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ORIGINAL ARTICLE

Gerard Groenewegen · Andries Bloem
Gijsbert C. de Gast

Phase I/II study of sequential chemoimmunotherapy (SCIT) for metastatic melanoma: outpatient treatment with dacarbazine, granulocyte-macrophage colony-stimulating factor, low-dose interleukin-2, and interferon-alpha

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Abstract A regimen of sequential chemoimmunotherapy (SCIT) was studied in a phase I/II study to analyze toxicity, anti-tumor and immunomodulatory effects in patients with previously untreated metastatic cutaneous melanoma. Treatment consisted of dacarbazine (DTIC) 800 mg/m^2 administered intravenously (i.v.) on day 1, followed by subcutaneous (s.c.) molgramostim (GM-CSF), 10 times 2.5 μg/kg on days 2 to 12, s.c. low-dose interleukin-2 (IL-2), 10 times 1.8 MU on days 8 to 18, and s.c. interferon-alpha-2b (IFN-α), 5 times 6 MU on days 15 to 20. Dosages were not escalated. Therapy was given in the form of outpatient treatment. Changes in T-lymphocyte phenotype and in soluble mediators were monitored during treatment. A total of 32 patients with stage IV melanoma were enrolled in the study. Treatment was well tolerated, without serious toxicity. In all cases, it could be given as outpatient treatment. Ten subjects out of the 31 patients evaluated showed an objective response, with 4 complete responses (CR) and 6 partial responses (PR); the response rate amounted to 32% (95% CI: 16–49%). Median survival of all patients was 8 months, with those patients who responded to treatment living longer than the non-responding group. Survival rate at 1 year was 22%. Monitoring of the effects of treatment revealed increased numbers of activated T-lymphocytes, both in the CD4 and in the CD8 subsets. The levels of soluble mediators such as sIL-2R and sCD8 were also increased. Changes were noted as early as the GM-CSF treatment period, and were observed to a further extent during IL-2 treatment. In the present study, it was found that this sequential chemoimmunotherapy regimen consisting of 4 agents (DTIC, GM-CSF, IL-2, IFN-α) has acceptable toxicity, can be administered on an outpatient basis, results in increased numbers of activated T-lymphocytes, and induces activity against metastatic melanoma that warrants further investigation.

Keywords Clinical trial · GM-CSF · Immune activation · Melanoma · Sequential chemoimmunotherapy

G. Groenewegen (✉) · G.C. de Gast
Department of Oncology, University Medical Centre Utrecht,
P.O. Box 85500, 3508GA Utrecht, the Netherlands
E-mail: g.groenewegen@azu.nl
Tel.: +31-30-2506265
Fax: +31-30-2523741

A. Bloem
Department of Immunology, University Medical Centre Utrecht,
P.O. Box 85500, 3508GA Utrecht, the Netherlands

Present address: G.C. de Gast
Division of Medical Oncology,
Antoni van Leeuwenhoekhuis/Netherlands Cancer Institute,
Amsterdam, the Netherlands

Introduction

Malignant melanoma with distant metastases remains a disease with a poor prognosis. A conventional chemotherapeutic regimen of dacarbazine (DTIC) offers palliative benefit to only a limited number of patients [17], with a response rate of approximately 20%. One of the approaches adopted for improvement of treatment results is the combination chemotherapy and immunotherapy. So far, the results of a meta-analysis [11] have suggested a benefit of DTIC plus interferon-alpha (IFN-α) over the use of a single agent (i.e. IFN-α), based on response rate. With more intensive regimens both toxicity and the response rates increase, though a survival benefit of the treatment combinations over chemo- or immunotherapy only has not been clearly demonstrated [14, 22]. Treatment results for metastatic melanoma therefore still need to be improved upon, with an accompanying reduction of toxicity.

In the study presented here, we tested a new regimen using granulocyte-macrophage colony-stimulating factor (GM-CSF) as immunomodulator and with ultra-low doses of interleukin-2 (IL-2). The treatment regimen consisted of DTIC, GM-CSF, IL-2 and IFN-α. Since

these agents were administered in sequence, we named this approach "sequential chemoimmunotherapy" (SCIT). A phase I/II study was performed to study toxicity and anti-tumor effects. An ex vivo monitoring program was included to detect the mechanisms involved in T-lymphocyte activation. The results revealed acceptable toxicity, clinical activity against metastatic melanoma, and increased numbers of activated T-cells as early as the GM-CSF treatment period, with further expansion during treatment with ultra-low doses of IL-2.

