Systemic side effects of isolated limb perfusion with tumor necrosis factor alpha
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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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Chapter IV

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.
Effects of isolated limb perfusion with TNF-α on systemic fibrinolysis

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®, 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 °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% NaHCO₃ 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. 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 I¹³¹ -labeled albumin [6]. After 90 minutes of perfusion, the
Effects of isolated limb perfusion with TNF-α on systemic fibrinolysis

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 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 Stabilyte Vacutainer® 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°C. Plasma was stored at -80°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.
Chapter IV

Results

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

![Figure 1](image1.png)

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).

![Figure 2](image2.png)

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](image-url)  
**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](image-url)  
**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.

![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.](image)

Mean PAP levels ranged from 200 to 1500 µg/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.
Effects of isolated limb perfusion with TNF-α on systemic fibrinolysis

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 invariably 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.

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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
Effects of isolated limb perfusion with TNF-α on systemic fibrinolysis

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 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 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 µg/m² 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 disproportionately 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.

References


