Targeting CD20 in Melanoma Patients at High Risk of Disease Recurrence

Alice Pinc1, Rajasekharan Somasundaram2, Christine Wagner1, Marcus Hörmann3, Georgios Karanikas4, Ahmad Jalili5, Wolfgang Bauer1, Patrick Brunner1, Katharina Grabmeier-Pfistershammer1, Melanie Gschaider1, Chiou-Yan Lai5, Mei-Yu Hsu5, Meenhard Herlyn2, Georg Stingl1 and Stephan N Wagner1,6

Melanomas contain distinct cell subpopulations. Several of these subpopulations, including one expressing CD20, may harbor stem cell-like or tumor-initiating characteristics. We hypothesized that patients at high risk of disease recurrence could benefit from an adjuvant anti-CD20 therapy. Therefore, we initiated a small pilot trial to study the effect of the anti-CD20 antibody rituximab in a group of melanoma patients with stage IV metastatic disease who had been rendered without evident disease by way of surgery, chemotherapy and/or radiation therapy. The major objective was safety, while secondary objectives were description of recurrence-free intervals (RFI) and overall survival (OS). Nine patients received rituximab at 375 mg/m² qw for 4 weeks followed by a maintenance therapy every 8 weeks. Treatment was discontinued after 2 years or with disease recurrence. Treatment was well tolerated. After a median observation of 42 months, the median neither of RFI nor of OS has been reached. Despite therapy that ended after 2 years, six out of nine patients are still alive and five of them are recurrence-free. Though the patient number is too small for definitive conclusions, our data may represent a first example of the potential therapeutic value of targeting CD20+ cell populations—at least for a subset of patients.

Received 3 November 2011; accepted 27 January 2012; advance online publication 21 February 2012. doi:10.1038/mt.2012.27

INTRODUCTION

Once melanoma has spread to visceral sites, the usual outcome is bleak with a median survival of 7–10 months and a 10-year survival rate of around 10%.1 While significant therapeutic progress has been achieved with the development of targeted and novel immunomodulatory therapies,2,3 the majority of clinical responses are still incomplete and/or disappointingly short-lived. A subgroup of metastatic melanoma patients may benefit from complete metastasectomy. These patients have a reported 5–7 months median recurrence-free interval (RFI) and, at best, a 19–21 month median overall survival (OS) as observed in large prospective clinical trials and retrospective analyses from extensive patient databases.4–8 After complete metastasectomy, immunotherapies aimed at preventing disease recurrence have shown some promise in small, uncontrolled prospective clinical trials5,9 and are currently evaluated in larger prospective trials. So far, however, no adjuvant treatment of stage IV melanoma patients has proved preferable to close observation aimed at early detection and surgical management of disease recurrence.11

Melanomas, like other malignancies, contain distinct cell subpopulations.12,13 Work on these subpopulations originated with the description of so-called cancer stem cells, first in hematopoietic and brain tumors and more recently in many other tumors (reviewed in refs. 13,14). With their exceptional capacity to self-renew and differentiate into diverse cell populations, these cells may be key to the functional heterogeneity of cancer15 and may thus have major translational impact. In melanoma, several subpopulations with the capacity of self-renewal, differentiation, tumorigenicity and/or drug resistance have been described,14,16–23 including one expressing the B cell marker CD20.19 CD20 was initially identified on a small percentage of human melanoma cells when cultured in embryonic stem cell medium and found on nonadherent spheres. These CD20+ melanoma cells followed the definition of cancer stem cells,24 i.e., they self-renewed and differentiated into several cell lineages. CD20+ melanoma cells were highly tumorigenic in vivo after xenotransplantation, indicating that these cells exhibit tumor-initiating capacity.18 Consistently, Schmidt et al. observed in a preclinical cell-based xenograft model an inhibition of growth and recurrence of highly tumorigenic human melanoma cells by specific targeting of the CD20+ subpopulation with autologous T cells genetically engineered to express a chimeric CD3ζ/CD20 antigen receptor.25

We hypothesized that melanoma patients at high risk of disease recurrence could benefit from an adjuvant therapy specifically targeting this tumor-initiating subpopulation. We have therefore initiated a small pilot trial to study the effect of the anti-CD20 antibody rituximab on disease recurrence in a group of melanoma patients.
with stage IV metastatic disease who had been rendered disease-free by way of surgery, chemotherapy, and/or radiation therapy.

