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Plasma Cytokine Analysis in Patients with Advanced Extremity Melanoma Undergoing Isolated Limb Infusion

Gina Shetty, BS¹, Georgia M. Beasley, MD², Sara Sparks, BS, MT, ASCP², Michael Barfield, MD², Melanie Masoud, BS¹, Paul J. Mosca, MD, PhD², Scott K. Pruitt, MD, PhD², April K. S. Salama, MD⁴, Cliburn Chan, MD, PhD³, Douglas S. Tyler, MD², and Kent J. Weinhold, PhD^{1,2}

¹Department of Immunology, Duke University, Durham, NC

²Department of Surgery, Duke University, Durham, NC

³Department of Biostatistics and Bioinformatics, Duke University, Durham, NC

⁴Department of Medicine, Duke University, Durham, NC

Abstract

Background—Preprocedure clinical and pathologic factors have failed to consistently differentiate complete response (CR) from progressive disease (PD) in patients after isolated limb infusion (ILI) with melphalan for unresectable in-transit extremity melanoma.

Methods—Multiplex immunobead assay technology (Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel, Millipore Corp., Billerica, MA; and Magpix analytical test instrument, Luminex Corp., Austin, TX) was performed on pre-ILI plasma to determine concentrations of selected cytokines (MIP-1 α , IL-1R α , IP-10, IL-1 β , IL-1 α , MCP-1, IL-6, IL-17, EGF, IL-12p40, VEGF, GM-CSF, and MIP-1 β) on a subset of patients ($n = 180$) who experienced CR ($n = 23$) or PD ($n = 24$) after ILI. Plasma from normal donors ($n = 12$) was also evaluated.

Results—Of 180 ILIs performed, 28 % (95 % confidence interval 22–35, $n = 50$) experienced a CR, 14 % ($n = 25$) experienced a partial response, 11 % ($n = 21$) had stable disease, 34 % ($n = 61$) had PD, and 13 % ($n = 23$) were not evaluable for response. Tumor characteristics and pharmacokinetics appeared similar between CR ($n = 23$) and PD ($n = 24$) patients who underwent cytokine analysis. Although there were no differences in cytokine levels between CR and PD patients, there were differences between the melanoma patients and controls. MIP-1 α , IL-1R α , IL-1 β , IL-1 α , IL-17, EGF, IL-12p40, VEGF, GM-CSF, and MIP-1 β were significantly higher in normal controls compared to melanoma patients, while IP-10 was lower ($p < 0.001$) in controls compared to melanoma patients.

Conclusions—Patients with unresectable in-transit melanoma appear to have markedly decreased levels of immune activating cytokines compared to normal healthy controls. This

further supports a potential role for immune-targeted therapies and immune monitoring in patients with regionally advanced melanoma.

After initial appropriate therapy, 2–10 % of patients with extremity melanoma will present with recurrent disease exhibiting single to several hundreds of tumor deposits between the primary site en route to the regional nodal basin in a pattern known as in-transit disease.^{1,2} Patients presenting with unresectable in-transit extremity melanoma are often treated with regional chemotherapy in the form of hyperthermic isolated limb perfusion (HILP) or isolated limb infusion (ILI), both of which deliver chemotherapy (usually melphalan) at far higher doses than can be achieved with systemic therapy.^{3–8} We currently utilize both HILP and ILI at our institution in the appropriate clinical setting, but we generally perform ILI as first-line therapy as a result of the lower rate of severe toxicity with ILI compared to HILP.^{3,4} Complete response (CR) rates after ILI are between 23 and 44 %, with a 30 % CR rate and 24-month median duration of response in our previously reported experience of 122 first-time procedures.^{3–7}

