MDSC as a mechanism of tumor escape from sunitinib mediated anti-angiogenic therapy

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ABSTRACT

Sunitinib is a receptor tyrosine kinase inhibitor (TKI) that is front-line therapy for metastatic renal cell carcinoma (mRCC). Its antitumor activity is related to its ability to block tumor cell and tumor vasculature cell signaling via several TKI receptors (i.e. vascular endothelial growth factor receptors VEGFRs, platelet-derived growth factors (PDGFs), and stem cell factors). Sunitinib also targets myeloid derived suppressor cells (MDSCs) significantly reducing their accumulation in the peripheral blood and reversing T cell (IFN-γ) suppression in both mRCC patients and in murine tumor models. This reduction in immune suppression provides a rationale for combining sunitinib with immunotherapy for the treatment of certain tumor types. Despite these encouraging findings, however, we have observed that sunitinib has variable impact at reducing MDSCs and restoring T cell function within the tumor microenvironment. Given the immunosuppressive and proangiogenic activities of MDSC, it seems plausible that their persistence may contribute to the resistance that develops in sunitinib-treated patients. While sunitinib reduced tumor infiltrating MDSCs in Renca and CT26-bearing mice, coinciding with strong to modest decreases in tumor size respectively, it was ineffective at reducing MDSCs (>35% reduction in Gr1+CD11b+) or tumor burden in 4T1-bearing mice. Persistence of intratumor MDSCs was paralleled by depressed intratumor T cell IFNγ response and increased GM-CSF expression. Additionally, in vitro and in vivo experiments showed that GM-CSF prolongs survival of MDSCs, thus protecting them from the 2 effects of sunitinib via a pSTAT5-dependent pathway. Although preliminary, there is evidence of intratumor MDSC resistance in some mRCC patients following sunitinib treatment.

Intratumor MDSC persistence and T cell IFNγ expression of MDSCs (5% of total digested cells). Thus far, tumors from 5/8 neoadjuvant patients showed suppressed T cell IFNγ expression. Additionally, intratumor MDSCs was paralleled by depressed intratumor T cell IFNγ response along with substantial expression of MDSCs (5% of total digested cells). Thus far, tumors from 5/8 neoadjuvant patients showed persistence of intratumor MDSCs and low T cell IFNγ production post nephrectomy in patients receiving sunitinib in a neoadjuvant setting are being compared to RCC patients undergoing nephrectomy without prior sunitinib treatment. Tumors from untreated patients showed suppressed T cell IFNγ response along with substantial expression of MDSCs (5% of total digested cells). Thus far, tumors from 5/8 neoadjuvant patients showed persistence of intratumor MDSCs and low T cell IFNγ production post nephrectomy, findings that parallel results from untreated tumors. In the remaining 3 neoadjuvant patients, intratumor MDSCs were detected at low levels which coincided with a T cell IFNγ response similar to that observed with normal donor peripheral T cells. GM-CSFs role in promoting MDSC survival in patient tumors is supported by the observation that GM-CSF is produced in short-term RCC cultures at levels capable of protecting MDSCs from sunitinib-induced cell death. Additionally, persistence of MDSCs also may be associated with increased expression of proangiogenic proteins, such as MMP9, MMP8, and IL-8 produced by tumor stromal cells or infiltrating MDSCs. Indeed our findings suggest that the most dominate MDSC subset in RCC patients is the neutrophilic population that produces proangiogenic proteins. We propose that the development of sunitinib resistance is partly mediated by the survival of MDSCs intratumorally, thereby providing sustained immune suppression and angiogenesis.

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1. Introduction

Targeting angiogenesis as a treatment for cancer is an approach with demonstrated utility in some tumor types including mRCC [1,2]. The pathogenesis of clear-cell RCC is partly the result of a common inactivation of the von Hippel–Lindau gene resulting in the over-expression of VEGF which promotes tumor-associated angiogenesis.
and tumor growth [3]. Small molecule inhibitors that target the VEGF signaling pathway have demonstrated activity in mRCC. One such inhibitor, sunitinib, currently front-line treatment for mRCC produces a 50% response rate and can improve progression-free survival when compared to IFNα [2]. Sunitinib is a multitargeted TKI that blocks signaling via VEGFRs, Flt3, stem cell factors (c-Kit), PDGFs, and colony-stimulating factor-1 receptors (CM-CSFRs) [4]. Sunitinib’s major activity is thought to be based on its inhibition of endothelial cells in the tumor vasculature, hence it was once hypothesized that resistance would be limited due to the drug’s targeting of genetically stable, non-transformed cells. [5,6]. However, sunitinib may also impair growth and viability of RCC tumor cells [7].