**RESULTS**

**Patient characteristics**

The study population consisted of nine patients (seven male, two female); baseline demographic and clinical characteristics are given in Table 1. All patients had clinical stage IV disease with metastatic lesions affecting at least two body sites. One patient presented with a history of M1a disease (distant skin, subcutaneous, or nodal metastases), two patients with a history of M1b disease (lung metastases) and six patients with a history of M1c disease (all other visceral metastases).

Three patients had had brain metastases. Most patients had received multiple systemic and/or localized therapies including (multiagent) chemotherapy, various immunotherapies, radiation therapy and/or chemoembolization before inclusion into the trial. One patient had undergone only complete metastasectomy (Table 1). Of note, eight patients reported a disease history with at least one episode where all metastatic disease was initially fully responsive to conventional therapies and/or grossly resected, but disease recurred over time. The length of each of these RFIs is given in Figure 1. Some patients had experienced several of these episodes during stage IV disease (Figure 1, patients #1, #2, #6, #9).

**Safety**

Rituximab treatment was well tolerated, there were no dose-limiting toxicities. The majority of nonlaboratory adverse event were NCI-CTC (v. 3.0.) grade 1/2 (n = 58; mostly respiratory disorders such as pharyngitis, rhinitis), the one grade 3 event (thrombosis requiring anticoagulation therapy) was judged to be treatment-unrelated. Laboratory adverse events were exclusively CTC grade 1/2 (n = 125; mostly liver function and hematology), there were no CTC grade 3/4 events. Serious adverse events did not occur, virus serology remained unchanged.

**Clinical results**

RFI and OS were defined as the time from initiation of therapy until documented recurrence of the disease and death, respectively. As of May 2011, the median follow up time for RFI and OS is 42 months. Despite therapy cessation after 2 years, six out of nine (66%) patients are still alive and five of them are recurrence-free. So far, the median neither of RFI nor of OS has been reached. The median of RFI is 42+ months (mean: 27.4+) as is the median of OS (42+ months, mean: 37.9+). One patient has been alive for 39+ months and showed only local recurrence of the disease. The other patients experienced a disease recurrence after 6–13 months of therapy and died from the disease later on (at 27–37 months, respectively). The duration of the RFIs following anti-CD20 therapy is given in Figure 2.

We are aware that the trial patients represent a nonstandard population, reflecting the heterogeneity of the disease along with the low frequency of standard therapies to induce complete remissions of established metastatic stage IV disease. In our patients, NED was observed after (poly-) chemotherapy or obtained by complete metasasectomy, occasionally in combination with local

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (years), gender</th>
<th>ECOG perf. status</th>
<th>TNM (AJCC 2002)</th>
<th>Metastasis</th>
<th>Number of sites</th>
<th>Sites</th>
<th>Prior therapies</th>
<th>Last therapy before rituximab</th>
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<tr>
<td>1</td>
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<td>2</td>
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<td></td>
<td>Surgery, HD-IFNα, DC-vaccination, chemotherapy (carboplatin)</td>
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</tr>
<tr>
<td>2</td>
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<td>1</td>
<td>M1c</td>
<td>4</td>
<td>Gastrointestinal tract, brain, lymph nodes</td>
<td></td>
<td>Gamma knife, surgery, LD-IFNα, DC-vaccination, polychemotherapy (TVP)</td>
<td>Polychemotherapy (TVP) + gamma knife (brain)</td>
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<td>M1c</td>
<td>2</td>
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<td>Surgery, chemoembolization of liver metastasis</td>
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<td>M1b</td>
<td>3</td>
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<td>Lymph node dissection, gamma knife, chemotherapy (dacarbazine)</td>
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<td></td>
<td>DC-vaccination, chemotherapy (dacarbazine, fotemustine), polychemotherapy (TVP)</td>
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<td>LD-IFNα, chemotherapy (dacarbazine), gamma knife, surgery</td>
<td>Surgery</td>
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</table>

