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Isolated Limb Infusion as a Model to Test New Agents to Treat Metastatic Melanoma

MICHAEL E. LIDSKY, MD*, PAUL J. SPEICHER, MD, BETTY JIANG, BS, MASAHIRO TSUTSUI, MD, and DOUGLAS S. TYLER, MD

Department of Surgery, Duke University Medical Center, Durham, North Carolina

Abstract

The limb model of in-transit disease can expand our understanding of treating melanoma because of the ease of obtaining tissue biopsies for correlative studies and the availability of preclinical animal models that allow validation of novel therapeutic strategies. This review will focus on regional therapy for in-transit melanoma as a platform to investigate novel therapeutic approaches to improve regional disease control, and help us develop insights to more rationally design systemic therapy trials.

Keywords

melanoma; in-transit; isolated limb infusion

INTRODUCTION

Melanoma is increasing in incidence faster than any other malignancy in the United States [1,2]. Of the 70,000 new cases reported in 2012, roughly half will present as disease located on an extremity, and even after appropriate local therapy, up to 10% of these patients will develop in-transit disease, defined as subdermal lymphatic metastases [3,4]. This pattern of advanced extremity disease occurring between the primary tumor and its draining lymph node basin is classified by the American Joint Committee on Cancer (AJCC) as either stage IIIB or IIIC, depending on regional lymph node involvement [3–5].

Patients presenting with unresectable in-transit melanoma confined to the extremity are often treated with regionally administered cytotoxic therapy in the form of hyperthermic isolated limb perfusion (HILP) or isolated limb infusion (ILI), both of which allow for the circulation of chemotherapy at doses much higher than can be administered systemically. The complete response rate at our institution after HILP and ILI is 55% and 30%, respectively. Despite differing complete response rates, the two forms of regional treatment are associated with similar long-term survival [6]. However, up to 40% of patients do not respond to this treatment strategy [6]. Extensive research efforts are, therefore, currently underway at our institution and others, in an effort to identify and test not only new infusional agents, but

*Correspondence to: Michael E. Lidsky, MD, Department of Surgery, Duke University Medical Center, DUMC3118, Durham, NC 27710. Fax: +1-919-684-6044; michael.lidsky@duke.edu.

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also to study the behavior and therapeutic response of melanoma to various agents under the controlled conditions of a regional therapy procedure. For the purposes of this review, we will focus on preclinical as well as clinical research that utilizes the platform of in-transit disease to investigate new therapeutic agents and strategies for treating advanced-stage melanoma.

GENOMICS OF IN-TRANSIT DISEASE

Substantial genetic heterogeneity in melanoma makes targeted treatment particularly challenging, but the multitude of tumors often found in a given patient with in-transit melanoma allows for serial biopsies and valuable genomic investigations. Not only is there heterogeneity between tumors of different patients, there also exists heterogeneity in primary tumors of the same patient and in tumors at different stages in the evolutionary pathway from primary to metastatic disease. The origins of mutated melanocytes are numerous; copy number alterations, mutated oncogenes, different DNA copy numbers and gene expression patterns, and differential regulation of genes are a wide array of known culprits [7]. Once a cancerous melanocyte develops, further genetic alterations occur throughout its clonal evolution and, as a result, distant metastases often have a heterogeneous genetic profile [8, 9]. In-transit melanoma, as both a physical and temporal intermediary between primary lesion and distant metastatic disease, provides us with a unique platform to observe early genetic changes in metastatic transformation. We see that in-transit tumors have greatest clonal similarity to the primary tumor, both in gene expression patterns and loss of heterozygosity [7,10,11]. Gene array studies at our institution have given additional insight into the relationships between primary, in-transit, and distant metastatic disease. Unsupervised hierarchical clustering of gene expression data of 1,000 genes from benign nevi, primary, in-transit, and distant metastatic disease showed that in-transit lesions from a given patient clustered together and had similar gene expression patterns [7].

Additional analysis of in-transit lesions identified two groups of in-transit melanoma, one subgroup more similar to a primary tumor and benign nevi group, and the other in-transit subgroup more similar to metastatic clusters. This finding again demonstrates the heterogeneity of melanoma overall and suggests differences in biological behavior. However, upon closer investigation of the biological processes related to the observed gene expression patterns of in-transit melanoma, subgroups with more homogenous features became apparent. For example, one subgroup had enriched expression of genes related to development, while another subgroup related to immune function. Furthermore, BRAF and NRAS mutation status were associated with dysregulation of particular oncogenic pathways [7]. In summary, primary tumors are genetically heterogeneous, but tumors that gain metastatic ability and produce in-transit lesions tend to have more homogeneous profiles. As we further divide in-transit disease into meaningful subgroups through better understanding of genetic expression patterns, targeted treatment strategies become increasingly important.

