Epigenetic therapy in allogeneic hematopoietic stem cell transplantation

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DNA methylation and other epigenetic phenomena appear to be relevant in the pathogenesis of several malignant disorders. DNA methyltransferases add methyl groups to cytosine-phosphate-guanine (CpG) islandsleading to gene promoter silencing. The DNA methyltransferases inhibitors azacitidine and decitabine have anti-tumor activity against a broad range of malignancies, but have been investigated mostly in myelodysplastic syndrome. In addition, these agents have immunomodulatory effects that are under investigation in the allogeneic stem cell transplantation scenario. Both drugs have been used in the perioperative period of allogeneic transplantations with varying degrees of success. It has been hypothesized that low dose azacitidine may increase the graft-versus-leukemia effect and have a role in the maintenance of remission after allogeneic transplantation for myeloid leukemias. It is also intriguing that this favorable effect might occur while mitigating graft-versus-host disease. Here we present a review of the rapidly growing field of epigenetic manipulation using hypomethylating agents in allogeneic transplantation.

Keywords: *Hematopoietic stem cell transplantation; DNA methyltransferase; Leukemia, myeloid; Epigenesis, genetic; Azacitidine; Immunologic factors*

Introduction

The loss of global DNA methylation was one of the earliest epigenetic abnormalities identified in cancer cells[1]. Several studies have shown DNA hypomethylation as a common feature of carcinogenesis[2]. On the other hand several investigators have indicated that DNA hypermethylation is a frequent phenomenon occurring across different cancer types[3]. CpG island promoter methylation may affect multiple pathways involved in apoptosis, cell cycle and DNA repair, for example[3]. Around 50% of tumor-suppressor genes have been reported to be silenced by aberrant DNA methylation of their promoters[4]. Interestingly, DNA hypermethylation is also involved in down-regulation of tumor suppressor micro-RNAs[5,6]. The mechanisms leading to aberrant DNA methylation are however not completely understood but may involve DNA instability, over expression of DNA methyltransferases(DNMTs), or environmental factors such as viral infections among others[4,7]. Disruption of epigenetic mechanisms provides a target for anticancer therapies.

DNA demethylating agents not onlyhave anti-tumor activity in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS), but also appear to have immunomodulatory effects[2,8,9]. This combination of anti-tumor activity and immune modulation along with a favorable toxicity profile makes these agents potential candidates for use in the transplantation setting.

Here we present a brief introduction to cancer epigenetics and review studies, which have evaluated the putative role of DNA demethylating agents in the allogeneic hematopoietic stem cell transplantation(allo-HSCT) setting. We do not address the potential role of histone deacetylase inhibitors in this setting, although this is another exciting avenue of investigation.

Epigenetics

Chromatin is made of basic repeating units, the nucleosomes, where packed DNA base pairs are wrapped around a histone octamer[2]. Epigenetics is defined as heritable changes in gene expression caused by mechanisms other than changes in the DNA sequence, while the sum total of all epigenetic information is called the 'epigenome'[10,11]. Gene silencing through epigenetic regulation involves a coordinated interplay of a number of processes which include, DNA methylation, histone modification, and nucleosome remodeling, among others[10]. For the purpose of this review, we will restrict our discussion to DNA methylation only.

DNA methylation

DNA cytosine methylation, where a methyl group is covalently bound to cytosine, occurs almost exclusively in a cytosine-phosphate-guanine (CpG) context, although non-CpG methylation has also been described, particularly in embryonic stem cells[12]. Most studies have revealed that

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genomic DNA methylation tends to spare CpG islands (sequences of DNA approximately 1,000 base pairs long, where the dinucelotide CpG is present at closer to its expected frequency, as opposed to other areas of vertebrate genome, whereitis depleted)[11]. The majority of gene promoters are located in these islands, and DNA methylation in this location silences their activity, thus repressing gene expression[11,13].

