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

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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.
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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).

![Subcutaneous Perfusion circuit diagram](image)

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% NaHCO₃ and 0.5 ml 5000 IU/ml heparin (Thromboliqine®). 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 (F₁⁺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 F₁⁺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 CaCl₂, 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, F1+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 F1+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](image-url)  
**Fig. 2** Geometric means of F1+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
Mean levels of TF (184 vs 145 fmol Xa/min/10^6 cells, 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.

F₁⁺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, F₁⁺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).
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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, F1+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 F1+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
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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.

References


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