Materials and methods

Clinical protocol

The study protocol was approved by the hospital's ethical committee prior to the start of the study, in compliance with all applicable regulations. Written informed consent was obtained from each patient before the start of treatment. All patients were treated at a single center, a university-based hospital. The study was designed as a phase I/II study to analyze toxicity, anti-tumor effects (response rate and survival), and the immunomodulatory effects of this regimen. The CTC (version 2.0) grading system for toxicity was used, being the most appropriate system at the time of analysis. A two-step design was used for the phase II part with a decision whether to continue accrual after 14 patients. Responses were analyzed according to WHO criteria. Duration of survival was assessed from the start of treatment.

Patient selection

All the patients included in this study had a histologically confirmed diagnosis of malignant cutaneous melanoma, and metastatic disease (AJCC stage IV). Treatment with a curative intent was not available. Inclusion criteria were as follows: performance status (Karnofsky ≥70%); age 18–70 yr; adequate renal function (estimated creatinine clearance >60 ml/min), bone marrow function (WBC $>3\times10^9$/l, platelets $>100\times10^9$/l) and liver function (bilirubin less that twice the upper limit of normal). Patients were excluded in the case of uncontrolled cancer other than melanoma, previous systemic treatment for metastatic melanoma, use of immunosuppressive medication, symptomatic brain metastases (only in the case of doubt was CT-scan performed), conditions affecting or being affected by the immune system, or known positive testing for HIV.

Treatment plan

A treatment cycle consisted of intravenously (i.v.) administered DTIC 800 mg/m^2 on day 1 at the hospital's daycare center. Further treatment was on an outpatient basis; instructions for subcutaneous (s.c.) injection were given to each patient and/or to a relative. From days 2 to 12, patients self-administered 2.5 μg/kg body weight GM-CSF (molgramostim; Schering-Plough, Maarssen, the Netherlands) 10 times s.c. From days 8 to 18, patients injected 1.8 MU IL-2 (aldesleukin; Chiron Europe, Amsterdam, the Netherlands) 10 times s.c. From days 15 to 20, 5 dosages of 6 MU IFN-α (IFN-α-2b; Schering-Plough) were given s.c. Dose escalation was not part of the study. During treatment, patients were seen once weekly at the outpatient clinic to be examined for toxicity and receive further medication. At day 22, a similar treatment cycle was started. After 2 cycles, a 2-week treatment-free interval was introduced, following which evaluation of anti-tumor effect was performed. In the case of an anti-tumor effect (stable disease or positive response) and good tolerance toward treatment, these patients were offered additional cycles, that started on day 64. Supportive care consisted of prophylactic use of ondansetron for DTIC-induced emesis, metoclopramide for bouts of nausea and vomiting, and acetaminophen against flu-like symptoms during the immunotherapeutical stage of the treatment.

Immunological monitoring

Blood was drawn before the start of treatment (day 1), and on days 8 (during GM-CSF), 15 (during IL-2), 20 (after IFN-α), 22 (prior to the start of the second cycle) and 36 (during the second cycle of IL-2). Soluble IL-2-receptor (sIL-2R) and soluble CD8 (sCD8) were assayed by ELISA. The procedure for analyzing activation antigens on T cells using flow cytometry has been described in detail elsewhere [19]. In short, two-color FACS analysis (FACScan; Becton Dickinson Immunocytometry Systems, BDIS) was performed on mononuclear cells obtained by Ficoll-Isopaque density centrifugation. Lymphocytes in the individual samples were identified by their light scatter properties ("lymphocyte gate"). Based on co-staining for CD45 and CD14, this lymphocyte gate was adjusted in such a way that it contained $>98\%$ lymphocytes. The presence of HLA-DR was analyzed on $CD3^+$ (all T cells), $CD4^+$ (helper T cells) and $CD8^+$ (cytotoxic T cells) cells. The sum of percentages of $CD4^+$ and $CD8^+$ cells equalled the percentage of $CD3^+$ cells within the lymphocyte population. CD28 and CD45RA and RO expression was studied on $CD4^+$/$CD8^+$ and $CD3^+$ T cells, respectively. Natural killer (NK) cells were defined as CD3-negative/CD(16 + 56)-positive. Flow data were analyzed using Cellquest software (BDIS) and calculated and expressed as the percentage positive cells compared to isotype- and subclass-matched control antibodies. All antibodies used for the flow cytometric analysis were obtained from BDIS. Absolute numbers of lymphocyte subtypes per volume of blood were used in further analyses.