Although the 30 % CR rate after ILI is generally higher than can be achieved with other therapies, the remaining patients (approximately 40 %) will have progressive disease (PD). Additionally, even though correcting the melphalan dose for ideal body weight has led to significant decreases in toxicity after ILI, approximately 20 % of patients will still have Common Terminology Criteria for Adverse Events (CTCAE), version 3, grade 3 or higher toxicity after the procedure.³ The ability to prospectively predict which patients will respond to ILI would allow for better patient selection so that only patients who are likely to benefit from the procedure are exposed to the risk. However, previous studies have failed to identify clinical, pathologic, pharmacokinetic, or tumor biology factors that predict clinical response to ILI.^{3–7,9} Immunologic factors have not been well studied in patients undergoing regional chemotherapy. However, there is increasing evidence that the mechanism to produce clinically meaningful responses can largely be attributed to immune activation for a wide range of melanoma therapies (cytotoxic chemotherapy, immune therapy, and protein targeted therapy).^{10–15} Multiplex analysis of plasma cytokines has been useful for both predicting response and monitoring therapy in metastatic melanoma patients treated systemically with interferon (IFN)- α 2b and interleukin (IL)-2.^{16,17}

We hypothesized that the immune profile determined by a panel of pretreatment plasma biomarkers might be different between those patients achieving a CR and those experiencing PD after ILI. The aims of this study were to compare the pattern of plasma cytokine levels in a subset of patients who experienced CR after ILI with those who had PD after ILI, and to compare the pattern of plasma cytokine levels in patients with in-transit extremity melanoma to those of healthy donors.

METHODS

A prospective database at Duke University Medical Center (DUMC) from 1995–2012 identified 180 melphalan based ILIs performed in 155 patients with in-transit melanoma. All patients were classified as American Joint Committee on Cancer stage IIIB, IIIC, or IV, and had any measurable out-of-field disease (above tourniquet position), if present, resected

before regional treatment.¹⁸ Response was evaluated 12 weeks postoperatively via the Response Evaluation Criteria in Solid Tumors for cutaneous lesions and toxicity was assessed continuously using CTCAE, version 3.¹⁹ This study and our prospective database were approved by DUMC's institutional review board.

ILIs were performed as previously described.⁷ After warming the extremity to 37.0 °C, chemotherapy (melphalan with or without dactinomycin) was rapidly infused into the arterial circuit and circulated for 30 minutes. Melphalan was dosed at 7.5 mg/L for the lower extremity and 10 mg/L for the upper; dactinomycin was dosed at 75 µg/L and 100 µg/L, respectively.⁷ Limb volume was calculated by integrating extremity circumference at 1.5-cm intervals up to the level of tourniquet placement, and chemotherapy dosing was corrected for ideal body weight on the basis of evidence that such modification reduces severe toxicity without altering CR rates.²⁰ Some patients were also enrolled onto phase I/II clinical trials combining ILI with a systemic targeted agent: 19 patients received 14 days of sorafenib with ILI occurring on day 7, while 30 patients received ADH-1 at least 4 hours before ILI and on postoperative day 7.^{21,22} The melphalan concentration in plasma from the infusion circuit was measured at 5, 10, 15, 20, 25, and 30 minutes by an improved assay based on a published high-performance liquid chromatography–fluorescence method by Ehrsson et al.²³

Melanoma patient plasma (6–8 ml) was collected immediately before ILI (after systemic therapy in 3 patients) from ACD anticoagulant Vacutainer tubes. Normal control plasma samples ($n = 12$) were used from a bank of normal controls and were drawn in compliance with Duke University Health System Institutional Review Board for Clinical Investigations. All blood samples were received on the same day they were drawn. All samples were processed following the current Duke Translational Research Institute-Immune Monitoring (DTRI-IM) Good Clinical Laboratory Practice (GCLP)-Compliant Standard Operating Procedures for Plasma Isolation and Cryopreservation.