While examination of molecular aspects of in-transit lesions as a group across patients provided interesting insight into their biology, the marked molecular similarity of in-transit lesions within a given patient was an important finding, as it implied that a single in-transit lesion from a patient could serve as a good surrogate molecularly for all the other in-transit

lesions in the same patient [12]. This finding helped demonstrate the utility of in-transit melanoma to serve as a tremendous research platform whereby researchers could have easy access to tumor tissue and perform sequential biopsies throughout the course of treatment. In this fashion, evaluation of targeted or immunologic therapies could assess not only if various target proteins or cells were present in the tumors prior to treatment, but also if these targets were appropriately altered by the therapy given. Furthermore, lesions could be biopsied at the time of disease progression to determine why treatment failure occurred.

PRECLINICAL MODELS OF REGIONAL THERAPY

In vivo models have become the gold standard for testing new therapeutic agents and strategies in the preclinical arena. In the study of in-transit melanoma, rodent models have traditionally been preferred, with the rat and mouse each having strengths and weaknesses for preclinical work (Fig. 1) [13–15]. There is not currently an immunocompetent rat model of melanoma, as rats do not get melanoma. As such, human xenografts are implanted in the hind limbs of nude rats to mimic in-transit tumors. This most typically requires subcutaneous injection of human melanoma cells in a physiologic suspension that also contains an extracellular matrix platform, typically matrigel [13,15]. After tumors reach a predetermined size, various systemic and regional treatment strategies can commence.

The rat model utilizes athymic nude females that are irradiated prior to inoculation to ensure adequate immunosuppression and maximize xenograft formation. After establishment of xenografts in the hind limb of the rat, ILI can be performed as previously described [13,15]. Briefly, utilizing an anesthetic combination of narcotics, ketamine and a potent inhalation agent, an inguinal incision is created and dissection is carried down to the level of the inguinal ligament. At that time, the femoral vessels are isolated, ligated proximally, and cannulated such that an infusion pump flows into the arterial system and the venous circuit drains by gravity into a reservoir. There are various nuances to the infusion, and some have advocated for oxygenation of the perfusate similar to perfusion techniques, with or without hyperthermia, while others perform the procedure in circuit with recirculation of the perfusate [14,16]. A proximal tourniquet is utilized to isolate the extremity from the systemic circulation. Upon completion of drug infusion, the extremity is perfused with normal saline to ensure that the cytotoxic agent is washed out, cannulas are removed, hemostasis is achieved, and the surgical incision is closed. Tumors are then observed longitudinally for response, and can be serially biopsied, or harvested at a given endpoint, for immunohistochemistry, molecular characterization, and so on.

The strengths of the rat model include the fact that it is technically feasible for surgeons and trainees, as well as many laboratory technicians, and is therefore easily reproducible. In addition, systemic therapies can be combined with regional chemotherapy as a means to alter the response to that agent [13,15]. However, the expense associated with a rat model of ILI is relatively high due to the cost of each animal and associated husbandry. In addition, while the immunodeficient nature of the rats allows for the study of human melanoma, it lacks the capability to investigate the immune response to regionally administered cytotoxic agents as well as to melanoma itself, a highly immunogenic disease.

The mouse model of ILI, on the other hand, addresses some of the shortcomings associated with the rat model [17]. Kim et al. [17] were the first to demonstrate the reproducibility and utility of using mice for ILI. Although more technically challenging than ILI in a rat, their group reported an acceptable learning curve of 15 mice until proficiency is reached, and the procedure was able to be performed by surgical trainees [17]. The cost per animal is also substantially less when compared to that of a rat. Perhaps the most significant strength of the mouse model for ILI is the fact that immunologic parameters can be observed because there is not only a murine melanoma that can be engrafted into an immunocompetent mouse, but also several genetically constructed immunocompetent murine melanoma models that will allow for the development of skin based melanoma with metastatic potential. Although human melanoma xenografts can be similarly used with nude mice, B16 melanoma is a widely used and easily attainable mouse melanoma cell line that can be utilized to create mouse melanoma tumors in an immune-competent mouse. Not only can immunologic agents such as anti-CTLA4 monoclonal antibodies, interleukin-2 (IL-2), or tumor necrosis factor-alpha (TNF α) then be combined with regionally administered cytotoxic agents, but the immunologic response to both tumor and the ILI itself can also be assessed, both systemically and in the tumor microenvironment.