Results

Patient characteristics

A total of 32 patients were included in this study; all received at least 2 cycles of the 4-agent treatment regimen. Patient characteristics have been summarized in Table 1. Only 2 patients received prior treatment in an adjuvant setting, 1 patient received an autologous tumour cell vaccine and one patient IFN-α in the context of the EORTC 18952 study. Median time from original diagnosis of skin melanoma to start of treatment was 1.5 yr (range: 0–20 yr). Disease extent has also been summarized in Table 1, including the stage according to the recent AJCC 2002 melanoma staging criteria [3].

Treatment characteristics

Patients started treatment between December 1994 and December 1997. A total of 97 cycles were completed. The median number of cycles per patient was 2, with a range of 2–8 cycles. Treatment could be given at the predetermined time in the planned dosages, without modifications or delay. In one patient, GM-CSF was omitted from cycles 3 and 4 after the observation of a skin rash without associated symptoms that was very likely to be related to the administration of this agent. The rash did not reoccur.

Table 1 Patient characteristics

Characteristic	No.	Percentage
Male/female	17/15	53/47
Age (median, yr)	50	
Range (yr)	20–67	
Performance status		
Karnofsky 100%, WHO 0	21	66
Disease stage (AJCC 2002)		
IVa	4	12.5
IVb	4	12.5
IVc	24	75
Site of metastatic disease		
Lymph node	18	56
Lung	15	47
Liver	12	38
Skin	12	38
Bone	6	19
Pleura	5	16
Other	11	34
No. of organs involved		
1	9	28
2	9	28
≥3	14	44
Mean	2.5	
Range	1–5	

Toxicity

Toxicity analysis was based on the first 2 cycles, since most patients received only 2 cycles, and those continuing treatment after cycle 2 did not show any new, additional, or cumulative toxicity (data not shown).

Treatment was generally well tolerated. Toxicity was either attributed to the chemotherapeutical or to the immunotherapeutical part of the treatment. As expected, DTIC caused nausea and vomiting, despite the prophylactic use of 5HT3-antagonists. To prevent immunosuppressive effects during treatment, DTIC was given without steroids. None of the patients experienced nausea greater than grade 1 and vomiting greater than grade 2 (CTC). The most frequently occurring types of toxicity are summarized in Table 2. Alopecia was not observed following DTIC treatment, not even after multiple cycles. Toxicity connected with immunotherapeutic agents mainly consisted of flu-like symptoms: chills, fever without infection, malaise and fatigue; these types of toxicity did not exceed grade 2 CTC. The flu-like symptoms could be partly controlled by acetaminophen. The decrease in the appearance of fever during the second cycle was ascribed to the more intensive use of this agent by the patients, as they had previously experienced such symptoms during the first cycle. Known IL-2-associated toxicity such as capillary leak syndromes or fluid retention were not noted: each patient was weighed daily to ensure the early detection of such disorders. IL-2 did cause minor local reactions at the injection site in nearly all patients. In all patients IFN-α was the strongest inducer of flu-like symptoms. Types and degrees of of toxicity have been summarized in Table 2. The one patient with cardiac toxicity had suffered from a myocardial infarction 5 years earlier. He developed an atrial flutter on day 12 of treatment, which reverted following quinidine treatment to normal sinus rhythm and was therefore graded as grade 3 toxicity. The entire treatment schedule could be given on an outpatient basis, and hospital admission was not necessary.