Plasma specimens were prepared for analysis in a 96-well plate utilizing a custom 13-cytokine Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel (Millipore Corp., Billerica, MA) following the kit-specific protocols provided by Millipore. Analytes were quantified using a Magpix analytical test instrument, which utilizes xMAP technology (Luminex Corp., Austin, TX), and xPONENT 4.2 software (Luminex). xMAP technology uses fluorescent-coded magnetic microspheres coated with analyte-specific capture antibodies to simultaneously measure multiple analytes in a specimen. After micro-spheres have captured the analytes, a biotinylated detection antibody binds to that complex. Streptavidin PE then attaches as a reporter molecule. Inside the instrument, magnetic beads are held in a monolayer by a magnet, where two LEDs are used to excite the internal micro-sphere dye and the dye of the reporter molecule, respectively. A CCD camera captures these images, which are then analyzed by Milliplex Analyst software (Millipore).

Concentrations of cytokines (pg/ml) were determined on the basis of the fit of a standard curve for mean fluorescence intensity versus pg/ml. Two quality controls were run with each assay (control 1, low level; control 2, high level.) All cytokines were found to fall within the quality control ranges except for control 1 of IP-10.

A nonparametric comparison (Mann-Whitney *U*-test) between cytokine levels was performed for normal vs. CR, normal vs. PD, and CR vs. PD with Bonferroni correction for multiple comparisons. A *p* value of 0.05 (0.00385 after Bonferroni adjustment) was considered significant. A post hoc power analysis was also performed with a 2-sided *t*-test (*p* = 0.004166667, Bonferroni correction for 12 cytokines).

RESULTS

From 2005 to 2012, we performed 180 melphalan-based ILIs. This includes patients who underwent multiple regional therapies (*n* = 25), as well as those who were treated with melphalan ILI plus concurrent systemic therapies (*n* = 59). Of 180 ILIs, 157 were evaluable for response as outlined in Fig. 1. CR was experienced in 50 patients (28 %, 95 % confidence interval 22–35), while 61 patients (34 %) had PD. The reasons 23 patients (13 %) were not evaluable were as follows: lost to follow-up (*n* = 11), less than 3 months after treatment (*n* = 5), aborted procedure (*n* = 2), and ILI performed for a pro-phylactic indication with no measurable disease at the time of ILI (*n* = 5). Fourteen patients (7.7 %) experienced grade 4 toxicities, including compartment syndrome necessitating fasciotomies (*n* = 11) and muscle necrosis requiring débridements (*n* = 3). Melphalan dose was corrected for ideal body weight in 90 % (*n* = 162) of the procedures. We previously reported that variables associated with having a CR or partial response after ILI included a lower melphalan dose (44.42 ± 13.39 mg in patients with disease that responded to therapy, vs. 50.96 ± 16.4 mg in patients whose disease did not respond to therapy) (*p* = 0.009), and having an infusion of the lower extremity (*p* = 0.001).³ We did not repeat the analysis of clinical predictors of response in the present study.

Pretreatment plasma was available for analysis for 23 CR patients and 24 PD patients. Table 1 lists the characteristics of patients undergoing ILI and normal, healthy controls (*n* = 12). Notably, patient characteristics and procedural variables are similar between the two groups, including age, disease stage, disease burden, and primary melanoma thickness. Similar to our previous results, the peak plasma concentration of melphalan during ILI was not different between CR (16.9 µg/ml) and PD patients (19.4 µg/ml). Two patients in the CR group also received systemic ADH-1 in addition to melphalan ILI, while one patient in the PD group received systemic sorafenib in combination with melphalan ILI. Kaplan-Meier survival curves in Fig. 2 found a trend toward a survival benefit for those achieving a CR compared to PD that was not statistically significant (*p* = 0.10).

The cytokines included in the analysis (Table 2) were selected on the basis of evidence suggesting these cytokines to be important in determining response to therapies and outcomes in melanoma (Fig. 3).^{16,17} Figure 4 displays results of the cytokine analysis. Among the 13 tested cytokines, there were no statistically significant differences in levels between CR and PD patients. Several of the melanoma patients had levels of cytokines below the limit of assay detection. In particular, of the total patients (CR 23, PD 24), the following had undetectable levels: IL-1Rα (CR 16, PD 18), IL-1α (CR 17, PD 13), IL-1β (CR 18, PD 20), and IL-12p40 (CR 16, PD 15). Nearly all other melanoma patients and all normal controls had measurable levels of the other cytokines.