PRECLINICAL STRATEGIES EXPLORED IN ANIMAL MODELS

For appropriately selected patients with in-transit melanoma, regional chemotherapy is a promising therapeutic option and is currently the focus of continued clinical research. Regional therapy requires vascular isolation of the affected area, after which chemotherapy is delivered at doses 10–20 times higher than what is tolerated systemically, with dosing based on affected limb volume. Debate continues as to whether regional therapy produces an overall survival benefit over other therapeutic modalities, but studies have consistently demonstrated a survival advantage in patients who have a clinical response [18–20].

To be considered as a potential drug for use in regional therapy, agents must not require systemic metabolic transformation to take on a biologically active form. Melphalan is an alkylating agent derived from phenylalanine and is the first line regional chemotherapy for most patients with melanoma. Critical to melanin synthesis, phenylalanine is preferentially taken up by melanocytes. Melphalan should, hypothetically, result in selective toxicity in melanocytes and melanin-containing melanoma cells. Used as a systemic agent, melphalan is ineffective despite these theoretical benefits, as the allowable dose is considerably lower than the oncologically effective dose. For regional therapy, on the other hand, it is possible to achieve this significantly higher critical dose, without substantial systemic toxicity.

A number of other therapeutic agents have been utilized, either alone or in combination with melphalan, for regional chemotherapy in melanoma. Dacarbazine (DTIC) is the mainstay of systemic treatment against melanoma, but was found to have inferior response rates compared to melphalan in the setting of regional chemotherapy. This observation is probably related to the fact that conversion of DTIC to its active metabolite requires enzymatic conversion in the liver [21,22]. Cisplatin originally held significant promise in numerous preclinical studies of regional chemotherapy. Early clinical reports of response rates to cisplatin were favorable, but substantial concerns over toxicity also arose [23]. Subsequent

studies later confirmed significant limb-threatening morbidity with the use of cisplatin, and consequently it is seldom used in regional therapy [24,25].

Temozolomide (TMZ), which is closely related to DTIC but does not require hepatic conversion to take on its active form, is one of the most recent chemotherapeutic agents to be studied in patients with in-transit melanoma. Preclinical studies in animal models reported superior delay of tumor growth compared to regional melphalan at equitoxic doses [26]. Specifically, the quintupling time and tumor regression rates were 29.8 days and 66.7%, respectively for TMZ, compared to 18.9 days and 16.7%, respectively, for melphalan [26]. These observations provided the rationale for a multi-institutional phase I clinical trial examining TMZ in patients with in-transit melanoma who did not respond to a melphalan based regional treatment which has just recently completed enrollment (discussed in more detail under clinical trials) [27]. Despite the identification of novel agents appropriate for regional administration, resistance to chemotherapy remains a poor prognostic factor for patients. Therefore, various strategies to augment the response to these agents have been identified and continue to be tested in preclinical animal models.

Use of Systemic Therapy to Augment Regionally Administered Cytotoxic Agents Targeting drug-resistance pathways

In efforts to identify mechanisms of drug resistance, glutathione (GSH) has emerged as a major protein associated with the melphalan resistance pathway [28–30]. Depletion of GSH has been shown to reverse resistance to this alkylating agent [31]. Butathione sulfoxime (BSO) was identified as a potent inhibitor of γ -glutamyl-cysteine-synthetase (γ -GCS) which governs the rate-limiting step of GSH synthesis, and impairment of this process subsequently re-sensitizes tumor cells to alkylating agents, such as melphalan [32–34]. With this knowledge, our group looked at GSH levels within tumor and monitored tumor response in a nude rat model [35]. Rats were treated with two doses of systemic BSO (intraperitoneal) separated by 12-hr, with the second dose given 3-hr prior to regionally administered melphalan [35]. Not only did BSO result in a reduction of GSH levels by 71.8%, the combination of this modulator with regionally delivered melphalan increased the tumor quadrupling time [35]. The major conclusion from this work was that by modulating and impairing GSH, BSO potentiates the cytotoxic effects of melphalan in the regional setting, without an increase in morbidity [35].

