Cancer Therapy: Clinical

Effect of Granulocyte/Macrophage Colony-Stimulating Factor on Vaccination with an Allogeneic Whole-Cell Melanoma Vaccine

Mark B. Faries, Eddy C. Hsueh, Xing Ye, Mary Hoban, and Donald L. Morton

Abstract

Purpose: The availability of a variety of immune response modifiers creates an opportunity for improved efficacy of immunotherapy, but it also leads to uncertainty in how to combine agents and how to assess those combinations. We sought to assess the effect of the addition of granulocyte/macrophage colony-stimulating factor (GM-CSF) to vaccination with a melanoma vaccine.

Experimental Design: Ninety-seven patients with resected melanoma (stage II-IV) were enrolled, stratified by stage, and randomized to receive a cellular melanoma vaccine with or without GM-CSF. The primary endpoint was delayed-type hypersensitivity (DTH) response to melanoma cells. Antibody responses, peripheral leukocyte counts, and survival were also examined.

Results: The GM-CSF arm showed enhanced antibody responses with an increase in IgM titer against the TA90 antigen and increased TA90 immune complexes. This arm also had diminished antimelanoma cell delayed-type hypersensitivity response. Peripheral blood leukocyte profiles showed increases in eosinophils and basophils with decreased monocytes in the GM-CSF arm. These immune changes were accompanied by an increase in early melanoma deaths and a trend toward worse survival with GM-CSF.

Conclusion: These data suggest that GM-CSF is not helpful as an immune adjuvant in this dose and schedule and raise concern that it may be harmful. Based on the discordant findings of an immune endpoint and clinical outcome, the use of such surrogate endpoints in selecting treatments for further evaluation must be done with a great deal of caution. (Clin Cancer Res 2009;15(22):7029–35)

The arrival of numerous immunomodulatory agents for use in clinical trials has renewed hope that the dramatic and durable regressions seen occasionally with immunotherapy might be experienced by a larger number of patients. The list of these new tools includes cytokines, Toll-like receptor agonists, antiregulatory agents such as anti-CTLA4 antibodies, and changes to the immunologic milieu through modifications of the host such as lymphodepletion. Due to redundant systems of control and regulation in the immune system, combinations of stimuli or modulators will likely be required to deliver consistent clinical benefit. However, rational strategies for designing and evaluating such combinations are not yet mature.

During the time that Canvaxin, an allogeneic whole-cell melanoma vaccine, was undergoing phase III trial evaluation, additional research was conducted in an attempt to enhance immune responses. Several trials evaluated the effect of the addition of various immunomodulators and adjuvants on immune endpoints. Here, we report the results of a randomized, open-label trial of the standard vaccine protocol with or without the addition of granulocyte/macrophage colony-stimulating factor (GM-CSF). These randomized data contribute to our understanding of the clinical and immunologic effect of GM-CSF on active immunotherapy.

GM-CSF is a leukocyte growth factor approved for use in leukopenic cancer patients and has been incorporated into numerous tumor vaccines. Its use is supported by a significant body of preclinical studies (1–4). In addition, GM-CSF has been used as a single agent in the adjuvant setting in melanoma and showed improved outcomes relative to historical controls (5). However, despite its common inclusion as a vaccine component, randomized trials examining the effect of GM-CSF on the immunologic and clinical effects of vaccines in cancer patients are sparse.
The first two doses of Canvaxin were admixed with the induction doses of BCG. Canvaxin was given every 2 weeks × 5 and then monthly × 4 to complete 6 months of immunization. A 50% dose reduction was employed for GM-CSF if patients experienced an absolute granulocyte count of >20,000/mm³. GM-CSF was held at the next dose if granulocyte counts increased >50,000/mm³. Toxicity was recorded using the National Cancer Institute Common Toxicity Criteria.

Assessment of immunologic response

**DTH testing.** Immunologic monitoring consisted of DTH skin tests and serum antibody measurements. DTH was done immediately before initiation of treatment and at the time of each vaccine dose. One tenth of the therapeutic dose of vaccine cells was used for DTH testing. Induration was determined at 48 h and read as the mean of the widest diameter of induration and the perpendicular diameter thereof. Control DTH response to nonmelanoma antigens was monitored by administering a PPD skin test to PPD-negative patients at monthly intervals until the patient became PPD positive or the seventh treatment. Blood samples were collected at baseline, at weeks 2, 4, 6, and 8, and at months 3, 4, 5, and 6 just before receiving vaccine for antibody and immune complex measurement.

