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Influence of pegylated interferon-α therapy on plasma levels of citrulline and arginine in melanoma patients

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Summary. The aim of this study was to evaluate the effect of pegylated interferon-alpha (PEG-IFN-α) on the plasma citrulline/arginine ratio, regarded as an index of nitric oxide (NO) synthesis, in patients with high-risk melanoma. Forty patients were randomly assigned to either PEG-IFN-α treatment (n = 22) or to observation only (control group, n = 18). The treatment group received 6 μg PEG-IFN-α/kg once a week during 8 weeks, followed by a maintenance dose of 3 μg/kg/wk. Blood was collected at different time points, plasma concentrations of citrulline and arginine were measured and the ratio of citrulline/arginine was calculated. Patients treated with PEG-IFN-α showed a significant decrease in the concentrations of citrulline and in the citrulline/arginine ratio during the whole study period, both compared to baseline values and to the control group. The data suggest that therapy with PEG-IFN-α results in a marked decrease in the synthesis of NO in melanoma patients.

Keywords: Interferon – Nitric oxide – Melanoma – Citrulline – Arginine

Introduction

Therapy with interferon-alpha (IFN-α) is used against a variety of diseases, amongst others renal cell carcinoma, melanoma, chronic myelogenous leukemia and chronic viral hepatitis. Unfortunately, treatment with this cytokine is associated with the development of a wide variety of side effects, both somatic (e.g. flu-like symptoms, fatigue, anemia, thyroid dysfunction) and neuropsychiatric (e.g. depression and anxiety) (Dusheiko, 1997; Dieperink et al., 2000; Trask et al., 2000; Van Gool et al., 2003a). Among the most disturbing toxicities are depressive symptoms and syndromes, which may occur in 15–40% of the patients in the course of this treatment (Dieperink et al., 2000).

Many signaling pathways and biochemical mechanisms are hypothesized to mediate the neuropsychiatric side effects of cytokine therapy. Influences of cytokines on the function of for instance the serotonergic system could underly the pathophysiology of cytokine-induced depressive symptoms and syndromes (Bonaccorso et al., 2002; Van Gool et al., 2003b, 2004). Cytokines are also known to induce inducible nitric oxide synthase (iNOS), one of the three subforms of NOS (Bogdan, 2001; Busse and Mülsch, 1990; MacMicking et al., 1997). The nitric oxide (NO) system has been hypothesized to be involved in depression (Van Amsterdam and Opperhuizen, 1999; Hurlock, 2001). NO has been shown to modulate the effects of monoaminergic neurotransmitters such as serotonin, noradrenaline and dopamine (Kiss, 2000; Prast and Philippu, 2001), and the activity of the hypothalamus-pituitary-adrenal axis (Schmidt and Walter, 1994; Van Amsterdam and Opperhuizen, 1999); both are thought to be involved in the pathogenesis of depression (Maes and Meltzer, 1995). The role of NO in the pathogenesis of depression is further supported by observations that the antidepressant paroxetine is reported to inhibit NO synthesis (Goodnick and Goldstein, 1998) and NOS inhibitors have antidepressant-like properties in rodents (Karolewicz et al., 1999; Harkin et al., 1999). In addition, in one study assessing a relatively small number of patients, plasma nitrate levels, an indicator of NO production, were elevated in depressive disorder, while these levels normalized

after successful treatment with antidepressants (Suzuki et al., 2001).

To our knowledge, only one study focused on the role of the NO metabolism in IFN-α-induced depression in humans (Suzuki et al., 2003). These investigators used plasma nitrate concentrations as a measure of *in vivo* NO production. They found an increase of nitrate levels in chronic hepatitis C patients who developed depression in the first four weeks of treatment with IFN-α. No change in plasma nitrate was seen in patients on IFN-α without depressive symptoms and in patients who developed a depression later in the course of treatment with IFN-α. Moreover, plasma nitrite levels – nitrite is the direct product of NO oxidation – did not change significantly in any group (Suzuki et al., 2003).