There were notable differences in cytokine levels between the melanoma patients (both CR and PD) and the normal, healthy controls that were studied ($n = 12$). MIP-1 α , IL-1R α , IL-1 β , IL-1 α , IL-17, EGF, IL-12p40, VEGF, GM-CSF, and MIP-1 β were statistically significantly higher in normal controls compared to melanoma patients (both CR and PD). In contrast, levels of IP-10 were significantly lower ($p < 0.001$) in normal controls compared to melanoma patients. CR ($n = 23$) also had lower levels of IL-6 compared to healthy controls ($p = 0.026$). Post hoc power calculations are listed in Table 3.

DISCUSSION

Regional chemotherapy continues to have a role in the treatment of unresectable in-transit melanoma and can offer durable response rates that are superior to other forms of therapy currently available. In this study, the CR rate of melphalan-based ILIs was 28 % ($n = 180$), which is similar to the largest published series of ILI ($n = 204$) from Kroon et al.⁶ However, the toxicity in our series is not insignificant, with 7.7 % (14 of 180) of patients experiencing limb-threatening toxicities. Thirty-four percent ($n = 61$) had no clinically measurable treatment response. Although many studies have attempted to identify markers that may preselect patients who will benefit from regional chemotherapy, no consistent criteria have been identified.^{3-7,9} We were likewise unable to define a panel of immunologic parameters from pretreatment patient plasma that would distinguish CRs from PD patients. However, CR and PD patients appeared to have similar profiles, which were dramatically different from normal controls.

Other studies have examined the immunologic profile of melanoma patients using multiplexed assays with slightly conflicting results.^{16,17} A 29-plex cytokine assay on plasma samples from 179 resected high-risk melanoma patients before adjuvant treatment and 378 age- and sex-matched healthy controls showed a statistically significant increase in concentrations of IL-1 α , IL-1 β , IL-6, IL-8, IL-12p40, IL-13, GM-CSF, MCP-1, MIP-1 α , MIP-1 β , IFN- α , TNF- α , EGF, VEGF, and TNF-RII in plasma of melanoma patients compared with controls.¹⁷ The same study also found that higher levels of IL-1 β , IL-1 α , IL-6, TNF- α , and chemokines (MIP-1 α and MIP-1 β) before therapy (IFN- α 2b) positively correlated with the duration of regression-free survival.¹⁷ Sabatino et al., using multiplex assays in patients with metastatic melanoma or renal cell carcinoma treated with systemic IL-2, found patients with higher levels of VEGF and fibronectin did not exhibit a response to IL-2 therapy, and elevated levels of these proteins were associated with a significantly worse overall survival.¹⁶

In our study, the panel of cytokines found to be important in predicting response to IL-2 and IFN- α 2b did not appear to be important in determining response to ILI.^{16,17} Notably, our samples were also stored for approximately 1 to 5 years, and this could have potentially altered analyte levels. Although our study is limited by a smaller number of patients (47 melanoma patients, 12 normal controls) and a heterogeneous population, with three patients receiving systemic therapy before ILI, patients with in-transit melanoma may have a different immunologic profile compared to other forms of advanced melanoma. By definition, in-transit disease represents local regional failure, and although approximately 50 % of patients with in-transit disease will have lymph node involvement, a small proportion

will never develop systemic disease, making this pattern of disease potentially unique from other types of metastatic disease.²⁴ Using tumor gene expression data, our group has previously found that distinct patterns of gene expression could distinguish in-transit melanoma lesions from primary and other metastatic melanomas.²⁵ Data from our current study suggest that patients with in-transit disease may be globally immunosuppressed, although this needs to be confirmed in a larger prospective data set. Certainly a pattern of immunosuppression in these patients would support the use of therapies directed at immune activation.