**Abbreviations:** DC, dendritic cells; HD, high-dose; IFN, interferon; LD, low-dose; M1a disease, distant skin, subcutaneous, or nodal metastases; M1b disease, lung metastases; M1c disease, all other visceral metastases. TVP, Temozolomide, Vinblastin, Carboplatin.
Patients with or without disease recurrence were comparable for the time period from onset of stage IV disease until initiation of anti-CD20 therapy [patients with recurrence (median: 21, range 2–36 months) versus without recurrence (median: 24, range 3–48 months)] and established prognostic factors such as performance status [patients with recurrence (50% each ECOG grade 0 and 1) versus without recurrence (40% grade 0, 60% grade 1)], history of brain metastases [patients with recurrence (25%) versus without recurrence (40%)], previous disease progression according to M category [patients with recurrence (1 × M1a, 1 × M1b, 2 × M1c) versus patients without recurrence (2 × M1b, 4 × M1c)], and age at initiation of anti-CD20 therapy [patients with recurrence (mean: 63 years) versus without disease recurrence (mean: 52.2 years, Table 1)].

Biomarkers of therapy
In an attempt to identify biomarkers of anti-CD20 treatment in melanoma patients or correlation with the clinical course, we analyzed several phenotypic parameters. For immunophenotyping, paired peripheral blood mononuclear cells samples obtained before and during therapy were available from eight trial patients (one patient excluded due to a sampling failure).

As expected, immunophenotyping of peripheral blood mononuclear cells showed a consistent loss of CD19+ B lymphocytes during anti-CD20 therapy, as demonstrated in samples collected at week 4 (Figure 3) and at 6 and 18 months (Figure 4). We did not observe any consistent changes in the absolute or relative numbers of CD3+CD4+, CD3+CD8+, CD16+CD56+, and CD4+CD25+CD127− lymphocytes during therapy, neither early at week 4 (Figure 3) nor later at 6 or 18 months (Figure 4).

Presence of CD20+ melanoma cells and lymphocytes in tumor samples
We had access to metastatic melanoma samples collected from seven patients before anti-CD20 treatment. These tissue samples were analyzed for the presence of the CD20+ melanoma cells and lymphocytes. CD20+ melanoma cells were identified by coexpressing the β3 integrin subunit, because the expression of β3 integrin is restricted to tumor cells in human melanoma tissues and CD20+ melanoma spheres have been shown to coexpress the β3 integrin subunit. CD20+ β3 integrin+ cells were observed in five out of seven tumor lesions (from three patients with and two patients without disease recurrence), in two samples (from one patient with and one patient without disease recurrence) these cells could not be detected. CD20+ β3 integrin+ cells were distributed within the tumors either as single cells or as small clusters (Figure 5), in each sample the frequency was below 1% of tumor cells.

CD20+ lymphocytes were present in each tumor sample, preferentially grouped in small clusters at the rim of the tumor (Figure 5). Sometimes we observed single CD20+ lymphocytes between tumor cells. Consistent with previous reports, the frequency of CD20+ lymphocytes ranged between around 5 and 20% of tumor-infiltrating lymphocytes. We also had the chance to collect a post-treatment tumor sample from a patient with disease recurrence. Here, neither CD20+ lymphocytes nor CD20+ melanoma cells could be detected.

DISCUSSION
Whether heterogeneity of melanoma cells in phenotype and function is following a deterministic model driven by small subpopulations of cancer stem cells or a stochastic model resulting from the same probability of virtually all tumor cells to generate distinct subpopulations, or rather both models via bidirectional interconvertibility is still a matter of debate. All models, however, are consistent with a new understanding of the complex biology of the disease as a dynamic process mediated by generation of sporadically present subpopulations through epigenetic changes and/or
microenvironmental factors that determine clonal dominance. Chemotherapeutic drugs, radiation treatment and presumably host immunity may impose a pressure to induce tumor-initiating subpopulations. Work on cancer stem cell niches further suggests that cytokines, soluble growth factors and extracellular matrix components such as osteopontin may provide a local microenvironment to sustain these subpopulations. As a result, patients who initially profited from conventional therapies will develop disease recurrence over time.