The aim of the present study was to investigate whether the synthesis of NO is affected during treatment of melanoma patients with the pegylated form of IFN-α (PEG-IFN-α) as well. PEG-IFN-α is more convenient to patients than standard IFN-α, because it has to be administered only once a week, while its biological activity is similar (Bukowski et al., 2002). As an index of NOS activity, we measured the plasma concentrations of citrulline and arginine by HPLC in the patients under study and calculated the ratio of citrulline/arginine. The amino acid citrulline, which is formed concomitantly with NO, is regarded a more stable marker of NO synthesis than measurement of the serum levels of nitrate/nitrite (Pall, 2002). However, it has to be mentioned that citrulline originates not only from NO production, but also from the action of the enzyme ornithine carbamoyltransferase. Furthermore, the enzyme argininosuccinate synthase is capable of citrulline catabolism in mammals. In view of the previously mentioned data, we hypothesized that in humans treatment with PEG-IFN-α would result in increased citrulline levels, while arginine levels may decrease.

Materials and methods

Subjects and procedures

Samples were obtained from patients participating in a trial of the European Organization for Research and Treatment of Cancer (EORTC 18991), evaluating the efficacy and toxicity of PEG-IFN-α-2b versus controls in high-risk melanoma patients. High-risk melanoma patients were defined as patients with histological documented regional lymph node involvement of a primary cutaneous melanoma or unknown primary. Patients underwent a full lymphadenectomy within 56 days prior to randomization. In case of a synchroneous primary tumor, this was completely removed as well. Patients were randomized in a 1:1 ratio to the treatment arm (8 weeks induction with 6 μg/kg/wk s.c., followed by a five years maintenance with 3 μg/kg/wk s.c.; the treatment group), or observation only (the control group). In patients assigned to the treatment group who experienced severe toxicities, the PEG-IFN-α dose was adjusted according to protocol. The study protocol excluded patients with organic mental disorders, with psychiatric disorders at baseline that could be exacerbated by PEG-IFN-α (e.g. depression) and with alcohol abuse. In addition, we excluded samples from patients on antidepressant, antipsychotic or antiepileptic drugs. Samples were considered ineligible after PEG-IFN-α was stopped. No samples were analyzed from patients with distant metastatic disease or a bad general condition e.g. due to cancer progression. This report concerns with the first 50 patients enrolled in the EORTC study in our center. Ten subjects had to be excluded, 7 patients because no samples were taken at all and 3 because of psychotropic drug use. Of the remaining 40 patients, 22 (11 males, 11 females, mean age 45.7 years, range 32–68) belonged to the treatment arm, and 18 (11 males, 7 females, mean age 46.3 years, range 26–67) to the observation group. Mean doses (SD) of PEG-IFN-α used were: at 4 weeks 5.70 (0.92), at 8 weeks 5.0 (1.4), at 3 months 2.9 (0.54) and at 6–8 months 2.9 (0.3) μg/kg/wk.

Blood samples were taken at baseline, at 4 and 8 weeks during the induction phase, and at 3 and 6–8 months. For practical reasons, it was not possible to obtain blood samples at fixed times or under fasting conditions. Some samples were missing due to administrative failure at baseline and during follow-up. Moreover, the number of available samples decreased in the course of time due to attrition (because of recurrent disease or treatment cessation). The study was approved by the local ethical committee and patients gave informed consent.

Amino acid analysis

EDTA blood was obtained by venipuncture and after immediate centrifugation (20 min at 2650 g) plasma was separated and frozen at −80 °C. For amino acid analysis plasma was deproteinized with 5-sulphosalicylic acid (6%, w/v) and amino acids were assayed by high-performance liquid chromatography using automated pre-column derivatization with o-phthaldialdehyde (Fekkes et al., 1995). The citrulline/arginine ratio was calculated by dividing the plasma level of citrulline by the plasma level of arginine.