Despite the significant improvement in treatment, mRCC patients ultimately develop resistance to sunitinib, via multiple proposed mechanisms that are still under investigation. There is growing evidence that MDSCs which accumulate in human and murine tumors, may play an important role in the general process of angiogenesis. VEGF, along with other tumor-derived products, blocks myeloid cell differentiation resulting in the accumulation of a heterogeneous group of immature myeloid cells (CD11b+CD14+CD33–null/null) and wild type (WT) BM were obtained as previously described [19].

In some experiments, MDSC subsets were isolated from human RCC tissue for analysis of expression of proangiogenic proteins by proteome profile array (PPA). After RCC tumors were digested, as previously described, the cells were stained with antibodies to CD33, HLA-DR, CD14, and CD15. The cells were sorted using a FACS Calibur into CD33+HLA-DR+CD15–CD14– (n-MDSC), CD33+HLA-DR+CD15+CD14– (m-MDSC), and CD33+HLA-DR+CD15+CD14+ (m-MDSC) subsets. Lysates were then made from the subsets, protein assays performed and equal amounts of protein used in a PPA (R&D Systems) [20].

2.4. MDSC analysis

MDSCs were analyzed from the PB and tumors of RCC patients. Surface staining for HLA-DR, CD15, CD14, and CD33 followed Fc block (human IgG at RT) for 30 min at 4 °C as described [19,20]. Samples were acquired on a BD FACSCalibur and analyzed using CellQuest software.

Murine MDSCs from spleen, BM and tumors were surface stained (CD11b and Gr1) as previously described. Intracellular staining of BM MDSCs for expression of pSTAT3 and pSTAT5 was performed as previously detailed [19].

2.6. Human tumors

RCC tissue was digested with collagenase, DNAse, and hyaluronidase (Sigma) for 30 min and sieve-filtered to obtain a single-cell suspension. A portion of cells were stained for MDSC and T cell models. Fourteen cell-free supernatants, or in supernatants derived from the long-term, clear-cell RCC line, SK-RC26b, (previously acquired from Dr. Neil Bander at Cornell Medical Center) in the presence or absence of sunitinib.

2.7. Analysis of growth factors and proangiogenic proteins

Cell-free tumor-conditioned media (TCM) from sunitinib-treated and untreated tumor cells were frozen at −80 °C and semiquantitative analysis of numerous cytokines, chemokines, and growth factors was done according to the human cytokine and angiogenesis proteome profile arrays (R&D Systems). Analysis was done using NIH image J software. For quantitative analysis of GM-CSF, plasma was assayed according to the Bio-Plex Pro custom assay (Biorad). Tissue lysates were brought to 1 mg/ml using 1xPBS with 0.5% BSA, and 50 ul was added to each well. A separate, standard curve was made using lysis

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buffer as diluent for the analysis of cell lysates. Data was acquired and analyzed on a Luminex device using the Biorad Bio-Plex System and Bio-Plex Manager 3.0 software.

2.8. Biostatistics

Murine experiment results were pooled from n≥3 and mean ± standard deviation was expressed. Treatment groups were compared using a t-test for two samples assuming equal variances. p-value < 0.05 was deemed significant. Statistical analysis of human MDSCs, T cells and clinical correlates was performed as described previously [19,20].

3. Experimental results and discussion

3.1. Sunitinib reduces splenic MDSC levels, restores T cell function, and can enhance the efficacy of immunotherapy in sunitinib-sensitive murine tumor models

Our findings using three mouse tumor models showed that sunitinib reduced MDSC levels and improved T cell responses systemically [19]. When mice bearing Renca (renal cancer), CT26 (colon cancer) and 4T1 (mammary tumor) were treated i.p. for 9 days with sunitinib (20 and 40 mg/kg), a highly significant reduction in splenic MDSC (Gr1⁺CD11b⁺) occurred to a similar degree in all three tumor models resulting in restoration of T cell type-1 IFN response when tumor-bearing splenic MDSC was added to activation cultures. (anti-Gr1Ab) and by near total suppression conferred to naïve T cells restoration of T cell function following mechanical MDSC depletion to T cell suppression in tumor bearing mice was evident by the proliferative T cell responses to normal levels observed with T cells from naïve non-tumor bearing (NTB) mice. The contribution of MDSCs to T cell suppression in tumor bearing mice was evident by the restoration of T cell function following mechanical MDSC depletion (anti-Gr1Ab) and by near total suppression conferred to naïve T cells when tumor-bearing splenic MDSC was added to activation cultures.

