6, 7, 8, 12, and 24 hours.
Samples were assessed for the presence of nitric oxide metabolites in two independent laboratories with similar results.

Statistical analysis
Data were analyzed using SPSS for MS WINDOWS (release 5.0). To analyze differences in heart rate, mean arterial pressure, cardiac output, systemic vascular resistance and TNF-α-levels before and after perfusion the Wilcoxon Matched-Pairs Signed-Ranks Test was used. A p-value < 0.05 was considered significant. To analyze the effect of perfusion on the sum of nitrate and nitrite an Analysis of Variance was carried out, comparing patients and controls.

Results

Clinical details, TNF-α levels and nitric oxide metabolites of the patients are summarized in the table, where the highest values of plasma TNF-α and the sum of nitrite and nitrate are shown.

Patient	Diagnosis	Heart Rate	Cardiac Index	Systemic Vascular Resistance	TNF- α	NOx
1	synovia sarcoma	120 (76)	5.1 (2.9)	585 (1072)	1393(43)	39.9 (22.2)
2	melanoma	100 (64)	5.5 (2.8)	334 (856)	38500(66)	35.4 (40.0)
3	synovia sarcoma	150 (75)	8.0 (3.8)	429 (890)	21065(12)	22.2 (22.2)
4	liposarcoma	145 (75)	9.4 (3.9)	296 (749)	134000(1)	28.8 (33.2)
5	schwannoma	140 (80)	5.8 (3.6)	572 (1119)	17109(5)	35.0 (42.0)
6	histiocytoma	150 (110)	5.8 (3.6)	650 (908)	51000(10)	19.0 (18.6)
7	histiocytoma	155 (88)	6.2 (4.0)	501 (1000)	8200 (1)	32.2 (23.6)
8	melanoma	120 (66)	4.4 (2.2)	627 (927)	267000(65)	34.6 (24.9)

Table 1: Clinical details, peak systemic TNF-α levels and nitric oxide metabolites. Tumor necrosis factor α is abbreviated as TNF-α, the sum of plasma nitrite and nitrate as Nox. Values are presented as highest value after perfusion (heart rate, cardiac index, TNF-α, NO_x), or lowest value after perfusion (systemic vascular resistance), while the value 1 hour before perfusion is given between brackets.

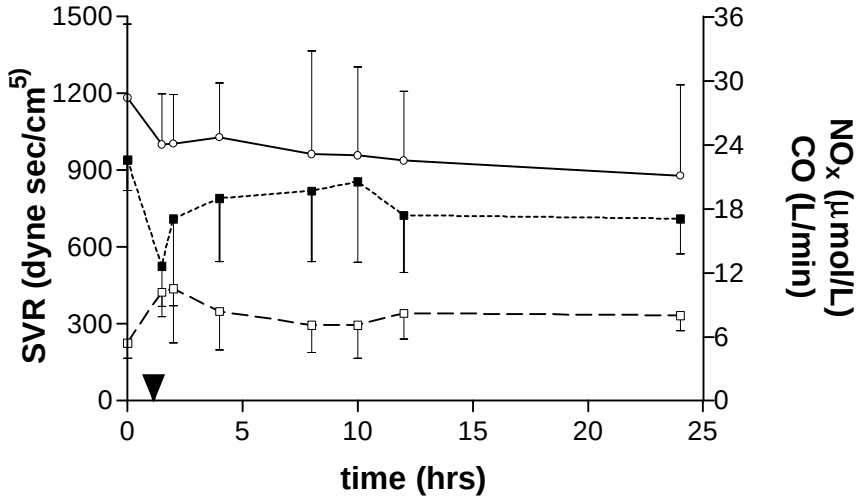

Fig. 1 **The black rectangles (dotted line) show the change in mean systemic vascular resistance (SVR) with time. The open rectangles (interrupted line) show the mean cardiac output (CO). The open circles (solid line) represent the mean sum of nitrite and nitrate (NO_x). Hyperthermic isolated limb perfusion was started at time point 0 and stopped after 1.5 hrs. as indicated by the large closed triangle. Error bars represent standard deviation.**

Figure 1 shows the course of mean systemic vascular resistance, cardiac output and mean nitrite + nitrate levels over time.

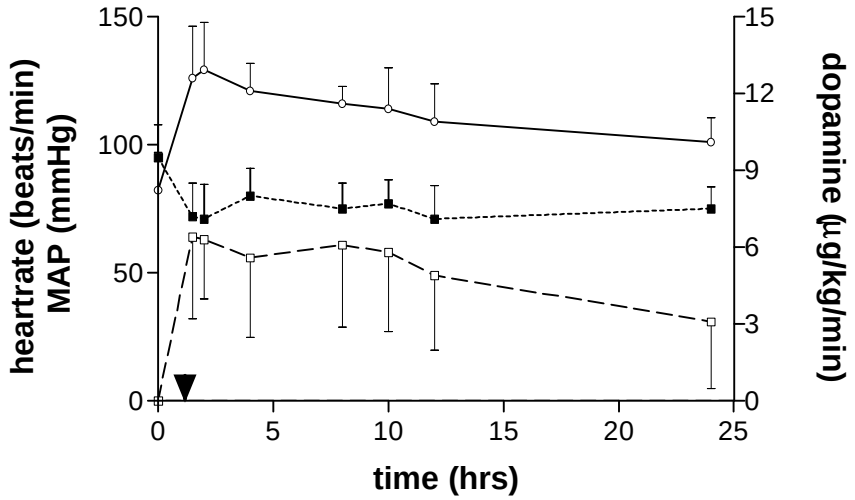

Fig. 2 **The black rectangles (dotted line) show the change in mean arterial blood pressure (MAP) with time. The open rectangles (interrupted line) represent the mean dose of dopamine administered. The open circles (solid line) show the mean heart rate. Hyperthermic isolated limb perfusion was started at time point 0 and stopped after 1.5 hrs. as indicated by the large closed triangle. Error bars represent standard deviation**.

Figure 2 shows the trend of mean values for heart rate, mean arterial pressure and dopamine requirements.