DNA methylation is mediated by DNMTs. This includes the maintenance of methylation by DNMT1 as well as *de novo* methylation during embryogenesis by DNMT3a, DNMT3b, and DNMTL[14,15]. DNMT2, on the other hand does not methylate DNA but instead is involved in methylation of aspartic acid transfer RNA[16]. In addition, recent studies have shown that DNMT3a and DNMT3b are also involved in DNA methylation maintenance[17].

Demethylating Agents

Several therapeutic strategies have been developed to induce epigenetic changes in cancer cells. These include DNMT and histone deacetylase (HDAC) inhibitors. Although several DNMT inhibitors (DNMTis) have been studied in pre-clinical and early phase clinical trials, only two, 5-Azacitidine (Azacitidine) and 5-Aza-2'-deoxycitidine (decitabine) have been approved by the Food and Drug Administration (FDA)in the United States for the treatment of MDS[2,18-24].

Mechanism of action of Azacitidine and Decitabine

Both azacitidine (5-Aza-CR) and decitabine (5-Aza-CdR) are prodrugs that are converted to their active triphosphate forms 5-Aza-CTP and 5-Aza-dCTP, respectively, after cellular uptake by a human concentrative nucleoside transporter 1 (hCNT1) [2,25,26]. 5-Aza-CR can be incorporated into RNA as well as DNA, whereas 5-Aza-CdR can only be incorporated into DNA[2]. The incorporation into DNA induces hypomethylation of the daughter DNA strands, while the incorporation into RNA causes ribosomal disassembly and disruption of protein translation[2]. Furthermore, it has been shown that the hypomethylating effect of decitabine is most evident at low concentrations that are effective in covalently trapping DNMT without cell-cycle arrest or cytotoxicity. At higher doses, decitabine is cytotoxic, inhibits DNA synthesis and induces cell-cycle arrest as a 'classical' chemotherapy agent[27].

Immunomodulatory effects of DNA demethylating agents

In addition to the cytotoxic effects, DNMTsappears toinduce phenotypic modifications ('maturation') of leukemic cells, including increased expression of HLA class I/II antigens and increased expression of tumor antigens. These changes, discussed below, potentially could increase susceptibility of malignant cells to immune surveillance mechanisms, such as the graft-versus-malignancy effect of allogeneic cells. In addition, DNMTi may mitigate graft-versus-host disease (GVHD) possibly by increasing the number of regulatory T cells (Tregs), or by another unknown mechanism.

Induction of terminal differentiation of leukemic blasts

Pinto et al. demonstrated the induction of morphological and functional differentiation of AML cells to mature elements following repeated exposure to decitabine[28]. Moreover, increased expression of class I human leukocyte antigens (HLAs) and HLA-DR in response to treatment with decitabine has been reported[29,30]. The increased expression of these antigens may induce a higher immunogenic potential of malignant cells thus rendering them susceptible to the graft-versus-leukemia effect (GVL) mediated by donor cells in allogeneic transplantations.

Up-regulation of major histocompatibility class 1-related chain B

Major histocompatibility (MHC) class 1-related chain A (MICA) and B (MICB) are polymorphic transmembrane glycoproteins that act as ligands for the immune complex receptor NKG2D expressed by natural killer (NK) cells, CD8 cytotoxic T-cells, and γδ-T cells. MIC is a critical component of target cell susceptibility for these cells[31-33]. Tang et al. demonstrated MICB up-regulation in cell lines following treatment with decitabine. This phenomena was accompanied by promoter DNA demethylation and DNA damage and significantly enhanced susceptibility of tumor cells to NK-cell mediated cytotoxicity[31].

Effects on natural killer cells

Interleukin-2 (IL-2) plays an important role in the development and expansion of effector T cells and maintenance of immune tolerance[34,35]. Promotion of immune tolerance by IL-2 is mediated through the generation and maintenance of Tregs, which are generally defined by $CD4^+CD25^+FOXP3^+$[36-38]. Zorn et al. demonstrated that administration of low dose recombinant IL-2 induced the expression of $CD4^+CD25^+FOXP3^+$ T cells *in vivo*[36]. These authors further demonstrated that while low dose IL-2 therapy induced the expansion of NK cells, it did not induce FOXP3 expression in NK cells. But when NK cells were pre-treated with varying concentrations of decitabine, IL-2 induced the expression of FOXP3 suggesting that FOXP3 gene was repressed in NK cells by DNA methylation[36]. Interestingly, Chan et al. demonstrated that NK cells use DNA methylation to maintain clonally restricted expressions of highly homologous killer immunoglobulin-like receptor (KIR) genes and alleles, and that decitabine induced KIR DNA hypomethylation and heterogeneous expression of multiple KIR genes[39].