Anti-tumor effects

Clinical evaluation of response was performed in 31 patients. The remaining patient developed symptoms indicating the presence of brain metastases during the second cycle. Extra-cranial disease was not re-evaluated. In 10 patients objective responses were observed, which lasted at least 1 month. Four patients experienced complete remission, 6 patients showed partial remission, giving a response rate of 32% (95% CI: 16–49%). Responses were observed at every metastatic site. Response rate per system was highest for pleural lesions (60%), followed by skin (38%), lung (31%), and lymph nodes (28%); the response rate for liver metastases was only 10%. In 5 patients, stable disease or minor regression was observed, while in 16 patients disease progression had already been noted at first evaluation. The site of disease progression in 6 out of the 10 responding patients was the brain, without any progression being observed in previously known tumor sites.

Survival data

The duration of survival was analyzed by life table statistics calculated from the start of treatment. Overall median survival was 244 days. Survival at 6 months was 66%; 1-year survival and 2-year survival were respectively 22% and 12%. The best survival was noted in the subset of patients with a complete response ($n=4$; median survival 1,440 days). For patients with a partial response ($n=6$), median survival was 315 days.

Monitoring data

A monitoring program for T-lymphocyte phenotype was performed to gain insight in the biological effects of the studied regimen. SCIT treatment caused a statistically significant increase, as measured in the peripheral blood, in the number of activated T-lymphocytes (CD3, CD4, CD8/HLA-DR double positive cells), with a minor increase in the number of NK cells. These results have been summarized in Table 3. The activation of T-lymphocytes could already be detected after 6 days treatment with GM-CSF. After the administration of IL-2, a further increase in the number of activated T-cells was noted. Cell numbers decreased during

Table 2 Toxicity during 2 cycles of SCIT

Most frequent types of toxicity		No. of patients with toxicity				
	CTC	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Vomiting		17/12[a]	13/18	2/2	0	0
Fever		8/16	12/9	12/7	0	0
Fatigue		8/9	17/16	7/7	0	0
Other types of toxicity						
		No. of patients and worst toxicity during 2 cycles of SCIT				
	CTC	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Hematology						
	Hemoglobin	27[b]	0	4	1	0
	Leukocytes	29	0	1	2	0
	Platelets	27	5	0	0	0
Liver						
	Bilirubin	27	4	1	0	0
	Alk. phos.	24	6	2	0	0
	ASAT	20	12	0	0	0
	ALAT	15	11	6	0	0
Renal						
	Creatinine	30	2	0	0	0
Cardiac						
	Dysrhythmia	31	0	0	1	0

[a]Number of patients in first and second cycle with specific toxicity
[b]Highest grade of toxicity of 2 cycles, presented as number of patients; *Alk. phos* alkaline phosphatase

IFN-α treatment, though they still remained elevated. The number of NK cells showed a significant increase only after the administration of IL-2. Measurements in the second cycle during treatment with IL-2 also showed increased cell numbers for activated T-lymphocytes. In a limited number of patients treated with chemotherapy only ($n=3$) or chemotherapy in combination with granulocyte-colony stimulating factor (G-CSF; $n=3$) for malignant disease other than metastatic melanoma, increased numbers of activated T-lymphocytes were not observed on day 7 after chemotherapy (data not shown). At the same time point of T-cell phenotypic analysis, additional signs of T-cell activation were noted in the markers soluble IL-2 receptor (sIL-2R) and soluble CD8 (sCD8). As shown in Fig. 1, significant increases were induced during GM-CSF treatment, with a further increase during IL-2 noted for both markers.

One of the concerns in immunotherapy is the lack of effect of immune reactivity due to the absence of co-stimulatory molecules on lymphocytes. The role of CD28 in the generation and survival of activated T-cells, both CD4 and CD8, has been reviewed recently [4]. Therefore the presence of CD28 on CD4 and CD8 cells was also monitored. It appeared that the number of CD4/CD28 and of CD8/CD28 double-positive cells increased significantly during treatment with GM-CSF, with a further increase during IL-2 administration, allowing necessary signaling via this molecule. These results are shown in Fig. 2. The changes are comparable to the changes in the number of CD4 and CD8 lymphocytes respectively.