Statistical analysis

Data were stored using SPPS software and analyzed using SAS software. Estimates of changes of parameters at follow-up compared to baseline were obtained using mixed model analysis of variance (mixed model ANOVA) after log transformation of the outcome variables. A two-way ANOVA model was specified in which the effects of group (2 levels), time (4 levels) and their interaction on the change from baseline of the outcome variables were analyzed. Group by time interaction effects with a statistical significance of $P > 0.10$ were eliminated from the model. Furthermore, adjustment was made in the model for the following covariates: the baseline measurement of the outcome variable at hand, sex, and age. No structure was imposed on the correlation of the residuals. The effects on the outcome variable considered, as estimated from the mixed model using a restricted maximum likelihood technique, were back-transformed and expressed as percent changes from baseline in adjusted geometric mean levels. The latter technique enabled us to deal with missing values.

Results

Due to reasons mentioned in the previous section, of the 40 patients included in this study, amino acid levels were determined in 32 subjects at baseline, in 27 at 4 weeks, in 25 at 8 weeks, in 26 at 3 months and in 17 subjects at 6–8 months. The mean concentrations (±SD) of citrulline and arginine, and the mean value of the citrulline/arginine-ratio at baseline in the treatment group

Table 1. Comparisons of percent changes from geometric mean baseline concentrations of citrulline, arginine and the citrulline/arginine-ratio during follow-up between PEG-IFN-α treatment group and control group

	Change (%)			
	4 weeks (n = 27)	8 weeks (n = 25)	3 months (n = 26)	6–8 months (n = 17)
Citrulline				
IFN-α vs. baseline	-22.8^{**} ($-32.8/-11.2$)	-21.8^{***} ($-30.7/-11.8$)	-19.4^{*} ($-31.0/-5.8$)	-21.0^{**} ($-31.4/-9.0$)
Controls vs. baseline	1.4 ($-10.6/15.1$)	2.7 ($-8.6/15.4$)	5.7 ($-8.6/22.4$)	3.7 ($-9.0/18.2$)
IFN-α vs. controls	-23.8^{**} ($-35.2/-10.2$)	-23.8^{**} ($-35.2/-10.2$)	-23.8^{**} ($-35.2/-10.2$)	-23.8^{**} ($-35.2/-10.2$)
Arginine				
IFN-α vs. baseline	17.3^{*} (2.0/34.9)	31.6^{***} (19.9/44.6)	23.6 ($-1.6/55.1$)	46.7^{**} (18.2/82.1)
Controls vs. baseline	14.9^{*} (1.6/29.8)	-10.5^{*} ($-19.4/-0.6$)	-7.4 ($-23.3/11.7$)	-1.8 ($-18.7/18.6$)
IFN-α vs. controls	2.1 ($-15.1/22.9$)	47.0^{***} (27.9/69.0)	33.5 ($-0.6/79.3$)	49.4^{*} (12.0/99.4)
Citrulline/Arginine ratio				
IFN-α vs. baseline	-32.5^{***} ($-43.8/-19.0$)	-41.0^{***} ($-49.6/-31.1$)	-27.7^{*} ($-44.4/-5.3$)	-40.6^{***} ($-55.6/-24.7$)
Controls vs. baseline	-11.6 ($-25.0/4.1$)	11.8 ($-6.0/33.1$)	8.2 ($-13.3/35.9$)	4.3 (15.1/28.1)
IFN-α vs. controls	-23.7^{*} ($-40.2/-2.5$)	-47.3^{***} ($-57.3/-33.3$)	-33.9^{*} ($-52.8/-5.2$)	-42.3^{**} ($-58.8/-22.1$)

Change (%) = estimation of percent changes (95% confidence interval) from baseline in geometric mean levels within groups as well as between groups, adjusted for baseline value, age and sex (baseline = 100%). Statistical significance: $^{*}0.005 < P < 0.05$; $^{**}0.0005 < P < 0.005$; $^{***}P < 0.0005$