Exploiting immunomodulatory effects of hypomethylating agents in the allogeneic transplantation setting

Possible mitigation of graft-versus-host disease by effects on regulatory T-cells

GVHD is one of the major complications of allo-HSCT and is mediated by donor T cells reacting against host antigens[40]. These T cells also play an important role in controlling a variety of critical steps after transplantation such as facilitating

engraftment of hematopoietic stem cells, immune reconstitution and elimination of residual disease, among others[41-43]. Tregs have been shown to mitigate GVHD by suppressing the early expansion of donor T cells[44].

Epigenetic modifications play a critical role in the locus coding for FOXP3, a forkhead transcription factor expressed in Tregs[37,45,46].

Sánchez-Abarca et al. demonstrated that azacitidine has profound effects on the activation and proliferation of T cells[9]. In their study, the addition of azacitidine to the cell culture significantly inhibited the activation and proliferation of T cells in a dose-dependent manner. Azacitidine-treated T cells produced significantly lower amounts of pro-inflammatory cytokines, tumor necrosis factor (TNF)-α and interferon (IFN)-γ compared with stimulated untreated T cells. Gene expression arrays revealed up-regulation of the FOXP3 and FOXO3a genes as well as genes involved in cell-cycle inhibition such as p27, p16, p53, and p73, whereas IFN and IL-10 genes were down-regulated. FOXP3 promotermethylation was decreased after prolonged drug exposure. These findings were also confirmed *in vivo* in a GVHD mouse model, where azacitidine treatment improved mice survival and decreased GVHD related scores. The most effective time for the administration of the drug to prevent GVHD was in the range of 2-4 days after transplantation when the alloreactive T cell expansion is maximal. Interestingly, no significant change in FOXP3 promoter methylation pattern or azacitidine-induced increase in Tregs was seen during the first four days of culture. However, longer exposure induced significant promoter demethylation and drove T cell differentiation towards a Treg phenotype. The authors concluded that azacitidine prevents the development of GVHD by inhibiting the early expansion of T cells, while delayed and prolonged exposure minimized the risk of GVHD by Treg expansion[9].

In another study, Choi et al. demonstrated that both azacitidine and decitabine induced FOXP3 mRNA and protein expression in $CD4^+CD25^+FOXP3^-$ humans (*in vitro*) and in mice[8]. They transplanted lethally irradiated Balb/c mice with T cell depleted bone marrow and conventional T cells along with azacitidine-treated Tregs, decitabine-treated Tregs or phosphate-buffered saline (PBS)-treated T cells. Mice receiving azacitidine or decitabine-treated Tregs became complete donor chimera, had improved survival and less clinical GVHD, compared to mice treated with PBS-treated T cells. To study the effect of the*in vivo* treatment of mice with demethylating agents after allo-HSCT, mice were transplanted with T cell depleted bone marrow following ablative irradiation. After recovery of the blood counts the mice were infused with MHC mismatched $CD4^+/CD8^+$ T cells on day +11. Mice were then treated with PBS, decitabine or azacitidine. While the mice treated with decitabine died due to excessive myelosuppression, the azacitidine-treated mice had high rates of donor engraftment and no detectable GVHD. Moreover, the authors also demonstrated maintenance of the GVL effect with azacitidine treatment. Interestingly they also indicated that decitabine treated Tregs from FOXP3 knockout mice were as suppressive as decitabine treated Tregs from FOXP3 wild-type littermate controls, suggesting that the suppressor function of decitabine or azacitidine treated Tregs is not dependent on FOXP3 expression and that expression of other candidate genes is likely modulated andis necessary for the suppressor function of decitabine or azacitidine-treated Tregs to occur[8]. In summary, the above studies would suggest that treatment with demethylating agents may have a role in GVHD prevention[8,9].