Similar findings have been reported by others. Indeed, sunitinib monotherapy was shown to reduce MDSC levels in a number of transplantable tumors but with variable impact on growth [7,19,21]. Sunitinib also delayed the onset of tumor induction and reduced the incidence and growth of tumors in a mouse mammary tumor transgenic model [22]. This reduction in MDSC was accompanied by the restoration of T cell function as evident by an improved T cell IFNγ response (4.8±0.8% n=15) compared to that of splenic T cells post sunitinib treatment was paralleled by depressed T cell IFNγ response (4.8±0.8% n=15) compared to that of splenic T cells (25.2±4.9%) [19].

The persistence of intratumor MDSCs in the sunitinib-resistant 4T1 model may be related to local production of GM-CSF. A PPA, followed by the quantitative Luminex assay, revealed that GM-CSF was selectively expressed in tumor tissue lysates but not in plasma from 4T1 TB mice, mirroring the resistance pattern of MDSCs in 4T1-bearing mice. Moreover, the sunitinib sensitivity of intratumoral MDSCs from Renca and CT26 TB mice coincided with low levels of GM-CSF in the tumor relative to 4T1 tumors. Additional studies with purified cytokines demonstrated that GM-CSF (1–10 ng/ml) was most effective at protecting BM-derived MDSC from the anti-proliferative and proapoptotic effects of sunitinib (1 μg/ml) [19]. Importantly,

Table 1

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administration of recombinant murine GM-CSF along with sunitinib to 4T1 bearing mice could partially render the normally susceptible splenic MDSCs resistant to sunitinib treatment and this coincided with diminished T cell production of IFNγ.

Additional studies aimed at defining the mechanism responsible for GM-CSF-mediated sunitinib resistance suggested involvement of pSTAT5 [19]. Others have shown that STAT3 activation in tumors and MDSCs promotes immune suppression and that sunitinib can inhibit pSTAT3 leading to tumor apoptosis and MDSC reduction [7,25]. Likewise, our studies showed that pSTAT3-promoting BM derived MDSC cultures (IL-6 and G-CSF) remained sensitive to sunitinib-mediated suppression. In contrast, pSTAT5+ promoting MDSC cultures driven with GM-CSF [19,26] showed sunitinib-resistance in vitro. pSTAT5’s involvement in GM-CSF-mediated MDSC protection was supported by the observation that STAT5ab (null/null) BM-derived MDSCs compared to WT BM-derived MDSCs were not protected from sunitinib-mediated apoptosis in the presence of GM-CSF. Thus, we propose that the presence of GM-CSF promotes MDSC resistance to sunitinib by providing an alternating signaling pathway which both sustains MDSC viability, and remains unaltered in the presence of sunitinib. The precise downstream events involved in this resistance are yet to be defined.

3.3. Sunitinib treatment of RCC patients reduces PB MDSCs and increases T cell function

In cancer patients, MDSCs have been defined as CD33+CD11b+HLADR+ cells, although this population is heterogeneous based on morphology and surface staining [27]. MDSCs in the PB of RCC patients (CD33+HLADR−) constitute 5.3% (±1.0; n = 50) of the mononuclear cell population compared to 0.99% (±0.29; n = 11) in healthy age-matched donors. An analysis of tumors from untreated (no sunitinib) RCC patients showed that MDSCs comprise 5.9±1.1% (n = 38) of the total tumor single cell suspension (Ko et al., unpublished). We find that there are at least three MDSC populations in the blood and tumor. The neutrophilic population (n-MDSC, CD15+CD33+HLADR) is the most abundant (56% of MDSC), followed by the lineage-negative depletion (anti-CD15 beads) from PB mononuclear cells significant to induce pSTAT5 in MDSCs isolated from PB mononuclear cells significantly improved T cell IFNγ production, and that the addition of positively selected CD15+CD33+ cells to isolated patient T cells restored T cell suppression [20].