Similar to the BSO-melphalan model is the concept of augmenting the response to TMZ, another regionally administered alkylating agent, with O⁶-benzylguanine (O⁶BG). O⁶-alkylguanine-DNA alkyltransferase (AGT) repairs O⁶-methylguanine bases resulting from TMZ therapy. Without AGT, mismatched bases due to methylation remain unrepaired, and growth arrest and apoptosis ensues [36,37]. In fact, elevated levels of AGT are found in melanoma tumors compared to nonmelanomatous tissue, as well as in metastases compared to primary tumors [38,39]. O⁶BG is a potent inhibitor of AGT, and when administered intraperitoneally, AGT levels decrease by 93.5% [27]. In our nude rat model using human melanoma xenografts, we delivered O⁶BG intraperitoneally as a single dose and multiple doses with concomitantly delivered regional TMZ [27]. Without modulation using O⁶BG, TMZ ILI led to a quintupling time of 23.8 days and 50% tumor regression [27]. However, a

single dose of O⁶BG with regional TMZ increased the quintupling time and tumor regression to 42.5 days and 83.3%, respectively, and multiple doses of O⁶BG with TMZ ILI improved these outcomes to 50.2 days and 100%, respectively [27]. These data suggested that the efficacy of TMZ ILI can be augmented by inhibiting AGT with systemic O⁶BG, and will need to be explored in a clinical trial.

Another regulator of the mismatch repair pathways is poly(ADP-ribose) polymerase (PARP). As such, for the reasons outlined above, PARP plays a role in transcription, the cell cycle, and programmed cell death [40,41]. Cells deficient in PARP are more susceptible to cytotoxic drugs such as alkylating agents, and transient inhibition of PARP has been shown to augment chemotherapy, including TMZ [42–44]. Using the PARP inhibitor, INO-1001, with regional TMZ, we evaluated mismatch repair-proficient and -deficient human melanoma xenografts for response to this combination strategy [45]. The result of this study, performed using our rat ILI model, was that PARP inhibition with systemic INO-1001 augmented the response to regional TMZ in mismatch repair-deficient melanomas. Compared to mismatch repair-proficient xenografts, which demonstrated tumor growth of 152.4% only 24 days after TMZ ILI, the combination of PARP inhibition with regional TMZ in mismatch repair-deficient tumors showed significant inhibition of growth, with only 22.6% increase in tumor size after 40 days [45]. In theory, identification of mismatch repair deficiencies on a preprocedural biopsy, not a generally common finding in melanoma, may identify tumors that would benefit from systemic PARP inhibition and regional TMZ, and this is now being explored in humans [46,47].

Augmenting drug delivery and efficacy of regionally administered cytotoxic agents

Regional therapy for melanoma provides a unique platform for treating melanoma because cytotoxic drugs can be administered at up to 10–20× the systemically tolerated dose. For the reasons cited above, melphalan is the most widely infused alkylating agent for treating in-transit melanoma, but response rates certainly have room for improvement [48]. While the dose of melphalan administered to the extremity is high, it is plausible that drug delivery at the level of the tumor remains poor. Therefore, strategies to augment drug delivery to melanoma tumors and/or improve drug response have been developed and tested in preclinical models as well as clinical trials [13,15,49,50].

The first of such concepts is that of altering angiogenesis and melanoma vasculature [15]. Melanoma, via vascular endothelial growth factor (VEGF), stimulates angiogenesis that is dissimilar from normal tissue, both functionally and structurally [51,52]. In fact, high levels of VEGF are associated with a disadvantageous prognosis that is thought to be secondary to an immature vascular network that impedes drug infiltration [53]. The monoclonal antibody against VEGF, bevacizumab (Avastin, Genentech, San Francisco, CA), is thought to antagonize such underdeveloped vessels common to solid tumors, including melanoma [54]. The response to bevacizumab is typically transient, however, with the long-term effect being a reduction in overall tumor vasculature that may decrease drug delivery [55]. Due to the fact that ILI is performed as a single treatment, it became an attractive platform to investigate the concept of normalizing tumor vasculature with bevacizumab to improve the delivery of regionally delivered cytotoxic therapy [15]. Using an established rat ILI model,

Turley et al. [15] performed melphalan ILI three days after systemic administration of bevacizumab. This treatment strategy in a preclinical in vivo model demonstrated decreased vascular permeability and tumor interstitial fluid pressure, both of which were theorized to allow for the observed increased in drug delivery, evidenced by increased DNA-melphalan adduct formation [15]. The most encouraging result was the improved tumor response, with a prolonged quadrupling time after the combined therapy [15]. These results have prompted the development of a phase I clinical trial protocol that is currently under review by our Institutional Review Board.