**Anti-TA90 IgG and IgM titers.** Serum samples were analyzed prospectively for IgG and IgM antibodies to TA90 glycoprotein antigen. TA90 was purified from urine of a melanoma and ELISAs were done according to standard procedures reported elsewhere (9–11). Briefly, TA90 was adsorbed to 96-well ELISA plates at 120 ng/well, and serum sample dilutions were added. Subsequently, the bound immunoglobulins were reacted with the alkaline phosphatase-conjugated F(ab) fragment of goat anti-human IgG or IgM (Sigma). Absorbance at 405 nm was assessed, and the antibody titer was defined as the reciprocal of the highest dilution resulting in an absorbance of 0.05 at 405 nm after subtracting the absorbance values of the controls.

**TA90 immune complex assay.** Serum was assayed for TA90 immune complex as described previously (9). Briefly, patient serum was incubated on ELISA microtiter plates coated with murine monoclonal antibody to TA90. After washing, plates were incubated with goat anti-human IgG. An absorbance of 0.41 was the upper limit of normal. Interassay variability has been measured previously at <15% (9).

### Table 1. Demographics of population

<table>
<thead>
<tr>
<th><strong>GM-CSF</strong></th>
<th><strong>No GM-CSF</strong></th>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
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</tr>
<tr>
<td>Female</td>
<td>15 (33)</td>
</tr>
<tr>
<td>Age (y)</td>
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<tr>
<td>&lt;60</td>
<td>29 (63)</td>
</tr>
<tr>
<td>&gt;60</td>
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<tr>
<td>American Joint Committee on Cancer stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>9 (20)</td>
</tr>
<tr>
<td>II</td>
<td>10 (21)</td>
</tr>
<tr>
<td>III</td>
<td>26 (57)</td>
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<tr>
<td>IV</td>
<td>11 (24)</td>
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<tr>
<td>Median Breslow thickness (mm)</td>
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<tr>
<td>3.25</td>
<td>4.25</td>
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<tr>
<td>Nodal</td>
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<tr>
<td>In transit</td>
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<tr>
<td>Mean no. lymph node positive</td>
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<tr>
<td>10 (21)</td>
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<tr>
<td>M1A</td>
<td>5</td>
</tr>
<tr>
<td>M1B</td>
<td>4</td>
</tr>
<tr>
<td>M1A+B</td>
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</tr>
<tr>
<td>Prior treatment* (all stages)</td>
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<td>Radiation</td>
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<tr>
<td>Chemotherapy</td>
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<td>Immunotherapy</td>
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<tr>
<td>Other</td>
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*Some patients underwent more than one treatment modality.
Statistical analysis. With a sample size of 96, the study had 80% power to detect a 30% difference in DTH response. Comparison of group mean values for laboratory correlates was done by \textit{t} test or Fisher’s exact test. For comparison of immune response parameters, logtransformation was used to normalize distributions. Comparisons between groups during the vaccination period were done using mixed procedure (SAS 9.1.3) for longitudinal analysis. Specific time points were compared using \textit{t} test. However, these latter evaluations are considered exploratory only due to multiple comparisons. Survival was estimated using the Kaplan-Meier method and compared using log-rank test. All statistical analyses were two-tailed.

Results

Patient population. Ninety-seven patients were enrolled. Three were screen failures and did not receive vaccine. Demographic characteristics of the 94 patients eligible for analysis were similarly distributed between the two treatment arms (Table 1).

DTH response to Canvaxin immunotherapeutic. There was no significant difference in mean induration to baseline DTH testing with the vaccine (GM-CSF 4.2 ± 4.8 mm versus no GM-CSF 5.7 ± 7.7 mm; \( P = 0.25 \), Fig. 1). However, there was a trend toward increased DTH in the non-GM-CSF arm by week 4, which persisted and became significant at 16 weeks (GM-CSF 7.1 ± 4.3 mm versus no GM-CSF 12.8 ± 12.3 mm; \( P = 0.01 \), \textit{t} test). By longitudinal analysis, DTH values after initiating vaccination were significantly greater in the non-GM-CSF arm (overall mean 8.4 versus 10.9 mm; \( P = 0.006 \); Table 2). There was no significant difference in maximal DTH response.

PPD response. Mean PPD induration showed a trend toward a disproportionate increase in at week 4 in the non-GM-CSF arm (GM-CSF 6.3 ± 8.0 mm versus no GM-CSF 11.4 ± 8.0; \( P = 0.03 \), \textit{t} test). Because subjects were no longer PPD tested after becoming positive, there are few data points after week 4. By longitudinal and log-rank analyses, the increase in PPD response was not statistically significant.