Finally, the balance between naive (CD3/CD45RA) and memory (CD3/CD45RO) T-cells was studied. The number of both cell types increased during treatment with GM-CSF, with a further increase during subsequent IL-2 treatment. The values for both cell types reached their maximal concentrations after IL-2 administration. Fig. 3 shows that the fraction of CD45RO (memory cells) in the combined population of CD3/

Table 3 Median numbers of T-lymphocyte subpopulations during treatment and level of statistical significance

Treatment	Day	Lymphocytes		CD3/DR		CD4/DR		CD8/DR		NK	
		N	*P*	*N*	*P*	*N*	*P*	*N*	*P*	*N*	*P*
Start-1	1	1,513	–	34	–	18	–	14	–	173	–
GM-CSF	8	2,764	< 0.001	138	< 0.001	57	< 0.001	39	0.007	207	0.264
IL-2 (1)	15	2,311	< 0.001	247	< 0.001	191	< 0.001	53	< 0.001	286	0.036
IFN-α	20	1,709	0.036	97	0.015	75	< 0.001	33	0.031	233	0.062
Start-2	22	1,892	< 0.001	56	0.052	37	0.016	24	0.44	234	0.086
IL-2 (2)	36	1,947	0.011	137	< 0.001	117	< 0.001	31	0.014	245	0.109

N Median number of cells of all patients, given as 10^6/l.
P Value of *P* in Wilcoxon signed-rank test for 2 related observations, as compared to start-1 (asymp. sign 2-tailed)

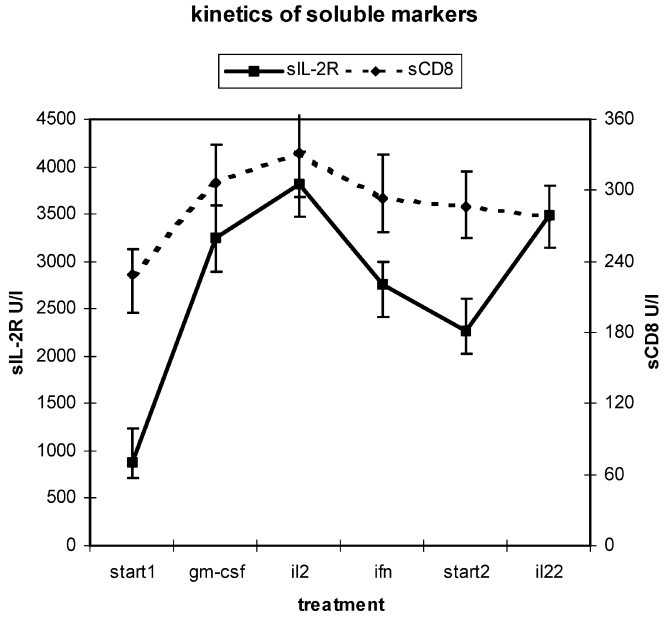

Fig. 1 Kinetics of soluble markers during treatment presented as mean values. All values after the start of treatment differ significantly from those at the start (Wilcoxon signed-rank test for two related observations, asymp. sign 2-tailed; $P \ll 0.05$). *Bars* represent SE of the mean