All patients developed a clinical picture of septic shock with hypotension, a significant increase in heart rate and cardiac index and a drop in systemic vascular resistance. They all needed volume resuscitation and dopamine in a dose of up to 6 mg/kg/min to maintain adequate blood pressure. Signs of sepsis abated within 12 hours and all patients could be discharged from the intensive care unit within two days following perfusion. Systemic TNF-α levels were very high during and immediately after the perfusion; they returned to baseline levels within 8 hours.

The clinical picture of septic shock in these patients is attributed to leakage of r-TNF-α from the perfusion circuit to the circulation of the patient, mainly through collateral blood flow. Leakage was confirmed by adding I^{131} -labeled albumin to the perfusate, up to 2% of which was subsequently localized in the central circulation of the patients. All patients showed a clinically and statistically significant rise in heart rate and cardiac index ($p < 0.01$) and a decrease in mean arterial pressure and systemic vascular resistance ($p < 0.01$). The rise in TNF-α-levels was statistically significant in all patients.

None of the patients showed any increase in nitrite or nitrate level at any time point. The mean value of the sum of nitrite and nitrate following perfusion in the 8 patients was 24,4 mmol/L (SD 5,9 mmol/L, 95% CI 23,4 - 25,4 mmol/L). This did not differ significantly from the mean value in 26 healthy controls of 23,9 mmol/L (SD 9,7 mmol/L, 95% CI 20,0 - 27,8 mmol/L).

Discussion

Nitric oxide is synthesized from L-arginine by nitric oxide synthase in many different cell types including endothelial cells. Two forms of nitric oxide synthase have been distinguished. In its constituent, calcium-dependent form nitric oxide synthase enzymatically produces small quantities of nitric oxide to act as an important modulator of vascular tone, in normal animals as well as in humans. In some animal models excessive amounts of nitric oxide can be produced by induction of a second type of nitric oxide synthase, the so-called cytokine-inducible nitric oxide synthase. Overproduction of nitric oxide by stimulation of this alternative, calcium-independent pathway is considered to explain the inappropriate vasodilation which is the hallmark of septic shock [8]. Endotoxin, but also cytokines like TNF-α are believed to induce nitric oxide synthase, leading to a sustained release of nitric oxide and catecholamine-refractory vasodilation. A rapidly expanding literature has implicated a role for cytokine-inducible nitric oxide synthesis in the pathogenesis of septic shock in animal models. Il-1, TNF-α and endotoxin induce nitric oxide synthase activity in vascular smooth muscle cells from rat aorta in vitro [9]. Studies in mice have shown that the administration of anti-TNF-α antibodies markedly reduces endotoxin-induced shock and nitric oxide synthesis in vivo [10]. Kilbourn and coworkers have induced hypotension in dogs by administering recombinant human TNF-α. N^G-monomethyl-L-arginine, a competitive inhibitor of nitric oxide formation from L-arginine, completely reversed this fall in blood pressure, which reappeared after the administration of excess L-arginine. Though levels of nitrite or nitrate were not measured directly, the authors conclude that excessive nitric oxide production mediates the hypotensive effect of TNF-α [3].

The role of a cytokine-inducible nitric oxide synthase in *human* septic shock is less clear. Cytokine-inducible nitric oxide synthase has been demonstrated in only a few human cell types in vitro, in contrast to the abundance of animal cell types shown to have this activity. TNF-α failed to induce nitric oxide in vascular smooth muscle cells from human saphenous vein [11]. On the other hand clinical studies in human bacterial sepsis have shown signs of increased nitric oxide production and a

correlation between the concentration of endotoxin in plasma, the plasma levels of nitrite and nitrate and the severity of circulatory shock [12, 13]. Furthermore L-arginine analogues that block nitric oxide production have been effective at increasing blood pressure in septic patients [14]. In patients treated with interleukin-2 plasma concentrations of nitric oxide metabolites have been shown to rise significantly [15]. On the other hand Ochoa and coworkers have described normal levels of nitrite and nitrate in trauma patients even when they developed signs of sepsis [16, 17].

In this study we have looked at the mechanism of vasodilation in cancer patients, who were treated with isolated limb perfusion with r-TNF-α and melphalan, by measuring plasma levels of TNF- α, nitrite and nitrate. Our study has several limitations. First, it may well be argued that plasma levels of nitrite and nitrate do not reliably reflect activation of the inducible form of nitric oxide synthase. Local levels of nitric oxide may have been sufficiently increased to generate profound vascular effects without producing a demonstrable rise of nitrite or nitrate in the plasma. Conclusive data would perhaps require a combination of plasma levels with other parameters such as excretion of nitrate in urine, nitric oxide levels in exhaled air, or accumulation of ^{15}N-nitrite and ^{15}N-nitrate in plasma and urine following administration of ^{15}N-labeled arginine. It should be noted however, that most of the scientific evidence for a pivotal role of nitric oxide in various types of vasodilation has been based on the demonstration of elevated levels of nitrite and nitrate in plasma or serum [12, 13, 15, 16, 18, 19]. Moreover, alternatives to measurement of plasma levels of nitrite and nitrate, like measurement of urinary excretion of nitrate, have not been shown to be better markers of the septic state. On the contrary, Jacob and coworkers have found that in trauma patients the mean plasma nitrate concentration on days with evidence of infection was significantly increased compared with days without active infection; mean urinary excretion of nitrate was *not* increased on infected days as compared with days without infection [17]. We feel that activation of the inducible nitric oxide synthase by r-TNF-α would at least be partially reflected in a rise of serum levels of nitrite and nitrate. Another limitation of our study is that we did not investigate the effect on hemodynamic parameters of nitric oxide inhibitors like N^G-methyl-L-arginine. Although important scientifically, this was considered to be unsuitable since it would imply treating our patients with two different investigational drugs: r-TNF-α and a nitric oxide synthase blocker. Furthermore, hypotension and shock in these patients could well be managed with conventional therapy and did not warrant the use of investigational drugs with possible harmful side effects. Potential drawbacks of the use of non-selective nitric oxide synthase blockers have been pointed out by a number of authors [20, 21, 22, 23, 24].