The recently identified CD20-expressing melanoma subpopulation is characterized by self-renewal, differentiation into several cell lineages and high tumorigenicity in cell-based in vitro and in vivo studies. Consistently, Schmidt et al. have described a highly tumorigenic human melanoma subpopulation that expressed high molecular weight melanoma-associated antigen/melanoma-associated chondroitin sulfate proteoglycan and contained a CD20+

Figure 4 Long-term effects of anti-CD20 therapy-induced changes in peripheral blood mononuclear cells. Long-term affects under therapy could be analyzed only in patients without disease recurrence. Percentages of CD19+ B lymphocytes, CD3+CD4+ and CD3+CD8+ T cells, CD16+CD56+ NK cells and CD4+CD25+CD127– regulatory T cells are shown for 3 patients (#1, open circle; #3; open square; #4: closed triangle) at 1, 6, and 18 months of therapy.

population with a CD44+CD61+CD24–CD34– cancer stem cell phenotype. The possible physiological and therapeutic relevance of this subpopulation is underpinned by the recent observation that anti-CD20 immunotherapy can inhibit growth and recurrence of human melanoma cells in preclinical xenograft models and, perhaps more strikingly, by our own observations in a subset of melanoma patients subjected to CD20-immunotargeting. As we are about to learn more about the biological significance of tumor cell subpopulations, we expect that their targeting will become an integral part of future therapeutic strategies, either aimed at the prevention of recurrence or at the elimination of established disease.

Our study represents a first example of the potential value of this strategy in the clinics, but also of its current limitations. Targeting a single subpopulation may not be sufficient to completely inhibit human melanoma growth in xenotransplantation models or to prevent recurrence in more than a subset of patients. In our trial, we could not differentiate between patients with disease recurrence from those without it by use of known prognostic factors. It can thus be concluded that the development not only of more effective (combination) therapies, but also of biomarkers for identification of patients who may potentially benefit from this kind of therapy is essential. Data from Schmidt et al. have given a first clue about the nature of a potential biomarker, i.e., the frequency of CD20+ melanoma cells in pretreatment tumor specimens. Consistently, we detected CD20+ cells in pretreatment melanoma lesions. Unfortunately, the low frequency of CD20+ melanoma cells, the small number of patients and the heterogeneity of the patient cohort did not allow any definitive statements beyond the purely descriptive observations provided.
We conclude that adjuvant immunotargeting of CD20 with mAbs offers an attractive and immediately available therapeutic option with an excellent safety profile, even in heavily pretreated melanoma patients. However, the patient cohort enrolled in this study is highly heterogeneous and this may have affected the observed clinical results. Thus, application of these initial and preliminary clinical observations through carefully designed trials is highly warranted and may open up a new perspective for a more effective and better tolerated treatment option for at least a subset of patients suffering from high-risk or metastatic melanoma.

MATERIALS AND METHODS

Patient eligibility criteria. Eligibility criteria were: age ≥18 years; biopsy-confirmed nodular metastatic melanoma, clinical stage IV according to AJCC 2002,44 no detectable disease after therapeutic intervention. Exclusion criteria were: prior treatment with an anti-CD20 antibody, ECOG performance status ≤2, radiation or chemo-/immunotherapy ≤4 weeks prior to study entry; LDH- and S100- or MIA-serology >upper limit of normal; active infection incl. HIV, hepatitis B and C infection; pregnant and lactating females; history of other invasive cancers within the past 5 years.

Study design. This study (EudraCT number: 2007-005125-30) was an open label, single-arm, investigator-initiated pilot phase I trial. All patients were enrolled at the Medical University of Vienna under a protocol approved by the institutional review board (457/2007) and the Austrian health authority. The study was conducted according to the principles embodied in the Declaration of Helsinki Principles and supervised by a Data and Safety Monitoring Board of the Medical University of Vienna. All patients provided written informed consent. All patients had had documented biopsy-proven clinical stage IV melanoma and all disease had to be either responsive to systemic or localized therapeutic interventions or grossly resected within 8 weeks before enrollment into the trial. At the start of rituximab treatment, all patients had no evidence of disease (NED) as documented by tumor imaging, physical examination and LDH- and S100- or MIA-serology >upper limit of normal. Patients with a history of successfully treated brain metastases could be included.