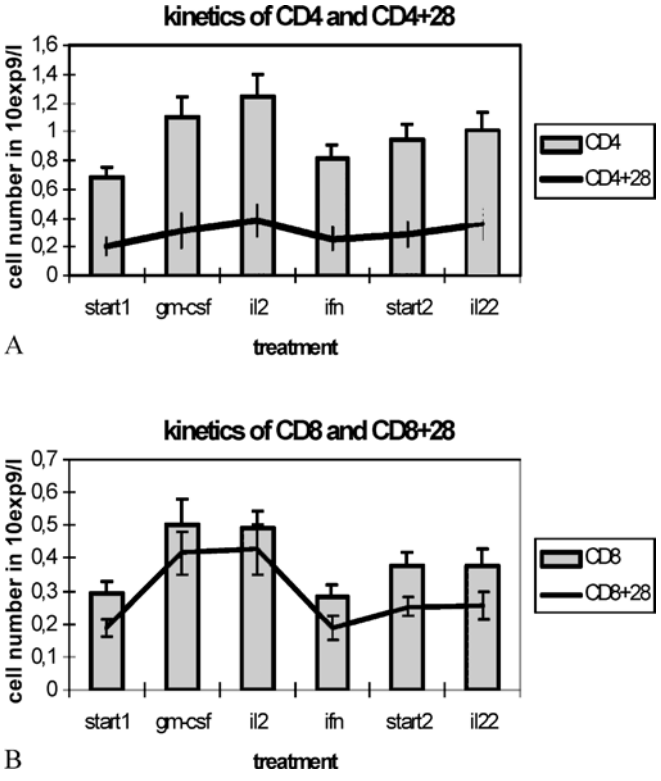

Fig. 2 Kinetics of CD4/CD4+28 (**A**) and CD8/CD8+28 (**B**) double-positive lymphocytes during treatment presented as mean values. All values after the start of treatment differ significantly from those at the start ($P \ll 0.05$; Wilcoxon test) except for CD8/CD28 in the *IFN* column. *Bars* represent SE of the mean

CD45RA and -RO cells became larger, revealing a stronger increase in this population compared to the naive cells. The difference was statistically significant (Wilcoxon test, one-sided; $P = 0.04$).

Since the patients were not selected for specific melanoma and/or HLA antigens, tumor specificity of the immune activation could not be studied.

Discussion

The study presented here aimed at investigating a new approach for the treatment of metastatic melanoma based on a combination of chemo- and immunotherapy. The regimen was based on the assumption that DTIC treatment results in the release of melanoma antigens, GM-CSF in the activation of dendritic cells to optimize antigen presentation, IL-2, with the ultra-low dosage selected, in the expansion of the activated T-lymphocyte population, and that IFN-α facilitates cytolytic activity by the upregulation of restriction elements on the melanoma cells. The study did not attempt to clarify these assumptions, though the monitoring program was included to obtain insight into these putative events. The clinical goal of the study was to improve treatment results for melanoma, and to reduce the toxicity of chemoimmunotherapy.

The use of DTIC has not been further discussed. It can be considered as a standard chemotherapeutic agent against melanoma. So far, available data do not reveal a detrimental effect of DTIC on the immune reaction [11]. This has also been suggested for other chemotherapeutic agents [16]. The available randomized studies do not show a clear advantage of chemoimmunotherapy over immunotherapy [15] or over chemotherapy [2, 13, 18, 21]. Still adopting the biochemotherapeutical approach, we added GM-CSF to the often used combination of IL-2 and IFN-α. This agent was selected for its known effect on antigen presentation, necessary for the initiation of the immune response, by its stimulating effect on dendritic cells [26, 27]. Studies in lymphoma patients have shown that this activating effect can also be observed in vivo, as measured by increased levels of soluble IL-2 receptor [9, 24] and by increased levels of soluble CD8 [9]. These observations have been confirmed in the present study, although in this case in patients with metastatic melanoma.

A limited number of studies have already used GM-CSF against melanoma, most of them as adjuvants in vaccination trials [26]. One of us used it in a concurrent approach with triple-agent immunotherapy [5]; in that phase I study, immune activation following combined therapy was observed, as well as indications of an antitumor effect. In the study presented here, GM-CSF has been used in a sequential approach to mimic the sequential events during the generation of an immune reaction. In another phase I study [25], escalating dosages of GM-CSF were added to a regimen of cisplatin, DTIC, tamoxifen, IL-2 and IFN-α. It was concluded that

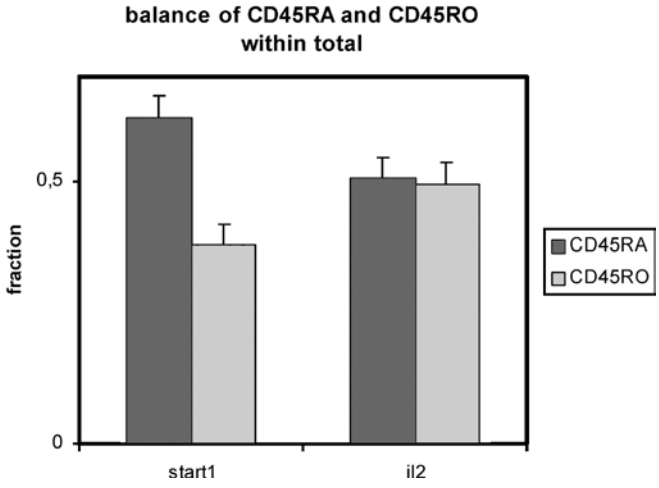

Fig. 3 Balance of CD3/CD45RA and CD3/CD45RO within the combined population at '*start1*' and during IL-2 treatment. *Bars* represent SE of the mean