Our findings in human subjects show that despite very high TNF-α levels and distinct signs of sepsis syndrome, increased nitric oxide-production could not be demonstrated in plasma. It is possible that, in human subjects, the presence of endotoxin is mandatory to trigger excessive nitric oxide production, despite high levels of TNF-α. The assumed absence of relevant amounts of endotoxin in our model may also explain the relative mildness and short duration of the clinical syndrome; animal experiments have shown that the toxicity of TNF-α is greatly enhanced in the presence of endotoxin. Endotoxin levels in blood were not measured in our study. Alternatively, there may be a nitric-oxide-independent pathway through which TNF-α induces vascular relaxation, possibly by direct activation of soluble guanylate cyclase. The existence of such a pathway was postulated earlier by Beasley and coworkers on the basis of their experiments in human vascular smooth muscle cells [11]. They could show that interleukin 1, TNF-α, interferon-γ and Escherichia coli lipopolysaccharide increased cyclic guanosine monophosphate in human saphenous vein vascular smooth muscle cells and that this effect was not reversed by adding L-arginine analogues. Moreover, analysis of nitric oxide synthase mRNA with the

use of polymerase chain reaction indicated that levels of mRNA for inducible nitric oxide synthase were low.

Alternatively, hypotension in the setting of isolated limb perfusion with TNF-α could also be explained by the generation of cyclooxygenase products like prostacyclin. Bernard et al. could show high levels of both prostacyclin and thromboxane A_2-metabolites in a group of patients with sepsis syndrome [25]. Calcitonin gene-related peptide is yet another possible mediator of hypotension in sepsis. It is increased in patients with sepsis and has been shown to be the most potent vasodilator and hypotensive agent in humans to date [26]. Adenosine triphosphate-regulated K^+ channels have also been shown to be important mediators of vascular smooth muscle tone [27]. These channels are activated by decreased intracellular adenosine triphosphate, by cytosolic acidosis and increased cytosolic lactate, conditions that may well have occurred in our patients. Activation of adenosine triphosphate-sensitive K^+ channels hyperpolarizes vascular smooth muscle and reduces Ca^{2+} entry into the cell, thereby inducing relaxation and vasodilation. These Ca^{2+} channels can also be downregulated by oxygen radicals produced by an oxidative burst of endothelial cells in response to the presence of endotoxin [28].

In conclusion, the results presented in this study do not support an important role for nitric oxide in producing vasodilation and shock in cancer patients treated with isolated limb perfusion with r-TNF-α, in whom a considerable leak of r-TNF-α from the perfusion circuit leads to high systemic levels of this cytokine. Further studies will be needed to clarify the exact mechanism of septic vasodilation in these patients.

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Chapter VII

Summary and concluding remarks

The main function of tumor necrosis factor alpha (TNF-α), a small polypeptide shared by all mammals, is probably protection against invading bacteria, parasites and viruses; killing of these microorganisms is facilitated in the presence of TNF-α. However, as its name suggests, TNF-α is also capable of killing tumor cells, in vitro as well as in vivo. This unique capacity has focused the attention on a possible role for this cytokine in the treatment of human cancer. It soon became apparent that its usefulness as such is seriously limited by its toxicity. In sensitive laboratory animals administration of even a very low dose of TNF-α will induce a sepsis like state, with hypotension, respiratory failure, hepatic necrosis and renal insufficiency. Higher doses may be fatal. Human clinical trials with TNF-α have confirmed its high toxicity, without showing any benefits in terms of tumor remissions.

Isolated limb perfusion was first described by Creech and Krementz in 1968. It is based on the assumption that the circulation of a limb can be isolated completely from the rest of the circulation, while the tissues of the isolated limb are kept viable with a perfusate, that is oxygenated by a bubble oxygenator. With a system like this, it would be possible to administer high concentrations of antineoplastic drugs to tumors of a limb, while sparing the rest of the body the detrimental effects of chemotherapy. Moreover, the temperature of the perfused limb could be maintained between 39 and 40^0C, which was expected to increase the antitumor effect of most antineoplastic agents. Many perfusions have been performed since, mainly with alkylating agents like melphalan, with variable results.

Lejeune and coworkers were the first to try recombinant TNF-α (r-TNF-α) in such an isolated perfusion system. They hoped to achieve sufficiently high concentrations of r-TNF-α in the tumor, with a systemic toxicity that would remain manageable. In their experiments, r-TNF-α was combined with melphalan, and with recombinant interferon gamma (r-IFN-γ), a cytokine that enhances the sensitivity of tumor cells to TNF-α by increasing the number of TNF-receptors on the cell surface. An impressive remission rate was achieved, especially in patients with locally irresectable sarcoma of a limb: 36% complete remissions and 51% partial remissions. In melanoma patients, the results also look promising, but formal testing of the efficacy of isolated perfusion with r-TNF-α for melanoma has not been completed. Systemic toxicity was manageable, but all patients required treatment in an intensive care unit for symptoms of sepsis.

The studies presented in this thesis exclusively deal with systemic toxicity; they do not report on remission- or survival rates. They were primarily undertaken to understand why patients treated regionally with TNF- α developed such serious systemic complications. The effects of TNF-perfusion on different organ systems are described in some detail. A better understanding of the toxicity of isolated limb perfusion with r-TNF-α might contribute to a better management of patients treated in this fashion. Because of the high remission rates that have been reported, their numbers can be expected to increase.

Apart from being a promising technique to treat some types of cancer, isolated limb perfusion with r-TNF-α presents an interesting model of sepsis and septic shock. In bacterial and fungal sepsis TNF-α is universally considered to be one of the prime mediators of shock and organ dysfunction. Cell wall products of these microorganisms, like lipopolysaccharide and peptidoglycan, stimulate macrophages

to produce excessive amounts of TNF-α. TNF-α directly interferes with cellular function but also sets of a cascade of inflammatory mediators that greatly enhance its deleterious effects. Clinical research of sepsis has traditionally been hampered by late diagnosis: once the syndrome is diagnosed the inflammatory cascade has fully developed and a plethora of damaging factors are at play. This makes it difficult to determine the role of any factor in particular. In isolated perfusion with r-TNF-α we have been able to show a considerable leak of r-TNF-α from the perfusion circuit to the general circulation. In fact, this type of treatment amounts to a 90 min. intravenous infusion of high doses of r-TNF-α, with a peak at the time when normal circulation is restored. Concentrations of systemic TNF-α are many times higher in these patients than those found in healthy volunteers after administration of what was considered to be acceptable doses of r-TNF-α. The pathology of the sepsis syndrome, induced by this cytokine, can thus be observed right from the start. Within its limitations, the model of isolated limb perfusion with r-TNF-α might prove useful in the study of sepsis.