(n = 15) were 26.5 ± 6.0 µmol/l, 57.2 ± 16.0 µmol/l and 0.491 ± 0.147, respectively. In the control group (n = 17) these values were 27.4 ± 7.3 µmol/l, 55.2 ± 12.2 µmol/l and 0.514 ± 0.167, respectively. Table 1 shows the percent changes (and 95% confidence intervals) of these parameters in both patient groups (treated or not treated with PEG-IFN-α) at all 4 time points compared to the respective baseline value, as well as the changes in the treatment group compared to the control group. These percent changes from geometric mean baseline levels were directly estimated from the model (through back-transformation), so being adjusted for the baseline covariates included in the model. The concentrations of citrulline decreased significantly at all time points in the patients treated with PEG-IFN-α during the whole study period, both compared to baseline and to non-treated controls. No changes in citrulline levels were observed in the non-treated controls. In the treatment group, arginine concentrations increased at 3 of the 4 time points compared to baseline, while in the control group these concentrations increased at 4 weeks and decreased at 8 weeks. The difference in arginine levels between the 2 groups (PEG-IFN-α vs. controls) reached statistical significance at 8 weeks and 6–8 months.

The ratio of citrulline/arginine was significantly decreased at all time points in the patients treated with

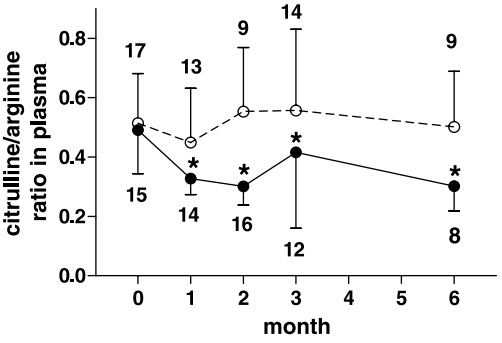

Fig. 1. Time-course of the citrulline/arginine concentration ratio in plasma of melanoma patients during treatment with PEG-IFN-α (●) and in an observation only group (○). The results are mean values ± SD. The figures in the graph represent the number of patients in each group. $^{*}p < 0.05$ vs. control group and vs. corresponding baseline value

PEG-IFN-α compared to both their baseline values and the control group. No significant changes were observed at any time point in the control group (Fig. 1).

Discussion

We observed a significant decrease of both citrulline levels and the citrulline/arginine ratio in plasma of melanoma patients during treatment with PEG-IFN-α, which suggests that the NOS activity is decreased during treatment with this cytokine. This result was rather unexpected and contrary to our hypothesis that treatment with PEG-IFN-α would result in increased NO synthesis. The latter hypothesis was based on the literature data on induction of iNOS by cytokines (Bogdan, 2001; Busse and Mülsch, 1990; MacMicking et al., 1997).

Several comments can be made concerning these unexpected findings. First, our method of measuring *in vivo* NO synthesis by the determination of plasma concentrations of citrulline and arginine is different from the methods mostly used, i.e. measurement of serum levels of nitrate or nitrite + nitrate. Perhaps our index of NO synthesis is less specific in this situation, since treatment with IFN-α might influence other metabolic pathways of arginine and citrulline as well, e.g. the activity of ornithine carbamoyltransferase, argininosuccinate synthase, arginase or the uptake of arginine by e.g. macrophages (Bogdan, 2001). Thus, it remains unclear whether altered citrulline levels result exclusively from decreased NOS activity or are also accounted for an increased activity of argininosuccinate synthase and/or a decreased activity of ornithine carbamoyltransferase. However, even though the measurement of nitrite + nitrate levels in body fluids is generally regarded as an index of NOS activity, basal levels of nitrite/nitrate in plasma and serum show great divergences (Tsikas and Frölich, 2004), probably because this determination is compromised by dietary sources, presence of nitrite/nitrate on plastic- and glassware and the chemical oxidation of NO to peroxynitrite by superoxide (Salter et al., 1996). Second, we measured an index of total NO synthesis, while cytokines regulate solely the expression and activity of iNOS (MacMicking et al., 1997). Moreover, in contrast to iNOS, the activity of the other two subforms of NOS, endothelial NOS (eNOS) and neuronal NOS (nNOS), is switched on by the elevation of intracellular Ca^{2+} levels and the binding of calmodulin (Bogdan, 2001). Since the three isoforms of NOS differ with respect to their regulation, the net effect of IFN-α therapy, hypothetically, might result in an inhibition of the conversion of arginine to citrulline and NO. An example of this is a recent study which shows that induction of iNOS restricts functional activity of both eNOS and nNOS in pig cerebral artery (Mathewson and Woodsworth, 2004). Third, the patients in this study are melanoma patients, which in contrast to the patients with hepatitis C studied by Suzuki et al. (2003) are lacking circulating immune complexes. In the absence of a viral or microbial costimulus type I interferons do not induce iNOS (Sharara et al., 1997). On the other hand, the group of Suzuki (2003) found no changes in the levels of nitrite, the first oxidation product of the unstable NO, in hepatitis C patients treated with IFN-α. Moreover, no change in plasma nitrate was seen in patients on IFN-α without depressive symptoms and in patients who developed a depression later in the course of treatment with IFN-α. Only in patients who developed a depression early in the course of treatment with IFN-α, an increase in nitrate levels was found. Thus, irrespective of whether it concerns hepatitis C or melanoma patients, the effect of treatment with IFN-α on the NO synthesis is probably not an increase in NO due to iNOS induction. Fourth, we used pegylated interferon instead of standard IFN-α, which was used in the other study (Suzuki et al., 2003). Although, PEG-IFN-α has similar or even more pronounced effects on tryptophan metabolism compared to standard IFN-α (Van Gool et al., 2004), the effect on the NO metabolism may be different.