Use of azacitidine to prevent and treat myeloid leukemia relapse after allogeneic hematopoietic stem cell transplantation

Demethylating agents in the treatment of acute myeloid leukemia/myelodysplastic syndrome relapse after allogeneic hematopoietic stem cell transplantation

Patients with acute leukemia or MDS that relapse after allo-HSCT have a poor prognosis. Therapeutic options for these patients are limited and the optimal management is controversial[47]. While a second allo-HSCT or donor lymphocyte infusion (DLI) offer long disease-free survival in a small subset of patients, treatment-related mortality (TRM) and relapse rates are high[48-50].

Demethylating agents have been used to treat recurrent disease after allo-HSCT, and the achievement of remission and complete donor chimerism have been reported[51,52]. Jabbour et al. treated 17 patients with low-dose azacitidine after allo-HSCT for AML/MDS as salvage (n=9) or maintenance (n=8) therapy[53]. Azacitidine was started at a median of eight months after allo-HSCT in patients with recurrent disease and at a median of two months when given as maintenance therapy. The response rate among patients with recurrent disease was 55%, but complete remission (CR) occurred mostly in indolent relapses with low bulk disease. Median CR duration in patients who received azacitidine as maintenance therapy was 17 months. The two-year event-free survival (EFS) and OS rates were 30% and 80%, respectively. Overall the drug was well tolerated and no extramedullary toxicities or increased risk of infections was noticed. In another study, Lübbert et al. treated 26 patients with AML/MDS in hematological relapse after allo-HSCT with repeated cycles of low-dose azacitidine (100 mg/day for three days) followed by DLI[54]. CR was seen in 16% of patients and another 50% had temporary disease control with stable mixed chimeras for a median duration of 72 days. Acute GVHD was seen in only three patients and the estimated two-year survival was 16%. This low survival rate was however similar to the results obtained with other interventions such as second allo-HSCT in this setting[55].

Although it is postulated that hypomethylating agents are more effective as such when given in lower doses, investigators have reported on the use of higher doses of decitabine to treat post-transplantation relapses. Ravandi et al. conducted a phase I trial of decitabine in 14 patients with advanced leukemia or transformed chronic myeloid leukemia (CML) who had failed allo-HSCT[56]. Doses were 100 mg/m^2 to 150 mg/m^2 given every 12 hours for five days, followed by infusion of stem cells from the original donor. The treatment was well tolerated, and disease response was seen in 57% of the patients with a median survival of 190 days. These doses were expected to be primarily cytotoxic, and it is unclear if a hypomethylating effect was present in light of the more recent knowledge discussed above.

Schroeder et al. showed the feasibility of prospectively administering azacitidine combined with DLI as first salvage therapy to treat relapsed AML/MDS after allo-HSCT[57]. Overall response rate was 30% and five of 30 patients achieved long-term CR. Acute and chronic GVHD were seen in 37% and 17% of the patients, respectively. Studies of demethylating agents use for the treatment of AML/MDS relapse are summarized in Table 1.

Table 1 - Demethylating agents used for the treatment of disease relapse after allogeneic hematopoietic stem cell transplantation

Study	n	Disease	Agent used	Response	PFS	Survival
Giralt et al.[52]	3	AML/ALL	Decitabine	CR 100%	-	1 patient alive at 160 days
Jabbour et al.[53]	17 Relapsed disease (n = 9) Maintenance therapy (n = 8)	AML	Azacitidine	*RR 55%	55% at 1 year	OS 90% at 1 year
Lübbert et al.[54]	26	AML/CMML	Azacitidine + DLI	Clinical benefit 66% CR 16%	-	OS 16% at 2 year
Ravandi et al.[56]	14	AML/CML	Decitabine + HSCT	57%	Median PFS 60 days#	Median survival 190 days
Schroeder et al.[57]	25	AML/MDS/ CMML	Azacitidine + DLI	RR 64% CR 20%	-	Median OS 184 days