GM-CSF treatment combined with biochemotherapy was a feasible approach, and that there might possibly be a dose-response relationship with GM-CSF in terms of clinical efficacy. The study by Vaughan et al. differed from our study regarding the GM-CSF schedule, as these authors had opted for a nearly concurrent dosage of the 3 immunotherapeutic agents. Indications of the beneficial effect of GM-CSF administration were obtained in an adjuvant setting of high-risk early melanoma where it was used as single agent and reduced the recurrence rate compared to historical controls [23]. GM-CSF was used in melanoma patients in a randomized phase II study, either alone or in combination with DTIC [20]. In neither arm was an anti-tumor response observed, making it difficult to assess the possible effects of GM-CSF. Additionally, GM-CSF was used to prevent neutropenia in topotecan-treated melanoma patients [12], but in that study the possible immune effects of GM-CSF were not addressed. To our knowledge, a sequential approach incorporating GM-CSF into immunotherapeutic schedules has not been studied before.

IL-2 was used in this study in an ultra-low dose. In many immunotherapeutic studies, IL-2 is used at the nearly maximum tolerated dose, thus explaining the observed toxicities. However, suggestions regarding the biological effects of IL-2 at low doses have been put forward by Hlaik et al. [8]. Also, if T-lymphocytes are activated, they acquire a high-affinity receptor for IL-2 [1, 10], thereby allowing effects at a low dose of the ligand. The observed increases in numbers of activated T-lymphocytes in this study support the possibility of using low doses of IL-2. The limited increase in numbers of NK cells can be explained by the small fraction of these cells carrying a high affinity IL-2 receptor. It must be assumed that GM-CSF was involved in prior activating events for these T-lymphocytes. That is indeed reflected by the observed effects in the monitoring program, both as regards the early changes in the number of activated T-cells, the co-stimulatory molecule CD28 and the memory lymphocyte population (CD45RO).

IFN-α was included in the treatment schedule to increase the sensitivity of the melanoma cells to the cytotoxic T-lymphocytes to be generated [6]. So far, this aspect has not been studied in vivo. Support for this approach is given by the observation that in this study, when skin lesions regressed during therapy, this occurred during treatment with IFN-α. Rapid disappearance of the lesions prevented a biopsy from being carried out. The specificity of the generated effector cells in the peripheral blood has not been studied in this protocol. Lack of the available technology, which meant that the treated patients were not selected for HLA-type, excluded these studies.

The clinical goal of the study was to improve treatment results in patients with metastatic melanoma, with a reduction in the toxicity of treatment. Regarding the latter in the studied regimen, this compared favorably to other chemo-immunotherapeutic regimens since treatment was administered entirely on an outpatient basis and could be given in the planned dosages and at the predetermined times, with good tolerance in the studied patients. Constitutional symptoms were present, though these did not exceed grade 2 CTC. Toxicities associated with high-dose IL-2 did not occur. Using standard dose escalation criteria from phase I studies, the use of escalated dosages might be feasible. This is presently being studied. These additional studies may also be able to provide insight into the relative contribution of each agent to the observed effects.

Regarding anti-tumor effects, this phase II study is difficult to compare with other studies. At first glance the response rate seems to be lower than in other studies, though as regards long-term survival, with all the limitations involved, this study compares well with other studies on metastatic melanoma. Only a subset of patients seems to benefit from the given treatment, considering the observation that most survival benefit is gained by patients who obtain the complete disappearance of all metastatic lesions. Patients with partial remissions do not greatly differ from those with lack of tumour regression. Future clinical studies should therefore also be directed at increasing the fraction of patients with long-term survival, most likely by increasing the complete remission rate. The frequent relapses involving the brain in long-term survivors, also observed in this study, will be a complicating factor.

In conclusion, a sequential regimen of DTIC, GM-CSF, IL-2 and IFN-α is tolerated, has an anti-tumour effect and results in immune activation in patients with metastatic melanoma. The best schedule for the immune response enhancing effect of GM-CSF in melanoma treatment remains to be elucidated. Given the observed anti-tumour effects in this study, further studies are warranted.

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