By studying plasma only, immunologic responses to melanoma and other tumors may not be detected.^{24,26–30} Recently, intratumoral immune infiltrates have shown to correlate with clinical outcomes in melanoma much more consistently than biomarkers obtained from peripheral blood.^{24,26–30} Specifically, the characteristics of tumor-infiltrating immune cells have been associated with clinical outcomes in metastatic melanoma patients.³⁰ Melphalan, the standard chemotherapy for use in regional chemotherapy, has been reported to up-regulate the costimulatory molecules CD80 and CD86 in tumor tissue.¹⁵ We are currently conducting a phase I trial of intra-arterial temozolomide via ILI for patients with disease that fails to respond to melphalan therapy; treatment with temozolomide can result in the translocation and expression of calreticulin, a signal that encourages dendritic cells to phagocytose antigens and cross-prime tumor antigen-specific T cells.³¹ We have thus begun to collect sequential tumor samples, lymph node drainage, and peripheral blood for future studies in an attempt to develop a more comprehensive profile of tumor–host immune interactions. Importantly, in-transit disease provides a model where sequential tumor biopsies before, during, and after treatment can be performed to monitor treatment immune-related effects.

Although pretreatment tumor, lymph node, and peripheral blood can provide prognostic information regarding treatment response, the dynamic changes that occur with treatment are frequently not adequately captured, and comprehensive immune monitoring during treatment can be difficult. Multiplex analysis of plasma from metastatic melanoma patients receiving IFN- α 2b therapy resulted in a significant decrease of plasma levels of VEGF, epidermal growth factor, and hepatocyte growth factor, but these changes did not correlate with regression-free survival.¹⁶ We did not examine sequential biomarker levels in our study, which may ultimately demonstrate differences between patients who experience CR after therapy and those with PD, especially given that in our experience, maximal tumor response occurs from 6 to 12 weeks.³ Additionally, we only examined levels of 13 cytokines; many other cytokines may be important. Although an effective immunoresponse is likely essential to long-term tumor control, the optimal method of comprehensive immune monitoring for patients receiving any therapy for melanoma remains to be defined.

In conclusion, there were no differences between the levels of 13 cytokine among patients with in-transit melanoma who experienced CR compared to those with PD after ILI with melphalan. However, both CR and PD patients in this study seem to have markedly decreased levels of immune-activating cytokines compared to normal healthy controls. This further supports a potential role for immune-targeted therapies and immune monitoring in patients with regionally advanced melanoma.

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FIG. 1.

Response in 180 ILIs performed at Duke University from 2005 to 2012

FIG. 2.
Kaplan–Meier survival curve comparing survival (days) of those with CR (*orange*, $n = 23$)
to those with PD (*green*, $n = 24$)

FIG. 3. Preplasma levels of selected markers in patients with CR (blue), PD (green), and healthy controls (purple). The top *p* value in each row compares mean levels in normal vs. CR, the middle *p* value is for normal vs. PD, and the bottom *p* value compares means of CR vs. PD

FIG. 4.

Box and whisker diagrams of the notable differences in 13 cytokines. Each cytokine is listed on the top of each graph; each row is labeled on the bottom. CR complete response ($n = 23$), PD progressive disease ($n = 24$), N normal control ($n = 12$). The p values are calculated by the nonparametric Wilcoxon test. Red text and asterisk denote that the p value falls below 0.00385 (Bonferroni correction for 13 comparisons)