The concept of augmenting the response to regionally administered chemotherapy has also been tested using the cyclic pentapeptide ADH-1, which has been shown to reduce tumor cell growth and tumor progression, both in vitro and in vivo [56,57]. This systemically administered molecule antagonizes N-cadherin, which is overexpressed in melanoma and instigates proliferation, migration, and survival [58–60]. The results of clinical trials using this strategy, which are detailed below, were inconsistent and prompted us to re-focus our attention back to the preclinical in vivo model to investigate the mechanistic differences underlying response to combined ADH-1 and melphalan therapy (unpublished data currently under review) [49,50]. In combining systemic ADH-1 prior to regionally administered melphalan or TMZ in rats, we observed a differential tumor response across xenografts. The majority of xenografts demonstrated an increase in vascular permeability that was associated with augmented delivery of melphalan but not TMZ to the tumors. In some tumors, ADH-1 actually augmented tumor growth, an effect which in some cases could not be overcome by the regional chemotherapy treatment. In the tumors exhibiting a poor response, increased activity of the PI3K-AKT pathway was found, which is known to shift the axis toward a pro-survival state [61]. Additionally, vascular permeability was altered without a change in tumor interstitial fluid pressure, which would augment the delivery of plasma protein-bound drugs such as melphalan.

Another strategy for augmenting the response of melanoma to cytotoxic agents is the administration of melphalan in an acidic environment, a concept introduced by Kelley et al. [14]. The foundation for this study was based on previous work by Siemann et al. [62] and Skarsgard et al. [63], who had demonstrated the effects of pH on a tumor's response to alkylating agents, as well as the relationship between pH, hypoxia, and drug uptake by the tumor as it pertains to melphalan. Using NIH1286 human melanoma cell xenografts in athymic nude rats, this group performed 10-min isolated limb perfusions [14]. Using a melphalan perfusate with a mildly acidic pH (6.6–6.9), they observed improved cytotoxic activity compared to melphalan at a physiologic pH [14]. Additionally, measurement of nitric oxide (NO) levels in the xenografts revealed pathologic levels of NO, high enough to elicit tumor damage [14]. The results of these studies, taken together, highlight the benefit of melphalan administered in an acidic environment, which results in increased NO levels, increased melphalan delivery, and subsequent improved tumor response in this preclinical model [14].

Altering signaling cascades and promoting apoptosis

Similar to improving drug delivery and augmenting drug response, apoptotic thresholds can be theoretically lowered to make a tumor more susceptible to cytotoxic agents. An activating mutation at codon 1,796 in the proto-oncogene BRAF is present in up to 60% of melanomas, and results in a valine → glutamine codon change (V600E), causing the constitutive activation of BRAF, and subsequent activation of its downstream targets, most importantly ERK [64–66]. The BRAF → MEK → ERK pathway is thought to drive melanoma cell proliferation and portend a poor therapeutic outcome [64–66]. The strong relationship between the activated BRAF–ERK pathway and high frequency of the BRAF mutation in melanoma has led to major efforts to target this pathway in the treatment of melanoma [67,68]. The first of such agents was sorafenib, the multikinase inhibitor that targets BRAF, among others. Sorafenib inhibits angiogenesis, blocks cell proliferation, and impedes cell survival [69]. Previous work has also shown that it induces apoptosis [70–72]. Despite these previous findings, melanoma responds poorly to sorafenib as a single agent [73]. However, the ability of sorafenib to augment the response of melanoma to clinically relevant chemotherapeutic agents was unknown.

To answer this question, our group performed an in vitro and in vivo study to investigate the role of combining sorafenib, which is known to induce apoptosis and inhibit tumor growth, with an agent that has cytotoxic activity [13]. We used the Duke Melanoma (DM) cell line DM443 which was reasonably sensitive to sorafenib in vitro, moderately sensitive to melphalan in both in vitro and in vivo studies, and essentially resistant to TMZ in vitro and in vivo. Sorafenib was clearly shown to augment apoptosis when given in conjunction with alkylating agents like melphalan and TMZ. In addition, using the rat ILI model, systemic sorafenib given in conjunction with regional alkylating agents was shown to not only decrease BRAF signaling as measured by pERK levels but also to improve xenograft responses.

Similar to BRAF is the proto-oncogene Src, which encodes a tyrosine kinase responsible for proliferation, survival, angiogenesis, invasion, and metastasis [74–77]. It is therefore hypothesized that Src inhibition may afford an alternative treatment strategy for melanoma and impede tumor progression. Dasatinib is a “dirty” inhibitor of not only Src, but also other protein kinases, including c-kit, which is present in up to 40% of certain melanomas [78,79]. While dasatinib has been explored in the systemic setting with less than encouraging results, our group has examined the efficacy of Src inhibition in a regional setting using our rat model (unpublished results) [80]. To perform this experiment, rats were given daily dasatinib by oral gavage for 6 days, with melphalan ILI after the third dose. In a human melanoma xenograft (DM443) sensitive to dasatinib in vitro and moderately sensitive to melphalan in vitro and in vivo, we observed significant prolongation in the doubling time when rats were treated with systemic dasatinib and regional melphalan (44.8 days), compared to dasatinib (21.3 days) or melphalan (24.7 days) alone. Systemic toxicity of dasatinib was moderate, so while these results are encouraging, a phase I clinical trial focusing on toxicities will be the next step in development of this combination strategy.