The major objective of this pilot trial was to determine safety, because rituximab—an immunosuppressive agent—was given to a vulnerable patient collective, namely (in most cases heavily) pretreated patients suffering from a highly immunogenic tumor. Secondary objectives were description of RFI and OS. Rituximab was administered at a dose and schedule established in follicular lymphoma patients,46 i.e., induction treatment with 375 mg/m² qw for 4 weeks followed by maintenance therapy with 375 mg/m² every 8 weeks. Treatment was stopped after 2 years or with recurrence of disease (as to tumor imaging, physical examination, or LDH- and S100- or MIA-serology >upper limit of normal performed during the follow-up period).
every 8 weeks). After 2 years, recurrence-free patients were followed only by physical examination and LDH-, s100-, MIA-serology every month as well as tumor imaging every 3 months. Patients with progressive disease received salvage therapies (including chemotherapy, radiation therapy, experimental vaccination) and were followed for survival.

**Study assessments.** Safety evaluations were conducted at baseline and at each visit thereafter and consisted of history taking and physical examination, CBC, serum biochemistry, baseline coagulation, coombs testing, quantitative immunoglobulins, complement C3/C4/CH50 levels and virus serology were evaluated at baseline and every 8 weeks in the first 2 years and graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 3.0. Radiologic assessments included complete tumor imaging (computed tomography scan or magnetic resonance imaging of chest, abdomen/pelvis, and brain or whole body positron emission tomography/computed tomography) and were performed every 8 weeks in the first 2 years, thereafter every 3 months (follow-up). Scans were read by the study-radiologist/nuclear medicine physician, who decided on continuation of the therapy.

**Immunological analyses.** Peripheral blood mononuclear cells were analyzed by four-color flow cytometry at indicated time points. B cells, CD4⁺ T cells, CD8⁺ T cells, NK cells and regulatory T cells were gated on a two-laser flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA) as CD19⁺, CD3⁺CD4⁺, CD3⁺CD8⁺, CD16⁺CD56⁺, and CD4⁺CD25⁺CD127⁻ lymphocytes, respectively. Fluorochrome-labeled monoclonal antibodies directed against CD3, CD4, CD8, CD16, CD25, CD4⁺CD25⁺CD127⁻ lymphocytes, respectively. Fluorochrome-labeled monoclonal antibodies directed against CD3, CD4, CD8, CD16, CD25, CD36, and CD127 were obtained from BD Biosciences. Percentages of positive cells were calculated.

**Immunostainings of pretreatment tumor tissues.** Immunostainings of pretreatment tumor tissues. Immunostainings of pretreatment tumor tissues were performed essentially as described. Briefly, formalin-fixed paraffin-embedded tissue sections were subjected to epitope retrieval by target retrieval solution (Dako, Glostrup, Denmark) and CD20 and β3 integrin expression visualized with fluorochromes Alexa633 and Alexa488, respectively. Antibodies were mouse monoclonal anti-CD20 (clone L26, Dako) and mouse monoclonal anti-β3 integrin (clone 23C6, BD Biosciences). Secondary antibodies were Alexa633-conjugated goat anti-mouse IgG2A and Alexa488-conjugated goat anti-mouse IgG1 (both BD Biosciences). Secondary antibodies were Alexa633-conjugated goat anti-mouse IgG2A and Alexa488-conjugated goat anti-mouse IgG1 (both BD Biosciences). Secondary antibodies were Alexa633-conjugated goat anti-mouse IgG2A and Alexa488-conjugated goat anti-mouse IgG1 (both BD Biosciences).

**ACKNOWLEDGMENTS**

We thank the patients and their families who participated in this trial; the clinical staff members of the Division of Immunology, Allergy and Infectious Diseases, Department of Dermatology, Medical University of Vienna; Drs. Ulrich Jäger, Winfried Pickl, and Franz Trautinger for serving in the Data and Safety Monitoring Board, Drs Peter Bauer, Claus Garbe, and Axel Hauschild for conceptual contributions and Dr Wolfgang Schreiner for statistical advice. This work has been funded by grants of the Vienna Hans Mayr-Fund and the Vienna Science and Technology Fund (WWTF) through project LS11-045 to S.N.W. We thank Wolfgang Schreiner for statistical advice. This work has been funded by grants of the Vienna Hans Mayr-Fund and the Vienna Science and Technology Fund (WWTF) through project LS11-045 to S.N.W. We thank Wolfgang Schreiner for statistical advice.