In **Chapter I** the clinical experience with r-TNF-α in the treatment of cancer is described. The purpose of this chapter is to familiarize intensive care staff with the concept of isolated limb perfusion with r-TNF-α, a new modality in cancer treatment, that has attracted considerable attention. The systemic toxicity of this type of treatment, and of cancer therapy with r-TNF-α in general, is reviewed. This review is based on Phase I and Phase II trials published in the English language, along with supportive documentation and data on 64 patients treated with r-TNF-α in our own institution. Guidelines are offered for the successful management of this type of patient. Treatment with r-TNF-α results in a characteristic clinical syndrome, which resembles the sepsis syndrome. Hypotension and respiratory failure are the main features of this syndrome. Toxicity is largely independent of the route of administration. Very high serum concentrations of TNF-α, if short-lived, can be less toxic than sustained low serum concentrations. Treatment of patients who have undergone isolated limb perfusion with high dose r-TNF-α is feasible and effective in a modern ICU setting, even if high serum concentrations of TNF-α, due to leakage from the perfusion circuit, cannot be avoided. Most patients can be discharged from the ICU within 24 hours.

Chapter II describes the post-operative course of 25 consecutive patients, who underwent hyperthermic isolated limb perfusion with r-TNF-α and melphalan, following pretreatment with r-INF-γ, as treatment for recurrent melanoma, primary nonresectable soft tissue tumors, planocellular carcinoma or metastatic carcinoma. It is a retrospective, descriptive study, relating systemic TNF-α levels with indices of disease severity. All patients developed features of sepsis syndrome and required intensive care treatment. Most patients recovered quickly with a median ICU stay of 2 days (range 1-25). Maximum systemic TNF-α levels ranged from 2284 to 83000 ng/L (median 25409 ng/L) and returned to baseline values within 8 hours. Despite these high levels of TNF-α no patient died in the ICU, although the patient with the highest TNF-α level developed multiple organ failure and required continuous venovenous hemofiltration for 16 days. Linear regression analysis showed a positive correlation between maximum TNF-α levels and systemic vascular resistance ($p<0.01$), cardiac index ($p<0.02$), lung injury score ($p<0.02$), prothrombin time ($p<0.02$) and activated partial thromboplastin time ($p<0.05$). It is concluded that hyperthermic isolated limb perfusion with r-TNF-α leads to high systemic levels of TNF-α, probably due to leakage of r-TNF-α from the perfusion circuit, mainly through collateral bloodflow. A sepsis like syndrome is seen in all patients. Despite high levels of systemic TNF-α, this sepsis syndrome is short-lived and recovery is rapid and complete in most patients.

Several investigators have reported that IFN-γ can alter TNF-α-induced effects in vitro. In **Chapter III** we have assessed in vivo effects of r-IFN-γ on r-TNF-α-induced activation of systemic blood coagulation in a non-randomized study in 20 consecutive cancer patients. Eight patients were treated with r-IFN-γ prior to and during hyperthermic isolated limb perfusion with r-TNF-α and melphalan (IFN-γ group). They were compared with twelve patients who did not additionally receive r-IFN-γ (non-IFN-γ group). Before start of perfusion, higher levels of TNF-α, prothrombin fragment 1 and 2 (F_{1+2}) and thrombin-antithrombin complexes (TAT) were found in the IFN-γ group. Fibrinogen and antithrombin III (ATIII) levels tended to be lower in this group. High TNF-α levels, due to leakage during perfusion, were associated with activation of coagulation in all patients, that became obvious after the end of perfusion, when heparin treatment had been antagonized. Activation, measured by increased F_{1+2} and TAT levels, was significantly stronger in the IFN-γ group. Monocytic tissue factor (TF) remained low, possibly due to shedding of TF positive vesicles and/or sequestration of TF positive activated monocytes against the vessel wall. In both groups F_{1+2} and TAT levels declined 24 hours after the perfusion, whereas monocytic TF increased to levels that were higher in the IFN-γ group. In conclusion, our data confirm a strong activation of coagulation induced by r-TNF-α in cancer patients. They suggest that r-IFN-γ may lead to a slight activation of coagulation and augments TNF-α induced procoagulant activity. These effects may be due to r-IFN-γ induced sustained monocytic TF activity.

The study described in **Chapter IV** was undertaken to determine the effects on systemic fibrinolysis of hyperthermic isolated limb perfusion with r-TNF-α and melphalan, with or without pretreatment with r-IFN-γ. Twenty patients were treated with r-TNF-α and melphalan; four patients, treated with melphalan only, served as controls. Of the twenty patients treated with both r-TNF-α and melphalan, eight received r-IFN-γ for two days before the perfusion and as a bolus into the perfusion circuit. A significant leak of r-TNF-α from the perfusion circuit to the systemic circulation was observed in all r-TNF-α treated patients (mean maximum TNF-α 87227 ng/L *versus* 31 ng/L in controls, $p<0.002$). In these patients, but not in controls, there was an almost instantaneous rise in systemic tissue plasminogen activator (t-PA) activity (from 0.26 IU/ml to 5.28 IU/ml in 90 min), causing activation of fibrinolysis. After a delay of 90 minutes, plasminogen activator inhibitor-1 (PAI-1) antigen rose to high levels in the r-TNF-α treated group (mean maximum PAI-1 1652 ng/ml *versus* 211 ng/ml in controls, $p<0.02$), associated with a sharp decrease of tPA-activity and a slower decrease of plasminogen-antiplasminogen complexes (from 5.28 IU/ml to 0.02 IU/ml in 2 h, and from 1573 μg/L to 347 μg/L in 22 h respectively). No additional effect of r-IFN-γ pretreatment on fibrinolysis could be demonstrated. These results suggest that in isolated limb perfusion with r-TNF-α and melphalan an initial activation of systemic fibrinolysis, induced by leakage of r-TNF-α from the perfusion circuit, is set off by a subsequent inhibition of the fibrinolytic system by PAI-1. This large increase in PAI-1 could place the patient at risk for deposition of microthrombi in the systemic circulation.