Manipulation of cytokines in regional therapy

The concept of coadministering an immune modulator with regional alkylating agents was introduced in the 1990s with TNF α for soft tissue sarcomas [81,82]. The synergism that was observed was determined to be secondary to a sixfold increase in tumor melphalan concentration compared to surrounding skin and muscle in a rat isolated limb perfusion (ILP) model, perhaps due to improved permeability in the tumor vasculature [16,83,84]. The benefit of this combination therapy did not hold up in clinical trials, however, with comparable response rates and increased toxicity in patients treated with TNF α [85]. Further efforts have since been undertaken to identify an agent that acts synergistically with melphalan with an equal or improved toxicity profile.

The first of such agents is IL-2. In their rat model of extremity soft sarcoma, Hoving et al. [86] demonstrated a similarly synergistic response when IL-2 was included in the perfusate with melphalan. Not only did tumors exhibit an increase in melphalan uptake compared to surrounding normal tissue, they also found an increase in TNF messenger RNA (mRNA), which suggests a role of macrophages in augmenting the tumor response when IL-2 is combined with melphalan [86]. Interestingly, unlike TNF α , the synergistic tumor response was observed without concomitant damage to the vasculature [86].

Histamine is yet another inflammatory and immune modulator that elicits vascular edema and subsequent extravasation of lymph proteins into the extracellular space. When combined with melphalan in a regional setting for soft tissue sarcoma, Brunstein et al. [87] demonstrated similar synergy to that observed with TNF α . Tumor regression, defined as at least a 25% decrease in tumor volume, was observed in 66% of tumors treated with histamine and melphalan ILP, with a complete response rate of 33% [87]. This is in comparison to only 17% tumor regression observed with either histamine or melphalan alone [87]. In addition, a twofold tumor-selective increase in melphalan uptake was observed, which was quadruple that of the normal surrounding tissue [87]. The mechanism for such synergism was found to be due to a directly cytotoxic effect on the tumor itself, as well as direct injury to the tumor vasculature, and indirectly by augmenting tumor-specific melphalan delivery [87]. This synergy has also been observed in combination with doxorubicin ILP, again demonstrating a tumor regression rate of 67% [88]. While these data pertain to extremity soft tissue sarcoma, the concept of synergizing the response to regionally administered alkylating agents is relevant to in-transit melanoma, and should be investigated in preclinical models.

THERAPEUTIC STRATEGIES BASED ON PATIENT RESPONSE DATA

Hypoxia

Hypoxia is believed to play a significant role in tumor behavior and response to therapy across numerous solid tumors. The degree and features of hypoxia present in the tumor microenvironment of melanoma, however, remain poorly defined. Using the compound EF5 ([2-(2-nitro-1-H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl) acetamide]) which binds to viable cells within hypoxic regions of tumors, our group has started to explore the degree and extent of hypoxia in melanoma tumors. Preclinical and early clinical work by our group

using this compound combined with optical spectroscopy in a nontherapeutic clinical trial has demonstrated significant variability in hypoxia among various melanoma lesions [89]. We are currently working to better understand the clinical and prognostic implications of these findings and the various immunologic features associated with hypoxia in melanoma. It is known that chronic hypoxia plays a significant role in tumor-specific immune responses, and tumor hypoxia has been shown to create an environment that attracts T regulatory cells which modulate the differentiation and function of immune cells specifically by inhibiting the tumor-specific T-cell response [90–93]. As such, tumor hypoxia in melanoma may be protective against a cytotoxic immune response, with impact on tumor progression and response.

Additionally, the considerable limb ischemia induced during ILI leads to a hypoxic and acidic tumor environment, which is believed to be significant in augmenting the cytotoxicity of melphalan [19,94,95]. Initial in vitro studies revealed that the combination of hypoxia and the subsequent acidic environment augmented the cytotoxic effect of melphalan [62]. How tumors respond to this acidosis and low oxygen tension in the clinical setting of ILI, however, is the subject of ongoing investigation. This work will help determine whether strategies that try to decrease tumor hypoxia and/or acidosis will be effective in not only increasing tumor responses but also helping generate an immune response to the regional treatment that may have a systemic antitumor effect on distant micrometastatic disease which is probably present in a majority of these patients.