Similar to what we observed in murine tumor models, sunitinib treatment of murine tumor models dramatically reduced the number of MDSCs (CD33+CD11b+HLADR− and CD15+CD33+HLADR−) in the PB (Day 28 of cycle 1) [20]. Analysis of patients receiving multiple cycles of sunitinib suggests that maximal reduction in MDSC numbers occurs after the 2nd cycle and remains depressed even after the 4th cycle, although there is some recovery in MDSC levels at this time (Finke, unpublished). This reduction in PB MDSCs correlated with a significant increase in T-cell IFNγ production after in vitro stimulation with anti-CD3/anti-CD28 antibodies (t = −0.66, p = 0.03) [20]. An additional report in RCC patients showed that the percentage of CD14+HLADR−/low MDSC was increased compared to normal donors which was reduced by sunitinib treatment. Interestingly the reduced frequency of dendritic cell subsets, myeloid-DC-1 (MDC-1) and myeloid-DC-2 (MDC-2), noted in RCC patients was restored to normal baseline levels with sunitinib treatment and the high MDC-1 frequency correlated with tumor shrinkage [32]. These finding suggest that sunitinib monotherapy in RCC patients can reduce immune suppression thereby providing a rationale for combining sunitinib with immunotherapy.

3.4. Evidence of MDSC resistance in some RCC patients receiving sunitinib is suggested by the persistence of intratumoral MDSCs and T cell suppression

Sunitinib treatment of metastatic patients with prior resection of primary tumors resulted in reduced MDSC frequency and restored T cell IFNγ response in PB mononuclear cells [20]. However, the impact of sunitinib on the persistence of intratumoral MDSCs and T cell function is not possible in this patient population. A phase II trial of neoadjuvant sunitinib by Rini et al., (in preparation) has involved treating patients with sunitinib where the primary RCC tumors were not amenable to resection. Neoadjuvant sunitinib led to downsizing/downtstaging of primary tumors, which allowed resection in over 40% of initially unresectable, primary, RCC tumors. Although not complete, an early analysis of tumors from neoadjuvant patients compared to untreated tumors shows variability in the ability of sunitinib to reduce MDSC levels and restore T cell IFNγ production within the tumor bed, a finding similar to those observed in our murine tumor models. Of the neoadjuvant tumors examined post-sunitinib treatment, (d = 9–25) 3/8 had very low levels of MDSCs (0.7%, 2% and 0.5% CD33+HLADR−) compared to MDSC levels in untreated tumors (5.3% MDSC) and significant T cell IFNγ production (27%, 15%, 10% IFNγ−) comparable to healthy donor PB T cells (18 ± 1.5%, n = 21). However, 5/8 patients showed suppressed T cell function (3.0%, 3.5%, 1.0%, 1.5% and 2% IFNγ−) similar to tumor-derived T cells from untreated RCC patients (8.7 ± 1.2%, n = 19). Of these 5 tumors, MDSC levels were available from 3 samples and all of the samples had high MDSC levels (4.9%, 10.2% and 4.2%), similar to untreated RCC patients.

Whether local production of GM-CSF is responsible for the persistence of MDSCs in some of the neoadjuvant patients is not yet established; however, preliminary data from short-term cultures (1–3 passages) of freshly isolated RCCs (n = 15) along with several established RCC lines (n = 6) showed that tumors can produce sufficient amounts of GM-CSF (887.9 ± 307.7 pg/ml) to protect MDSCs from sunitinib-induced apoptosis ([19] and Ko et al., unpublished). Moreover, the concentration of GM-CSF detected in tumor cell line supernatants was sufficient to induce pSTAT5 in MDSCs isolated from the PB of RCC patients. A role for GM-CSF in promoting MDSC accumulation in sunitinib-treated TB hosts is supported by studies implicating GM-CSF in MDSC expansion [33] and by the observations that vaccine strategies employing high-dose GM-CSF promoted MDSC expansion and reduction in T cell immune responses [34,35].