PFS: progression-free survival; OS: overall survival; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; CR: complete remission; RR: response rate; CMML: chronic myelomonocytic leukemia; CML: chronic myeloid leukemia; HSCT: hematopoietic stem cell transplantation; MDS: myelodysplastic syndrome; DLI: donor-lymphocyte infusion

*Response rate in patients treated for relapsed disease

#For patients achieving response

Minimal residual disease-based preemptive therapy after allogeneic hematopoietic stem cell transplantation

Detection of minimal residual disease (MRD) after allo-HSCT is associated with increased risk of hematologic relapse[58-60]. Platzbecker et al. monitored 59patients prospectively for decreasing CD34 cell chimerism, a harbinger of impending relapse[61]. In case of decreasing chimerism, azacitidine was to be given at the standard dose of 75mg/m^2/day subcutaneously, for seven days, for a total of four cycles every 28 days. Of the 19 patients evaluable for response one month after the 4th cycle, tenshowed complete clearance of MRD defined as an increase of CD34$^+$ donor chimerism >80%. Hematologic relapse occurred ultimately in 13 of the patients, but was delayed for approximately a median of seven months after initial decrease of CD34 donor chimerism to <80%. Grade III-IV neutropenia and thrombocytopenia were common however.

Demethylating agents for the prevention of disease relapse after allogeneic hematopoietic stem cell transplantation

Recurrences often occur early after allo-HSCT, a period of time when bone marrow function is very susceptible to myelosuppression.If one is to propose a maintenance of remission strategy, careful determination of a "tolerable" dose is necessary since most patients may not be able to receive usually prescribed doses. In addition, lower doses may actually be associated with improved hypomethylating effects as discussed above[62]. We thus hypothesized that low-dose azacitidine would decrease relapse rates after allo-HSCT in patients with relapsed refractory AML/MDS, and treated 45 patients who had high-risk MDS/AML and were in CR at day + 30 after allo-HSCT[63]. Azacitidine was given for one to four 30-day cycles. Each cycle consisted of drug administration subcutaneously for five days, starting on the sixth week after allo-HSCT at one of five dose levels (8, 16, 24, 32, or 40 mg/m^2). DNA methylation was assessed using bisulfite pyrosequencing[64]. Azacitidine did not affect engraftment. In this dose-escalation study, the dose of 32 mg/m^2 for four cycles was chosen, while thrombocytopenia prevented escalation to 40 mg/m^2. At a median follow-up of 20.5 months, the one-year EFS and OS were 58% and 77%, respectively, in a cohort of mostly refractory, relapsed patients. The grade II-III and grade III acute GVHD rates were 27% and 9%, respectively. However, most acute GVHD started before starting azacitidine and patients with severe GVHD were excluded from the trial, so no firm conclusions regarding acute GVHD could be made. The probability of developing chronic GVHD was however decreased significantly with longer azacitidine treatments. Interestingly, no change in peripheral blood mononuclear cells or global DNA methylation was noticed in this study.

Goodyear et al. confirmed the tolerability of low-dose azacitidine early after allo-HSCT, and demonstrated an increase in the number of Tregs within the first three months after transplantation, as well as an induced cytotoxic CD8(+) T-cell response to several tumor antigens (such as Wilm's tumor antigen1). Twenty-seven patients were treated with a reduced-intensity regimen and a T-cell depleted graft[65].

We recently performed a matched control analysis of chronic GVHD incidence comparing patients who received low-dose azacitidine for relapse prevention, versus allogeneic HCT recipients that did not receive the drug. In this analysis, reported in abstract form only, use of azacitidine led to a significant reduction in chronic GVHD rates[66].