Regional Therapy and the Systemic Immune Response

A growing body of evidence implicates an altered immune response in the development of melanoma, and clinically meaningful responses to many therapies may be tied to immune activation [96–100]. While alterations such as reduced secretion of chemokines, low T-cell infiltration, and active immunosuppression allow tumor cells to escape immune destruction in the tumor microenvironment, patients with melanoma may also differ from healthy patients in their systemic immune background [101–105]. Data from 180 patients who had undergone melphalan-based ILI suggests that patients with melanoma are globally immunosuppressed compared to normal healthy patients [106]. In this cohort, cytokine levels (MIP-1a, IL-1Ra, IL-1b, IL-1a, IL-17, EGF, IL-12p40, VEGF, GM-CSF, and MIP-1b) were significantly higher in normal controls than melanoma patients, while IL-10 was lower.

Regional therapy, however, has been shown to generate an immune response both systemically and in the tumor microenvironment, which may work in parallel with the cytotoxic effects of chemotherapy to induce an antitumor response. Within the tumor microenvironment, melphalan has been reported to up-regulate the co-stimulatory molecules CD80 and CD86 to produce T-cell proliferation in response to tumor antigens [107]. Additional studies of peripheral blood have demonstrated alterations in T-cell stimulation, NK cell activity and sICAM-2 [108,109]. Furthermore, expression of type III TGF- β receptor (T β RIII), which is believed to inhibit cell migration and invasion, is down-regulated during the progression of many human cancers. Low levels of T β RIII expression have been shown to be associated with inferior results after ILI and overall poor prognosis [110].

Furthermore, RNA microarray analysis on tumor specimens before melphalan ILI demonstrated higher levels of leukocyte infiltration and T-cell regulating gene activation when comparing patients who completely responded to those who progressed after therapy. Expression of the regulatory T cell protein IDO was also found to be markedly lower in pretreatment tumor biopsies of complete responders as compared to biopsies of tumors from patients who progressed after regional therapy.

This interplay between regional treatment and immune activation has led to plans for the aforementioned clinical trial using neoadjuvant ipilimumab prior to melphalan-based ILI. Ipilimumab, a human IgG1-kappa monoclonal antibody against CTLA-4, allows for increased T-cell activation. It is our expectation that immune activation prior to ILI will result in improved clinical response compared to either therapy alone for in-transit melanoma of the extremity. A trial at the Memorial Sloane Kettering Cancer Center is already exploring the use of this compound after a melphalan-based ILI (clinicaltrials.gov: NCT01323517).

THERAPEUTIC CLINICAL TRIALS OF REGIONAL THERAPY FOR ADVANCED EXTREMITY MELANOMA

TNF α : ACOSOG Z0020

Early studies suggested TNF α as a potential therapeutic agent in regional therapy, particularly in combination with interferon- γ , but use of TNF α -based regimens has been hampered by toxicity [111]. The 2006 ACOSOG Z0020 trial set out to study the efficacy of melphalan plus TNF α in HILP, and enrolled 133 patients from ten participating centers, randomized to therapy with standard melphalan or combined melphalan with TNF α . This study demonstrated only comparable response rates (26% versus 25%), but was terminated after interim analysis revealed significantly increased toxicity with the addition of TNF α [85]. Some critics have argued that the 3-month response endpoint was too early to demonstrate a meaningful difference in response rates between the two groups, however at a subsequent 6-month analysis there was still no statistically significant difference between groups. Furthermore, a recent single-institution study undertaken at the University of Maryland also failed to demonstrate improved progression-free survival with TNF α compared to standard melphalan therapy [112].

ADH-1/LPAM ILI

The combination of systemic ADH-1 with standard melphalan ILI held much promise for patients with advanced extremity melanoma after preclinical studies demonstrated considerable improvements in tumor response, even in particularly melphalan-resistant tumors [113]. In a phase I trial that combined ADH-1 given on days 1 and 8, with melphalan ILI on day 1, Beasley et al. [49] reported that not only was this treatment strategy safe and tolerable, it resulted in better than anticipated tumor responses. Fifty percent of the patients experienced a complete response and no patients suffered dose-limiting toxicity. These encouraging results prompted a phase II multi-center study for patients with stage IIIB or IIIC extremity melanoma. The 45 patients enrolled in this multi-institutional study demonstrated a 38% in-field complete response rate, but with a median duration of response

of only 5 months. As there was no significant difference in regional progression-free survival between melphalan plus ADH-1 and melphalan alone, it was subsequently determined that combining ADH-1 with regionally administered melphalan provided no benefit compared to standard melphalan ILI [20].