While citrulline levels decreased at all time points in the patients treated with IFN-α, arginine levels showed an increase at all but one time point (only not significant at 3 months). The changes in the arginine levels in the control group were different to those in the treated group, i.e. an increase at 4 weeks, a decrease at 8 weeks and no change at 3 and 6–8 months (Table 1). Although the reason for these differences seen in the control group is unknown, one should not overrate this finding, because the confidence intervals at these time points are fairly high.

The finding of the present study, which suggests that patients treated with IFN-α have a lower NO synthesis compared to a control group, seems in line with our previous findings of increased neopterin plasma levels in the same group of patients, which might compromise the synthesis of tetrahydrobiopterin, because these pterins share the same precursor molecule (Van Gool et al., 2004). Tetrahydrobiopterin is important for the stabilization of the eNOS dimers necessary for enzymatic activity. Moreover, eNOS activity in this cohort of patients may also be lower, because of both a less functioning serotonin (5-HT) system – activation of eNOS can be induced by platelet-derived mediators such as 5-HT and ADP – and a

decreased amount of platelets due to treatment with IFN-α (Andrew and Mayer, 1999; Van Gool et al., 2004).

Limitations of this study are 1) the absence of psychiatric measurements in our cohort of patients and 2) that our method used to determine NO production is not only a rather indirect method, but also hard to compare to previous studies measuring plasma nitrate levels. The first point indeed is a serious flaw of the present study, but unfortunately cannot be dealt with, because these melanoma patients were seen by oncologists who performed no formal assessment of psychiatric status. Besides, only 2 of the 22 treated patients were diagnosed with a major depression upon psychiatric referral, which number is too small for any statistical analysis. Possibly in concordance with our data, two studies argue against the association of increased NO production with depression. One study investigated NOS activity in polymorphonuclear leukocytes of depressive patients by estimating nitrite content and these investigators reported a decrease in the NO synthesis in the patients as compared to the controls (Srivastana et al., 2002). The other study reported significantly lower levels of both plasma nitrite/nitrate and platelet eNOS activity in subjects with major depression compared with healthy control subjects (Chrapko et al., 2004).

The main message and conclusion of the present study is that in our cohort of melanoma patients treatment with PEG-IFN-α results in a decrease in the plasma levels of citrulline and in the ratio of citrulline/arginine, which we speculate could reflect diminished NO synthesis. We are currently performing a longitudinal study in which serial biochemical parameters measured in patients treated with (PEG-) IFN-α will be related to serial assessments of psychopathology. Results of the latter study may give an answer to the question whether or not the inhibition of NOS is in some way involved in the development of neuropsychiatric disturbances induced by this cytokine.

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