Anti-TA90 antibody response. Three antibody titers were measured: anti-TA90 IgM, anti-TA90 IgG, and an adsorbed anti-TA90 IgG. The last was done due to possible retention of bovine serum albumin in the vaccine preparation. Adsorption of serum had a significant effect on IgG values but not in IgM. Both IgG assays are presented due to differences between groups seen in the nonadsorbed samples, although these responses are likely due to vaccine-specific but not tumor-specific antigens.

There were no significant differences in any antibody titer at baseline. The GM-CSF arm showed increased IgG responses in the nonadsorbed assay (significant at 8, 12, and 20 weeks). By the longitudinal analysis of log-transformed values, this difference was a strong trend (\( P = 0.059 \); Fig. 2A). There were no differences in the adsorbed assay values (Fig. 2B). During treatment, IgM titers were generally higher in the GM-CSF arm (Fig. 2C). Both maximal IgM (GM-CSF 803 ± 623 versus no GM-CSF 565 ± 549; \( P = 0.015 \)) and mean values at 8 weeks (GM-CSF 662 ± 588 versus no GM-CSF 415 ± 509; \( P = 0.047 \)) were higher with GM-CSF. Using longitudinal analysis of the log-transformed values, comparison of all on-treatment IgM values showed a trend toward increase in the GM-CSF arm (\( P = 0.086 \)).

The TA90 immune complex assay showed the most marked difference between groups with a rapid and significant increase in TA90 immune complex levels by week 4, which persisted throughout the study period (overall mean GM-CSF 1.1 versus

<table>
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<th>Table 2. Immune parameters</th>
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<td>Pre-vaccination</td>
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<tr>
<td>GM-CSF</td>
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<td><strong>DTH to vaccine</strong></td>
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<tr>
<td>IgM</td>
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<td>IgG (adsorbed)</td>
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<td>IgG (nonadsorbed)</td>
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<td>Absolute eosinophil</td>
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<td>Absolute basophil</td>
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*\( P \) values are by \textit{t} test for pretreatment and by longitudinal analysis during treatment.
Longitudinal analysis showed the on-treatment comparison to be significant ($P = 0.01$).

**Peripheral leukocyte counts.** WBC counts and differentials were measured before each vaccine administration after the acute increase following GM-CSF dosing had abated. Hemoglobin and platelet counts were similar between groups throughout (data not shown). Total WBC and profiles were similar at baseline ($P > 0.05$ for all baseline values; Fig. 3). There was an increase in total WBC in the GM-CSF arm at weeks 2 and 8 and an increase in the non-GM-CSF arm at week 16 ($P = 0.04$) but no significant difference by longitudinal analysis ($P = 0.23$). There was an increase in mean absolute neutrophil count in the GM-CSF arm, which was most marked at week 2 (GM-CSF 4.8 versus no GM-CSF 3.1) but was not statistically significant ($P = 0.19$). Mean absolute lymphocyte counts were similar between arms ($P = 0.59$). The GM-CSF arm also had lower monocyte and higher eosinophil counts than the non-GM-CSF arm ($P = 0.008$ and 0.014, respectively). Basophil counts trended higher in the GM-CSF arm ($P = 0.13$).

**Adverse event profile.** Adverse event profiles were similar between the two arms, although grade 1 or 2 fatigue and injection site reaction/pain were more common in the GM-CSF arm (Supplementary Table).

**Survival.** The study was not powered to definitively assess survival differences, but examination of these data revealed a surprising result. There was an excess of early recurrences and deaths in the GM-CSF arm resulting in a significantly decreased survival in that group when the results were analyzed at 2 years ($P = 0.002$). With longer follow-up, the curves have become closer and only a trend remains ($P = 0.097$; Fig. 4).

**Discussion**

GM-CSF has been explored as an adjuvant to numerous vaccines. Strategies include coadministration of recombinant GM-CSF and transfection of vaccine or bystander cells for *in vivo* cytokine production and are supported by preclinical studies showing improved immunogenicity and plausible biological mechanisms of antitumor activity (1–4).

Our data show effects of the cytokine on the immunologic milieu of the host including increased eosinophils and basophils and decreased monocytes in the peripheral blood. These changes were accompanied by enhanced humoral and diminished cellular responses, and both IgM and IgG seem to have

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**Fig. 2.** Antibody response measures. A, mean titer of anti-TA90 IgG. B, mean titer of anti-TA90 IgG in the absence of adsorption with bovine serum albumin. C, mean titer of anti-TA90 IgM. D, mean measure (absorbance) of TA90-IgG immune complex. *, $P < 0.05$; **, $P = 0.06$, t test at each time point.
been affected. These immune endpoints were accompanied by a troubling trend toward reduced survival in the GM-CSF arm. All of these findings are in keeping with the limited randomized clinical trial data preceding this report.