Sorafenib/LPAM ILI

Similarly, although the preclinical data surrounding Sorafenib, a first generation BRAF inhibitor, was promising in initial animal models, a multi-center phase I clinical trial failed to replicate these encouraging results [114]. This dose escalation trial was conducted to evaluate the safety profile of such combined therapy, as well as to gain insight into the antitumor benefits of melphalan ILI in the setting of systemic sorafenib. After enrolling 20 patients at Duke University and Memorial Sloan Kettering Cancer Center, a maximum tolerated dose of sorafenib given in conjunction with ILI was identified. Unfortunately, this combination was associated with significant toxicity that was not seen in earlier rat models. Furthermore, only 15% of the cohort demonstrated a complete response to this treatment strategy, which did not compare well to historic results of standard melphalan ILI [114]. Correlative studies demonstrated that inhibition of the BRAF signaling pathway, as measured by inhibition of the phosphorylated forms of ERK and MEK that occurred in our preclinical models and was necessary for synergistic activity, was not occurring at the dosages used and tolerated in the phase I clinical trial. Given the combination of poor response and high toxicity, future efforts in this area will be focused on more specific second generation BRAF inhibitors, such as vemurafenib.

Temozolomide ILI

Temozolomide (TMZ) as an oral agent is believed to offer some clinical benefit to patients with metastatic melanoma, but the recent development of an intravenous formulation of the drug has generated considerable excitement. Unlike its closely related sister drug DTIC, TMZ does not require hepatic conversion to its active metabolite (MTIC) [27]. The IV formulation of the drug was recently FDA approved, and a multi-center phase I clinical trial has just been completed at Duke University, The University of Texas MD Anderson Cancer Center, and Moffitt Cancer Center. Final analyses of this trial are underway and the results being prepared for publication. In this first ever use of intra-arterial TMZ for melanoma, the maximum tolerated dose was established, and TMZ was found to exhibit an improved safety profile compared to traditional LPAM. Of perhaps more importance, a subset of patients (10%) demonstrated a complete response to TMZ despite having progressive disease after a previous melphalan-based ILI. Given the minimal toxicity and demonstrated efficacy even in some cases where LPAM has failed, TMZ warrants further study for use in regional therapy in order to optimize individual patient response.

Ipilimumab ILI

Newer targeted immunotherapies are now setting the stage for future treatments in patients with in-transit melanoma. Perhaps showing the most potential is ipilimumab, an anti-CTLA-4 antibody that has aroused widespread enthusiasm recently in the treatment of systemic metastatic melanoma [115]. An ongoing phase II clinical trial (NCT01323517) by the Memorial Sloan Kettering Cancer Center group is currently evaluating the use of

adjuvant ipilimumab starting 1–3 weeks after ILI for in-transit melanoma, the results of which are expected in mid-2015. Additionally, in light of data suggesting that the host immune response varies considerably between early and late disease [116,117], a multi-institutional trial based at Duke University utilizing neoadjuvant ipilimumab prior to ILI is currently in the planning phase.

Cisplatin ILI

Cisplatin was originally thought to have significant potential as a therapeutic agent in regional therapy, as it does not require metabolic conversion to take on an active form. Early animal data and initial clinical studies reported promising response rates using cisplatin during isolated limb perfusions [118,119]. Subsequent studies, however, failed to demonstrate improved efficacy, and were hampered by substantial toxicities [24]. Because of this, cisplatin has largely been abandoned as a choice in regional therapy for advanced extremity melanoma.

SUMMARY

Regional therapy strategies such as ILI provide a unique platform in oncology, particularly in the study and treatment of in-transit melanoma. Patients with in-transit disease often have multiple discrete tumors, allowing for multiple biopsies to be performed over time which allows for exciting investigation into the correlative science of novel treatment strategies. Additionally, ILI provides a unique setting to study how the tumor microenvironment affects chemotherapeutic delivery and response. Ongoing investigations continue to demonstrate the important role of regional therapy as a tool for exploring the underlying biology and future treatments of melanoma, both for in-transit lesions and ultimately for patients with systemic disease.

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FIGURE 1.

Animal model for isolated limb infusion. **A:** Schematic of rat ILI. **B:** Human melanoma xenograft on the hind limb of a nude rate. **C:** Cannulation of the femoral artery and vein of a nude rate. **D:** Schematic of a mouse ILI (permission secured from Elsevier on 22 July, 2013; License Number: 3194380127954) [17]. **E:** Human melanoma xenograft on the hind limb of a nude mouse. **F:** Cannulation of the femoral artery and vein in a nude mouse.