Chapter V describes renal function parameters in 11 cancer patients, who underwent 12 perfusions. Three patients, perfused with melphalan only, served as controls. All patients treated with r-TNF-α developed a sepsis syndrome and needed volume replacement and inotropes to remain normotensive; controls had an uneventful postoperative course. Creatinine clearance decreased transiently on the day of perfusion in both groups (mean preperfusion clearance 118 ml/min, mean postperfusion clearance 68 ml/min, $p<0.02$, $n=15$). Follow-up measurements of renal plasma flow and glomerular filtration rate in 9 r-TNF-α treated patients did not suggest permanent damage. One patient became hypotensive and developed transient multiple organ dysfunction with renal failure needing hemofiltration. In r-TNF-α treated patients, but not in controls, a transient increase in clearance of β_2

microglobulin (49 vs. 8171 ml/min, $p<0.001$) and urinary excretion of phosphate (12 vs. 48 mmol/24 hrs, $p<0.05$) was seen, compatible with proximal tubular dysfunction. These data suggest that hyperthermic isolated limb perfusion with melphalan decreases glomerular function, whether or not r-TNF-α is added to the perfusion circuit. Extension of the treatment regimen with r-TNF-α may result in additional proximal tubular dysfunction. If hypotension can be avoided this deterioration in renal function seems to be transient, with full recovery within weeks.

Finally, **Chapter VI** aims to analyze the mechanism of vasodilation and circulatory shock in patients who are treated with isolated limb perfusion with melphalan and r-TNF-α for locally advanced malignant tumors. The role of nitric oxide, if any, was determined by measuring plasma nitrite and nitrate levels. Eight consecutive patients developed sepsis syndrome due to leakage of r-TNF-α from the perfusion circuit to the systemic circulation. Despite the presence of very high systemic TNF-α levels during and immediately after perfusion and definite signs of hyperdynamic circulatory shock (increased heart rate, increased cardiac index, decreased systemic vascular resistance) nitrite and nitrate levels, measured in plasma at several time points, were not elevated. The hypothesis that, in humans, TNF-α induces vasodilation and shock through activation of inducible nitric-oxide synthase and subsequent formation of excessive quantities of nitric oxide is not substantiated by our results. Normal nitric oxide metabolite levels were found in the presence of high TNF-α levels and shock. Other mechanisms that do not involve the nitric oxide pathway are likely to play a role in the generation of hypotension and septic shock in this setting

The experience with isolated limb perfusion with r-TNF-α, laid down in this thesis, has confirmed the feasibility of this type of treatment. The question whether r-TNF-α has a role in anticancer therapy has not been specifically addressed in this work. However, it can be assumed that the usefulness of r-TNF-α as an antineoplastic agent will be favorably influenced if its therapeutic index could be increased. Improvement of postoperative care is one way to achieve this. We speculate that with the current standard of anesthesia and postoperative intensive care, even higher dosages of r-TNF-α than have been studied thus far, can be tolerated by most patients. For treatment of malignancies not located on a limb, intravenous administration of r-TNF-α in dosages exceeding what has been considered the maximum tolerable dose (300 $\mu g/m^2$ over 30 min.) can be considered. Even more important will be the quest for safer TNFs; mutant TNFs that selectively bind to the 55 kd TNF-receptor (TNF-R1) share the anticancer potential of native TNF-α but have less pro-inflammatory activity.

Finally, basic research in sepsis will eventually supply us with drugs that can abrogate many of its deleterious effects. These drugs will be equally effective to counteract systemic toxicity in cancer treatment with r-TNF-α. The challenge here will be to reduce toxicity without reducing antitumor efficacy. Conversely, drugs that reduce toxicity in a model of isolated perfusion with r-TNF-α may be useful in the treatment of sepsis.

Chapter VIII

Samenvatting

Tumor necrosis factor alpha (TNF-α) is een klein eiwit dat bij alle zoogdieren voorkomt. Het heeft een functie in de afweer tegen bacteriën, virussen en parasieten. Zodra deze organismen het lichaam binnentreden gaan bepaalde afweercellen TNF-α produceren. Daarnaast lijkt het ook een stof te zijn waarmee het lichaam zich beschermt tegen kanker. Al in de vorige eeuw bereidde de chirurg William Coley aftreksels van bacteriën,waarmee hij ongeneeslijk zieke kankerpatiënten inspoot: bij een aantal van hen zag hij na deze behandeling een sterke teruggang in grootte van de gezwellen. Helaas was het middel soms erger dan de kwaal. Toch bleef de gedachte dat bacteriën, direct of indirect, een factor tegen kanker konden produceren onderzoekers bezighouden. In 1975 lieten Carswell en zijn medewerkers zien dat muizen, die werden ingespoten met endotoxine (een bestanddeel van de celwand van sommige bacteriën) een stof in hun bloed maakten, die werkzaam was tegen kanker. Wanneer bloed van deze muizen werd ingespoten bij andere muizen, die een gezwel in de huid hadden, zagen de onderzoekers die gezwellen verdwijnen. Zij concludeerden hieruit dat inspuiten van celbestanddelen van bacteriën aanleiding geeft tot de vorming van een (overdraagbare) stof die een tumor-dodende werking heeft. Deze stof, waar ook Coley's resultaten voor een belangrijk deel aan moeten worden toegeschreven, noemden zij "tumor necrosis factor". Met het vorderen van de biologische technologie werd het uiteindelijk mogelijk het TNF-α zuiver in handen te krijgen en bovendien in voldoende hoeveelheden om er experimenten bij mensen mee uit te voeren.