TABLE 1

Characteristics of CR, PD, and normal controls in plasma analysis

Characteristic	CR (<i>n</i> = 23)	PD (<i>n</i> = 24)	Normal (<i>n</i> = 12)
Median age, y	67	66	34
Sex, <i>n</i> /% female	17/68 %	11/48 %	7/58 %
AJCC stage			NA
IIIC	10	11	
IIIB	13	11	
IV	—	2	
Primary melanoma			NA
Median thickness, mm	2.45	3.35	
<i>n</i>	16	17	
Disease burden, <i>n</i> /% high	14 (60 %)	15 (63 %)	NA
Time from initial diagnosis of melanoma to ILI, d			NA
Median	819	938	
Range	56–3580	204–6016	
Grade 3 or higher toxicity, <i>n</i> (%)	5 (22 %)	3 (13 %)	NA
Corrected for IBW, <i>n</i> (%)	21 (91 %)	22 (92 %)	NA
Peak melphalan concentration, µg/mL			NA
Median	16.9	19.4	
Range	14.7–20	16.6–26.4	

ILI isolated limb infusion, CR complete response, PD progressive disease, NA not applicable, AJCC American Joint Committee on Cancer, IBW ideal body weight

TABLE 2

List of studied cytokines

Name	Abbreviation	Alternative names	Description
Macrophage inflammatory protein 1 α	MIP-1 α	CCL3, Small-inducible cytokine A3	Involved in inflammatory response and in recruitment and activation of leukocytes
Interleukin-1 receptor antagonist	IL-1RA	IL-1F3, DIRA, IRAP	Inhibitor of pro-inflammatory responses. Inhibits IL-1, IL-1 α , and IL-1 β . Regulates various IL-1 immune and inflammatory responses
Interferon gamma-induced protein 10	IP-10	CXCL10, Small-inducible cytokine B10	Chemoattractant for monocytes, macrophages, T cells, natural killer cells, and dendritic cells. Inhibitor of angiogenesis
Interleukin-1 β	IL-1 β	Catabolin	Involved in inflammatory response, cell proliferation, cell differentiation, and apoptosis
Interleukin-1 α	IL-1 α	IL-1F1	Involved in immunoresponse, inflammatory response, and hematopoiesis
Monocyte chemoattractant protein-1	MCP-1	CCL2, Small-inducible cytokine A2	Chemoattractant for monocytes, macrophages, memory T cells, natural killer cells, and dendritic cells to sites of injury, infection, and inflammation
Interleukin-6	IL-6	BSF-2, IFN- β 2	Involved in inflammatory response and maturation of B cells
Interleukin-17	IL-17	IL-17A, CTLA8	Chemoattractant of monocytes and neutrophils to sites of inflammation
Epidermal growth factor	EGF	HOMG4, URG	Cell growth, proliferation, differentiation, and survival
Interleukin-12 subunit β	IL-12p40	IL-12B	Inducer of Th1 cell development. Inhibits some biological activities of IL-12
Vascular endothelial growth factor	VEGF		Stimulates vasculogenesis, angiogenesis, and endothelial cell growth
Granulocyte-macrophage colony-stimulating factor	GM-CSF		Stimulates production and differentiation of hematopoietic cells to granulocytes, monocytes, and macrophages
Macrophage inflammatory protein 1 β	MIP-1 β	CCL4, Small-inducible cytokine A4	Chemoattractant for monocytes and macrophages. Activates human granulocytes

TABLE 3

Post hoc power analysis with two-sided *t*-test and $p = 0.004166667$ (Bonferroni correction for 13 cytokines)

Cytokine	CR vs. normal	PD vs. normal	CR + PD vs. normal
MIP-1a	0.74	0.76	0.87
IL-1RA	0.43	0.44	0.57
IP-10	1	1	1
IL-1B	0.2	0.27	0.32
IL-1a	0.51	0.41	0.6
MCP-1	0.98	0.68	0.96
IL-6	0.07	0.14	0.14
IL-17	0.69	0.71	0.83
EGF	0.96	0.96	0.99
IL-12P40	0.31	0.24	0.38
VEGF	0.78	0.86	0.92
GM-CSF	0.39	0.41	0.53
MIP-1B	0.26	0.39	0.44

CR complete response, PD progressive disease