3.5. Tumor and MDSC production of proangiogenic proteins may contribute to sunitinib resistance in RCC patients

MDSCs are known to promote angiogenesis and tumor progression via a STAT3-dependent pathway [12,13]. In fact MDSCs producing BV8 (VEGF homologue prokineticin-2) appear to promote resistance to anti-VEGF antibody treatment. However, there is little information on the impact of proangiogenic products on sunitinib resistance in RCC patients with the exception of IL-8 [36], Immunostaining of pretreatment human RCC tissue revealed that high IL-8 staining was associated with sunitinib non-responders, while low IL-8 staining was associated with clinical response. Moreover, in an RCC xenograft model, treatment with sunitinib mimicked the resistance observed in humans and co-treatment with anti-IL-8 antibody restored sensitivity of TB mice to sunitinib [36].
Our preliminary analysis of tumor tissue lysates shows an apparent increase in expression of proangiogenic proteins (MMP9, MMP8 and IL-8) in neoadjuvant sunitinib-treated patients with persistent high MDSC levels (3/5 patients), relative to levels detected in neoadjuvant patients with low MDSC levels (n = 2) and even to the levels detected in the control tumors (n = 7). Although greater numbers are needed, the increase in tumor tissue levels of MMP9, MMP8 and IL-8 in the 3 neoadjuvant patients where MDSCs persisted suggests that MDSCs contribute to the production of an acquired alternate-type proangiogenic profile that may be enhanced by sunitinib treatment. Indeed, using MDSC subsets isolated from human RCCs via fluorescence-activated cell sorter (FACS), our PPA analysis of 50 angiogenic proteins demonstrated that n-MDSCs expressed high levels of MMP9, MMP8 and to a lesser extent IL-8 when compared to the other two subsets. Clearly, additional neoadjuvant tumor samples are needed to determine whether there are two subsets of RCC patients, one sensitive to sunitinib with low MDSC numbers and low expression of proangiogenic proteins (MMP9, MMP8, IL-8) and another resistant to sunitinib with persistent levels of MDSCs and elevated levels of proangiogenic proteins.

While IL-8 likely contributes to sunitinib resistance, its mechanism of action remains unclear. Interestingly, we have shown that increased plasma levels of IL-8 in mRCC patients during sunitinib treatment correlated with a significant decrease in progression-free survival (PFS) relative to patients where the IL-8 levels decreased or were unchanged from pre-treatment values (Varella, L. et al., in preparation). One potential explanation for the inverse correlation between plasma IL-8 levels and PFS is that enhanced IL-8 production in the tumors promotes accumulation of MDSC, thus leading to enhanced alternative proangiogenic pathways (MMP9/MMP8/IL8), thereby enhancing tumor growth. IL-8 is a chemotactic for neutrophils, and likely n-MDSCs, that binds to the chemokine receptor CXCR2 [37]. We have shown that over 53% of n-MDSCs and 20% lin-/m-MDSCs infiltrating RCCs express this receptor. Since IL-8 is a chemokine with potent proangiogenic activity, the up-regulation of IL-8 may induce proangiogenic pathways that allow the tumor to escape the anti-angiogenic activity of sunitinib-mediated VEGFR blockade. Additional studies are needed to test whether sunitinib resistance in RCC patients is linked to increased numbers of intratumoral MDSCs and increased expression of proangiogenic proteins, such as MMP9 and IL-8. In addition, the mechanism behind selective persistence and protection of MDSC in resistant RCC tumors compared to the peripheral blood requires further investigation.

It will be of interest to determine whether MDSCs from blood and tumors of RCC patients promote angiogenic activity in the RCC xenograft model. However, we have shown that incubation of normal donor blood with tumor-conditioned media (TCM) from melanoma and RCC lines, activates neutrophils and monocytes to display a greater ability to suppress T cell production of IFN-γ and RCC lines, activates neutrophils and monocytes to display a donor blood with tumor-conditioned media (TCM) from melanoma xenograft model. However, we have shown that incubation of normal and tumors of RCC patients promote angiogenic activity in the RCC blood requires further investigation.

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3.6. Summary

Our findings and those of others implicating the involvement of MDSC in tumor escape via immune suppression as well as anti-angiogenic drug resistance. Understanding the mechanisms which are involved in tumor resistant to sunitinib, including defining the contribution of MDSCs in this process, may provide new strategies for reducing resistance to this TKI and possibly other TKIs.


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