De resultaten van de eerste experimenten met TNF-α bij mensen vielen echter tegen. De meeste patiënten werden ernstig ziek na toediening van het middel en maar zelden werd een gunstige reactie op het gezwel gezien. Waarschijnlijk waren er nog hogere doseringen TNF-α nodig om effect op de kwaadaardige gezwellen te kunnen hebben, maar dergelijke doseringen werden voor de mens te giftig geacht. De verschijnselen die deze patiënten toonden deden overigens sterk denken aan wat men kon waarnemen bij patiënten die een ernstige infectie doormaakten. Inmiddels was komen vast te staan dat TNF-α een centrale rol speelt in de reactie van het lichaam op een ernstige infectie. Het fungeert daarbij als pro-inflammatoir eiwit, hetgeen wil zeggen dat het aan het begin staat van een cascade van stoffen die ontstekingsverschijnselen geven. Bij een ernstige infectie produceert het menselijk lichaam grote hoeveelheden TNF-α, waarschijnlijk meer dan nuttig is voor de afweer tegen de binnendringende microorganismen. De toestand die dan ontstaat wordt aangeduid als "sepsis", en wordt gekenmerkt door een lage bloeddruk, een verminderde hartwerking en onvoldoende bloeddoorstroming van de organen. Deze symptomen worden ook gezien bij kankerpatiënten die met hoge doseringen TNF-α worden behandeld.

TNF-α raakte als kankerbestrijdend middel op de achtergrond totdat Lejeune en zijn medewerkers besloten het op een andere manier toe te passen. Zij maakten daarbij gebruik van het principe van geïsoleerde regionale perfusie. Deze techniek was al eerder toegepast, maar nog niet met TNF-α. Het principe van geïsoleerde perfusie is eenvoudig: door de bloedsomloop van een arm of van een been volledig te scheiden van de bloedsomloop van de rest van het lichaam is het mogelijk gezwellen aan armen of benen met hoge doseringen geneesmiddelen te behandelen, zonder dat de rest van het lichaam te lijden heeft onder de giftigheid van die middelen. Zo'n volledige isolatie wordt bereikt door de aanvoerende en de afvoerende bloedvaten van een arm of een been aan te sluiten op een hart-long

machine, die het bloed in de geïsoleerde extremiteit niet alleen rondpompt maar ook van zuurstof voorziet (zie afbeelding bladzijde 12) Aan dit geïsoleerde circuit kunnen dan hoge doseringen geneesmiddelen worden toegediend. Lejeune en zijn medewerkers waren de eerste die daar, naast de gewone middelen tegen kanker, TNF-α aan toevoegden. Omdat dit keer wel hoge doseringen konden worden gebruikt (de bloedsomloop van het lidmaat was immers ontkoppeld van de rest van het lichaam) waren de effecten op bepaalde vormen van kanker veel beter dan in vroegere experimenten. Tegelijk bleek echter dat de patiënten ook van dit type behandeling ernstig ziek werden, zij het dat hun ziekteverschijnselen niet lang duurden en goed te behandelen waren op een Intensive Care afdeling.

Dit proefschrift bevat een aantal artikelen waarin beschreven wordt wat er met de patiënt gebeurt tijdens zo'n geïsoleerde perfusie met TNF-α. De effecten van deze behandeling op het gezwel vormen geen onderwerp van dit onderzoek.

In **Hoofdstuk I** wordt een overzicht gegeven van de ervaring die is opgedaan met TNF-α als anti-kanker middel. De nadruk ligt daarbij op de geïsoleerde perfusietechniek. Het overzicht is zowel op literatuurgegevens gebaseerd als op de eigen ervaring met een serie van 64 perfusiepatiënten. Alle patiënten, die zo'n behandeling ondergingen toonden dezelfde verschijnselen, zij het dat de ernst van patiënt tot patiënt varieerde. Lage bloeddruk en falen van de ademhaling waren de belangrijkste kenmerken. Met moderne Intensive Care mogelijkheden konden bijna alle patiënten, die een geïsoleerde perfusie met TNF-α hadden ondergaan de dag na de operatie weer worden teruggeplaatst naar de gewone verpleegafdeling. De manier van toedienen van het TNF-α maakt voor de bijwerkingen overigens weinig uit: ernstige algemene vergiftigingsverschijnselen zijn niet alleen beschreven wanneer TNF-α via de bloedbaan wordt ingespoten, maar ook bij onderhuidse toediening, bij toediening in de spier en bij toediening rechtstreeks in het gezwel.

In **Hoofdstuk II** wordt het ziektebeloop van 25 perfusiepatiënten meer in detail beschreven. Het bleek dat tijdens en direct na de perfusie een deel van de ingespoten TNF-α uit het geïsoleerde lidmaat weglekte naar de centrale bloedsomloop. In het bloed werden daarbij concentraties van TNF-α gemeten die vele malen hoger waren dan de concentraties die bij ernstige bacteriële infecties worden aangetroffen. Binnen 24 uur was de concentratie overigens weer tot normale waarden gedaald. Ook de klinische verschijnselen waren dan grotendeels voorbij. Uit eerder onderzoek is bekend dat TNF-α tot vaatverwijding leidt, de long beschadigt en de stollingsneiging van het bloed vergroot. In het onderzoek dat in dit hoofdstuk wordt beschreven kon worden aangetoond dat er een verband bestaat tussen de hoogte van de gemeten TNF-α concentratie in het bloed (dus de mate van lekkage) enerzijds, en de vaatverwijding, longbeschadiging en stollingsactiviatie anderzijds. Uit het onderzoek wordt geconcludeerd dat tijdens geïsoleerde perfusie met TNF-α ook in de rest van de bloedsomloop hoge concentraties TNF-α worden gevonden, en dat dit waarschijnlijk wordt verklaard door lekkage vanuit het niet volledig geïsoleerde lidmaat of door nog vrijkomen van TNF-α uit de extremiteit na herstel van de circulatie. Door deze lekkage treedt een ziektebeeld op dat sterke gelijkenis vertoont met een ernstige bacteriële infectie, maar korter duurt. Met adequate zorg herstellen de meeste patiënten binnen 24 uur.

In **Hoofdstuk III** wordt nader ingegaan op de activatie van de stolling die optreedt wanneer patiënten worden behandeld met regionale perfusie met TNF-α. Er wordt een vergelijking gemaakt tussen 8 patiënten die naast melfalan en TNF-α ook nog interferon-gamma (IFN-γ) kregen en 12 patiënten die alleen melfalan en TNF-α ontvingen. Van IFN-γ is bekend dat het de werking van TNF-α op gezwellen versterkt. Uit het onderzoek blijkt dat de IFN-γ groep, die twee dagen voor de perfusie was gestart met onderhuidse toediening van IFN-γ, direct voorafgaand aan de perfusie al tekenen van een geactiveerde stolling had. Tijdens de perfusie trad er in beide groepen een sterke activatie van de

stolling op, maar deze was meer uitgesproken in de IFN-γ-voorbehandelde groep. In het hoofdstuk wordt de veronderstelling geopperd dat toediening van IFN-γ een bepaald type witte bloedcel, de monocyt, aanzet tot het produceren van "tissue factor", een stof die de stollingscascade in gang zet.