Use of demethylating agents prior to transplantation

In order to asses if the use of demethylating agents may increase the toxicity of the conditioning regimen or otherwise affect transplantation outcomes, Padua Silva et al. studied 17 MDS patients that underwent allo-HSCT after prior therapy with decitabine[67]. Engraftment was seen in 16 patients and 100-day TRM was seen in only one patient. The authors concluded that the transplantation-related toxicity did not seem to be increased by prior use of decitabine. Others have reported similar survival after allo-HSCT in patients who did or did not receive azacitidine prior to allo-HSCT[68]. In addition, a trend towards decreased early relapse was seen in patients who received azacitidine prior to allo-HSCT (Table 2). The small number and the heterogeneous nature of the patients prevent a clear conclusion at this point regarding whether the use of these agents prior to allo-HSCT improves transplantation outcomes. Similarly, it is unclear if MDS patients should be transplanted at best response after azacitidine or decitabine, or after failing these drugs[69].

Table 2 - Demethylating agents used prior to allogeneic hematopoietic stem cell transplantation

Study	n	Disease	Agent used	Response	PFS	OS
De Padua Silva et al.[67]	17	MDS	Decitabine*	CR 76%	-	64% alive at median follow-up of 12 months
Field et al.[68]	54	MDS/CMML	Azacitidine#	-	41% at 1 year	47% at 1 year
Lübbert et al.[70]	10	AML/MDS/ CMML	Decitabine	-	33% relapse	30% alive$ □
McCarty et al.[71]	25	MDS/AML	Azacitidine	-	NR at median follow-up 1 year	NR at median follow-up 1 year
Kim et al.[72]	19	MDS	Decitabine (n = 9) Azacitidine (n = 10)	-	-	68% at 2 year

PFS: progression-free survival; OS: overall survival; AML: acute myeloid leukemia; CR: complete remission; CMML: chronic myelomonocytic leukemia; MDS: myelodysplastic syndrome; NR: not reached

*Preparative regimen; Fludarabine + Busulfan (n = 8); Fludarabine + Melphalan (n = 9)
#Preparative regimen; Fludarabine + Busulfan (n = 54)
$At +1, +10, +26 months after transplantation

Table 3 - DNA demethylating agents used as part of conditioning regimen

Study	n	Disease	Agent used + Conditioning regimen	Response	PFS	OS
Giralt et al.[52]	4	CML/AMML	Decitabine + (Bu/Cy)	CR 50%	-	3 patients alive*
De Lima et al.[73]	23	AML/ CMML/ALL/ CML	Decitabine + Bu/Cy	CR 91%	Median PFS 8.9 months	Median survival 17.2 months

PFS: progression-free survival; OS: overall survival; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; CR: complete remission; CMML: chronic myelomonocytic leukemia; CML: chronic myeloid leukemia; AMML: acute myelomonocytic leukemia; Bu: busulfan; Cy: cyclophosphamide

*At 167, 129, and 109 days post-transplantation

In the past, we investigated decitabine as part of the transplantation conditioning regimen. Decitabine was added to busulfan and cyclophosphamide, followed by HLA identical sibling donor allo-HSCT for high-risk patients with acute leukemia and chronic myelomonocytic leukemia (CMML)[73]. The regimen was well tolerated with a 100-day TRM of 9%. The incidence of acute and chronic GVHD was 18% and 40%, respectively. The median survival for the entire group was 17.2 months with 26% patients alive and disease free at a median of 3.3 years from transplantation. This trial, however, investigated cytotoxic doses of decitabine (Table 3) and this approach has not been pursued by other investigators.

Future directions

The immunomodulatory and cytotoxic effects of agents capable of epigenetic manipulation deserve further investigation in the realm of allogeneic transplantation. As an example, we and others are launching a phase I trial investigating the oral formulation of azacitidine in the prevention of relapse of AML/MDS in this setting. Other potential ideas include studies of GVHD prevention and treatment of GVHD with decitabine or azacitidine.

Clinical investigators will be charged with determining efficacy and toxicities of these agents, but it is an intriguing possibility that one might be able to pharmacologically separate GVHD from GVL. This hypothesis is under active investigation in different forms and approaches by several groups around the world[74].

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