Randomized trials of GM-CSF have been conducted in non-cancer vaccines, such as hepatitis B vaccination (12, 13). Consistent with our data, these studies showed enhanced humoral responses with the addition of GM-CSF.

In contrast to the infectious disease results, there are few published randomized studies of GM-CSF in cancer immunotherapy. Nonrandomized GM-CSF data have appeared promising in comparisons with historical controls (5), but this comparison has been questioned due to large potential confounders between the compared populations. Small trials have been reported but have not shown consistent immune or significant clinical effects (14–16). Hamid et al. performed a randomized, three-arm trial of peptide vaccination. Two arms received a sustained-release formulation of interleukin-12 and a third arm received soluble interleukin-12 and GM-CSF (17). They showed significantly increased cellular immune responses by DTH and ELISPOT in the arms not receiving GM-CSF. Interestingly, the risk of relapse was greatest in the GM-CSF arm as well, although not by a statistically significant amount. These immune and clinical differences were attributed to the interleukin-12 formulation rather than GM-CSF because there were no preclinical data supporting an adverse effect of GM-CSF.

Accrual to one large cooperative group randomized trial using GM-CSF and peptide vaccination has been completed, but final clinical results have not been reported.

Over the last several years, data have emerged to suggest potential mechanisms for an adverse effect of GM-CSF on tumor immunity. GM-CSF receptors are present in vascular endothelial cells, suggesting the possibility of facilitated tumor growth (18–20). Another potential mechanism is induction and activation of myeloid-derived suppressor cells. In mice, these cells are fairly well characterized as CD11b^+GR1^+. In humans, several candidate marker profiles have been identified including Lineage^+HLA-DR^-, and CD11b^+CD14^-CD15^+ cells (21, 22).

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**Fig. 3.** Mean peripheral blood counts of each treatment arm at the indicated time points. *, P < 0.05; **, P = 0.06, t test at each time point.
Such cells may produce immunosuppressive factors including transforming growth factor-β and lead to activation of regulatory T cells. Myeloid-derived suppressor cells also appear to have a role in inducing vascular endothelial growth factor secretion, and this role depends at least in part on the presence of GM-CSF (23). Increases in myeloid-derived suppressor cells have been linked to diminished antitumor T-cell responses. The frequency of circulating myeloid-derived suppressor cells correlates directly with stage in solid tumors and increases in the setting of GM-CSF treatment (24, 25). Together, these findings suggest a mechanism connecting GM-CSF with diminished DTH response and early recurrence.

GM-CSF dose appears to be critical in determining the immunologic effect. Several trials using lower doses of GM-CSF (<80 μg/d) have shown improvements in immune T-lymphocyte responses (26–29), whereas higher dose trials have shown either no effect or a decreased response (29–33). As reviewed by Parniani et al., the threshold for an adverse effect appears to be ~100 μg/d (29). Above this dose, myeloid-derived suppressor cells may be recruited in substantial numbers. Our trial, which was designed well before this potential adverse effect was known, used a dose well above the threshold, at ~400 μg/d (mean bovine serum albumin 1.97 m²). Route of administration and dose interval are also important in determining the area under the curve for GM-CSF plasma concentration, which can be variable even with consistent dosing (34). If future trials use GM-CSF, this important dose-response relationship needs to be taken into account.

Immunotherapy trials in the adjuvant setting have generally relied on surrogate immunologic endpoints including antibody titers, DTH skin testing, and in vitro cellular response assays. The antibody and DTH responses used here have been in use for many years and enjoy a high level of correlation to clinical outcomes (11, 35). Numerous phase II trials have shown not only an effect of vaccination on immune measures but also a correlation of immune response to survival. Such clinical correlation is relatively uncommon for surrogate endpoints, many of which have either mixed or no correlation with survival. However, despite this prior record, in this trial, a positive effect on one surrogate endpoint was accompanied by an “increase” in early recurrence and a concerning survival trend. This raises general questions about the interpretation of immunologic data and their use in directing development of immunotherapies. The current trial also suggests the utility of a functional cellular response assay, DTH, as an endpoint. Although this endpoint may be considered outdated, it is both in vivo and functional and has a well-established track record in experienced hands. Not only has DTH been correlated with survival, but also changes in DTH have now been correlated with changes in survival. This is the first report of a randomized trial showing such a linkage of immunologic and clinical endpoints with the addition of an immunomodulator. Alternative surrogate immune endpoints are certainly reasonable and necessary, but interpretation of such measures should be done cautiously.

Disclosure of Potential Conflicts of Interest

D.L. Morton, ownership interest, CancerVax Corporation.

References

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