Hoofdstuk IV behandelt de effecten van geïsoleerde perfusie met TNF-α op het afbreken van stolsels: de fibrinolyse. De verhouding tussen stolling en fibrinolyse wordt bij gezonde individuen zo geregeld, dat er enerzijds geen spontane bloedingen optreden en er anderzijds geen vaatafsluitingen ontstaan door spontane stolselvorming. In Hoofdstuk III was al beschreven dat bij perfusiepatiënten de stolling geactiveerd is. In Hoofdstuk IV wordt een groep van 20 patiënten, die werd behandeld met melfalan en TNF-α vergeleken met een controlegroep die alleen melfalan in het perfusiecircuit kreeg toegediend. De TNF-α behandelde groep toonde tijdens perfusie een snelle stijging van het "tissue plasminogen acitvator" (t-PA), een eiwit dat de fibrinolyse activeert. Een dergelijke t-PA stijging werd niet gezien in controlepatiënten, die een perfusie met alleen melfalan ondergingen. Enige tijd na deze stijging werd in de TNF-α behandelde groep echter een veel grotere stijging van het "plasminogen activator inhibitor" (PAI-1) gezien, een verbinding die het t-PA en daardoor de fibrinolyse remt. In Hoofdstuk IV wordt de suggestie geopperd dat door deze sterke remming van de stolselafbraak, gevoegd bij de al eerder aangetoonde activering van de stolling, een verhoogd risico op trombose en vaatafsluiting kan ontstaan bij patiënten, die behandeld worden met een TNF-α perfusie.

In **Hoofdstuk V** wordt onderzocht wat de invloed van perfusie met TNF-α op de nierfunctie is. Uit dierproeven was al bekend dat inspuiten van TNF-α vaak tot nierinsufficiëntie aanleiding geeft. Daarbij is het niet duidelijk of de nier nu wordt beschadigd door de lage bloeddruk die ontstaat na toediening van TNF-α of dat er ook sprake is van een direct toxisch effect van TNF-α op de nier. In dit hoofdstuk worden 11 patiënten, die werden behandeld met melfalan en TNF-α vergeleken met 3 controlepatiënten, die alleen melfalan in het perfusiecircuit kregen toegediend. In beide groepen werd op de dag van perfusie een verslechtering van de nierfunctie waargenomen die dus kennelijk moet worden toegeschreven aan de procedure en niet aan de TNF-α. Bij een aantal patiënten werd de nierfunctie ook nog nauwkeuriger gemeten met behulp van radioactief gemaakte stoffen. Deze techniek laat toe iets te zeggen over de bloeddoorstroming van de nier ("renal plasma flow) en de filtratiewerking ("glomerular filtration rate"). Er bleken geen belangrijke verschillen te bestaan tussen vóór en na perfusie gemeten waarden, zodat kon worden geconcludeerd dat er geen blijvende schade aan de nieren was ontstaan als gevolg van de perfusie met TNF-α. Het is van belang hierbij te vermelden dat bij alle patiënten de bloeddruk op peil kon worden gehouden, behalve bij één patiënt, die uiteindelijk een nierinsufficiëntie ontwikkelde. Aanvullend werd nog gekeken naar de urine uitscheiding van β_2 microglobuline, een eiwit dat in de normale situatie na filtratie bijna volledig wordt teruggeresorbeerd in de nierbuizen. In de met TNF-α behandelde groep nam de uitscheiding van dit eiwit sterk toe, in tegenstelling tot datgene wat in de controlegroep werd gezien. Dit zou kunnen wijzen op een tijdelijke beschadiging van het eerste gedeelte van de nierbuizen (de proximale tubuli) door TNF-α. Samenvattend is de conclusie van dit hoofdstuk dat geïsoleerde perfusie met TNF-α wat de nierfunctie betreft goed wordt verdragen en dat TNF-α mogelijk een specifiek beschadigend effect heeft op de proximale tubuli.

Tenslotte wordt in **Hoofdstuk VI** onderzoek gepresenteerd dat probeert opheldering te verschaffen over het mechanisme van de vaatverwijding die wordt gezien bij geïsoleerde perfusie met TNF-α. In proefdieren is aannemelijk gemaakt dat TNF-α een vaatwand enzym kan aanzetten (het "nitric oxide synthase"), dat het aminozuur arginine omzet in het citrulline. Hierbij komt een kort levende, maar zeer reactieve stof vrij, het nitric oxide (NO). Dit NO werkt ontspannend op de gladde spieren in de

vaatwand en leidt zo tot sterke vaatverwijding. Of het TNF-α bij de mens soortgelijke effecten heeft is minder bekend. In het perfusiemodel worden hoge concentraties TNF-α gevonden terwijl er ook meestal sprake is van vaatverwijding. Om deze reden is gezocht naar aanwijzingen voor overmatige NO-productie bij deze patiënten. Omdat NO vluchtig is, werden metingen verricht van nitriet en nitraat, de stabiele omzettingsproducten van NO. Bij geen van de 8 onderzochte patiënten kon een toename van nitriet of nitraat worden aangetoond, terwijl er wel degelijk sprake was van vaatverwijding en lage bloeddruk. In Hoofdstuk VI wordt dan ook geconcludeerd dat de vaatverwijding die ontstaat onder invloed van TNF-α perfusie niet via vorming van NO loopt. Het precieze mechanisme is nog onbekend.

Het onderzoek dat in dit proefschrift is beschreven onderstreept nog eens dat behandeling van kankerpatiënten met geïsoleerde regionale perfusie met TNF-α goed uitvoerbaar is. De patiënten worden weliswaar ziek, met symptomen die doen denken aan een ernstige bacteriële infectie, maar met goede zorg herstellen zij over het algemeen snel en volledig. Op grond van de ervaring opgedaan met dit type behandeling kan de veronderstelling worden geuit dat, met moderne behandelingsmethoden, een hogere dosering TNF-α verdragen kan worden dan tot nog toe werd aangenomen. Misschien moet zelfs worden overwogen de eerder uitgevoerde experimenten met in de bloedvaten toegediende TNF-α te herhalen, maar dan met hogere doseringen en in volledig gecontroleerde omstandigheden

Op grond van de overeenkomsten die er bestaan tussen patiënten, die een ernstige infectie doormaken en patiënten, die met TNF-α perfusie worden behandeld, lijkt het waarschijnlijk dat geneesmiddelen die werkzaam blijken te zijn op het gebied van sepsis ook werkzaam zullen blijken op het terrein van perfusie en vice versa. Onderzoek bij patiënten, die met TNF-α perfusie worden behandeld, zoals beschreven in dit proefschrift, kan op die manier een bijdrage leveren aan het vergroten van inzicht in het mechanisme van ontsteking, waardoor uiteindelijk betere behandelingsmogelijkheden voor sepsis beschikbaar zullen komen.

Dankwoord

Proefschriften komen tot stand doordat een aantal mensen heeft kunnen samenwerken. Dit proefschrift vormt hierop geen uitzondering. Van de velen die mij hebben geholpen wil ik een aantal apart vermelden.

Ik ben dank verschuldigd aan mijn promotores, en dat niet alleen omdat zij hun taken als promotor naar behoren hebben vervuld. Mijn eerste promotor, Prof. Dr. H. Schraffordt Koops, heeft als een van de eersten in Nederland het belang van perfusie met TNF-α ingezien, zonder welk voorbereidend werk dit proefschrift nooit ontstaan zou zijn. Mijn tweede promotor, Prof. Dr. R. van Schilfgaarde, is degene geweest, die de visie heeft gehad om een professionele Intensive Care afdeling voor chirurgische patiënten te ontwikkelen, met fulltime intensivisten, die zelfstandig kunnen werken binnen het chirurgische bedrijf. Aan hem dank ik een goede en stimulerende werkplek. Zonder dat zou dit proefschrift evenmin zijn ontstaan.

Dr. Armand R.J. Girbes ben ik dank verschuldigd omdat hij, als mijn directe collega, veel bijgedragen heeft aan de wetenschappelijke attitude binnen onze Intensive Care afdeling. Daarnaast heeft hij zich direct met de manuscripten bemoeid, waardoor ze zeker aan kwaliteit hebben gewonnen. Dr. Harald J. Hoekstra heeft als “onco-locomotief” positief bijgedragen aan het tempo waarin de in dit proefschrift gebundelde artikelen gestalte hebben gekregen. Dr. Jan. van der Meer heeft een onmisbare bijdrage geleverd aan twee hoofdstukken in dit boek, waarvoor ik hem veel dank verschuldigd ben.

De leden van de promotiecommissie, te weten Prof. Dr. H.J. ten Duis, Prof. Dr. N.H. Mulder en Prof. Dr. L.G. Thijs (VU, Amsterdam) dank ik voor de snelle manier waarop zij zich van hun taak hebben gekweten. De aanwezigheid van Prof. Dr. L.G. Thijs geeft mij bijzondere voldoening wegens zijn eminente positie binnen mijn eigen vakgebied.

Drs. John Maring is bij het schrijven en bij het maken van dit proefschrift mijn steun en toeverlaat geweest. Ik prijs mij gelukkig met zo’n creatieve en loyale collega te hebben mogen samenwerken. Het cliché dat zonder hem dit proefschrift niet tot stand zou zijn gekomen, moet in dit geval letterlijk worden opgevat. Dit werk is vooral een project van ons samen geweest. Graag spreek ik hier de hoop uit dat onze samenwerking een vervolg kan krijgen, ook na zijn aanstelling binnen de afdeling Anesthesiologie.

Prof. Dr. Maarten J.H. Slooff is uiteindelijk niet mijn promotor geworden in de academische zin, maar wel in overdrachtelijke zin. Hij is een van degenen binnen de chirurgische kliniek geweest die mij heeft “voortbewogen” door zijn nooit aflatende enthousiasme, zijn gevoel voor rechtvaardigheid en zijn oprechte persoonlijke belangstelling. Ik heb veel van hem geleerd, als dokter en als mens.

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Dr. Piet C. Limburg en Dr. Han Moshage hebben tot degenen behoord, die mij met kritische vragen op het rechte spoor der wetenschap hebben trachten te houden. Hun bijdrage heeft mij in staat gesteld het hier beschreven onderzoek aan te scherpen en nauwkeuriger te formuleren.

Dr. André B. Mulder wil ik dank zeggen voor de plezierige manier waarop Hoofdstuk II en IV tot stand zijn gekomen; veel van de daarin ten toon gespreide kennis komt eigenlijk van hem.

Drs. Robert J. van Ginkel is degene geweest die, uit hoofde van zijn KWF-fellowship, heeft gezorgd voor registratie en sampling van alle perfusie patiënten. Door zijn inspanningen werden veel resultaten mij op een presenteerblaadje aangereikt.

Mijn paranimfen, Drs. H. Delwig en Drs. J. C. Paling (Hans en Hans), wil ik danken omdat zij die taak op zich hebben willen nemen. Veel belangrijker is nog dat zij er tijdens mijn onderzoeksactiviteiten voor hebben zorg gedragen dat de “observed mortality’ van onze patiënten de “predicted mortality” niet heeft overschreden. Zij hebben veel opgeruimd wat ik in de klinische zorg heb laten liggen. Daarin hebben zij laten zien dat onze staf een team vormt. Het doet mij veel plezier dat allen bij deze promotie betrokken zijn, voor en achter de tafel.

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Het verpleegkundige personeel van de Intensive Care van de chirurgie ben ik erkentelijk voor de medewerking die zij aan het onderzoek hebben gegeven. Zij leveren niet alleen goede zorg, maar vormen een onmisbare schakel in het doen van patiëntgebonden onderzoek.

Mijn vrouw Mieke is gelukkig nooit de zuil geweest waarop ik mijzelf aan de academische wereld moest kunnen tonen, maar zonder haar was ik al lang van mijn voetstuk gevallen. Suzanne, Emma en Sophie: toch nog lakschoenen!