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SHORT COMMUNICATION

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The chemokine CCL21 protects normal marrow progenitors from Ara-C cytotoxicity

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Abstract *Purpose*: Chemokines are a family of small proteins that regulate leukocyte infiltration into inflamed tissue and play key roles in the pathogenesis of many diseases. Some chemokines can also reversibly inhibit the proliferation of hematopoietic progenitors. We have previously found that the chemokine CCL21 (Exodus-2/SLC/6Ckine/TCA4) is a potent inhibitor of the proliferation of normal hematopoietic progenitors. In this study we sought to determine whether this inhibition of proliferation could be therapeutically exploited by protecting normal marrow progenitors from the cytotoxicity of the S phase-active chemotherapeutic agent Ara-C. *Methods*: Untreated and CCL21-pretreated mice were given doses of Ara-C that are toxic to marrow myeloid progenitors. The recovery of these myeloid progenitors was analyzed by colony formation assays. *Results*: It was found that pretreatment with small doses of CCL21 prevented the death of normal murine marrow progenitors from the toxic effects of Ara-C. *Conclusions*: The chemokine CCL21 may be able to prevent Ara-C myelosuppression during acute leukemia induction chemotherapy, and thereby decrease morbidity and mortality of such therapy, and shorten hospital stays.

Keywords Chemokine · Ara-C · Myelosuppression · Leukemia · Myeloid progenitors

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Introduction

Chemokines are a related group of small proteins that mediate all leukocyte migration. They play critical roles in the pathogenesis of many human diseases, from AIDS to atherosclerosis to autoimmune disorders [1, 2, 9, 11, 12, 13, 17]. We and others have previously reported that many but not all chemokines can reversibly inhibit normal hematopoietic progenitor proliferation by blocking progression through the cell cycle [3, 4, 5, 6, 8, 14, 15]. This reversible inhibition is seen in both murine and human marrow progenitors, and both in vitro and in vivo. We have also recently reported that CCL21 is a potent negative regulator of normal marrow and chronic myeloid leukemia, but not acute myeloid leukemia (AML), progenitor proliferation [8, 16]. We and others originally isolated CCL21 as the most potent stimulator of T-cell and NK chemotaxis yet described [15, 19, 20, 21]. CCL21 is critical for the proper homing of T cells to secondary lymphoid tissues for antigen presentation [20, 21].

Ara-C is the most widely used agent in the treatment of AML. Induction of remission of AML with Ara-C-based regimens has significant morbidity and mortality from infection and hemorrhage due to prolonged myelosuppression. Reducing the length or severity of the myelosuppression of normal hematopoiesis during AML induction chemotherapy is therefore an important goal. In this study it was demonstrated that CCL21 inhibition of normal progenitor proliferation can suppress hematopoietic progenitor death from Ara-C treatment.

Materials and methods

Hematopoietic progenitor assays

Hematopoietic colony formation assays were performed essentially as described elsewhere [5, 6, 8]. Murine femur bone marrow on-adherent mononuclear cells were assessed for granulocyte-macrophage (colony-forming unit-granulocyte-macrophage, CFU-GM), erythroid (burst-forming unit-erythroid, BFU-E) and

multipotential (colony-forming unit granulocyte, erythroid, monocyte, megakaryocyte, CFU-GEMM) progenitor cells. Briefly, bone marrow cells were plated at 2.5 to 5.0×10^4 cells/ml in 1% methylcellulose culture medium in the presence of 1 U/ml recombinant human erythropoietin (Amgen Corporation, Thousand Oaks, Calif.), 10% v/v pokeweed mitogen mouse splenocyte conditioned medium, 50 ng/ml recombinant murine Steel factor, and 0.1 mmol/l hemin (Eastman Kodak, Rochester, N.Y.). Colonies were scored after 7 days of incubation in an atmosphere containing 5% CO_2 and lowered (5%) O_2. Absolute numbers of progenitors were calculated from the nucleated cellularity per femur and the number of colonies formed per number of cells plated. This controls for individual differences in marrow cellularity. For each data point three mice were individually analyzed in triplicate, and compared with three saline-injected controls, also analyzed in triplicate for each data point. Data were statistically analyzed using Student's *t*-test.

On day 0 individual mice were given 5 μg CCL21 intravenously, followed 3 h later by 500 mg/kg Ara-C subcutaneously. Both the chemokine pretreatment and the Ara-C injections were repeated once 3 h later. The chemokine and Ara-C dose and timepoints were chosen based on previous studies [5, 6, 7, 8, 10].

The percentage of progenitors in S phase at the time of chemokine treatment was analyzed by tritiated thymidine kill assays as previously described [6, 8]. Chemokine inhibitory activity has been shown to only be effective against progenitors progressing through S phase of the cell cycle [6, 8]. Mice were treated and sacrificed according to IACUC-approved protocols.

Results

The absolute numbers of each progenitor type per femur for CCL21-treated mice and simultaneously analyzed control mice were compared for each time-point (Table 1). On day 1 the Ara-C-alone mice showed a decrease of 76% in CFU-GM, 59% in BFU-E, and 59% in CFU-GEMM compared to control mice. The Ara-C/CCL21 mice had statistically significantly more of each progenitor type per femur left on day 1 after treatment. The Ara-C/CCL21 mice had a decrease of 51% in CFU-GM ($P=0.033$ compared to Ara-C-alone mice), 17% in BFU-E ($P=0.014$), and 21% in CFU-GEMM ($P=0.034$) compared to control mice.

On day 3 the Ara-C-alone mice had 71% fewer CFU-GM, 37% fewer BFU-E, and 61% fewer CFU-GEMM compared to control mice. The Ara-C/CCL21 mice had more of each progenitor type per femur surviving on day 3 after Ara-C treatment. The Ara-C/CCL21 mice had 63% fewer CFU-GM ($P=0.08$ compared to Ara-C-alone mice), 3% fewer BFU-E ($P=0.005$), and 44% fewer CFU-GEMM ($P=0.03$) compared to control mice.

On day 5 the Ara-C-alone mice had 34% fewer CFU-GM, 55% fewer BFU-E, and 63% fewer CFU-GEMM compared to control mice. The differences between the Ara-C-alone and the Ara-C/CCL21 mice had largely disappeared by day 5. The Ara-C/CCL21 mice had 26% fewer CFU-GM, 60% fewer BFU-E, and 66% fewer CFU-GEMM as compared to control mice. None of the mice in either the Ara-C or the Ara-C/CCL21 group died.

We and others have previously found that chemokines such as CCL21 reversibly inhibit hematopoietic progenitor progression through the cell cycle [6, 8]. Ara-C is specifically cytotoxic against progenitors transiting S phase. Therefore, the percentages of progenitors actively progressing through S phase of the cell cycle in the Ara-C-alone mice and the Ara-C/CCL21 mice were compared with that in control mice (Table 2). On day 1 the Ara-C-alone mice had no CFU-GM progressing through S phase as compared to 31% in control mice, 3% of BFU-E in S phase as compared to 34% in control mice, and no CFU-GEMM in S phase as compared to 29% in control mice. The Ara-C/CCL21 mice had 53% CFU-GM in S phase ($P<0.0001$ compared to Ara-C-alone mice), 59% BFU-E in S phase ($P<0001$), and 55% CFU-GEMM in S phase ($P<0.0001$).

On day 3 after treatment the Ara-C-alone mice had 37% CFU-GM progressing through S phase as compared to 26% in control mice, 38% BFU-E in S phase as compared to 20% in control mice, and 30% CFU-GEMM in S phase as compared to 29% in control mice. The Ara-C/CCL21 mice had 46% CFU-GM in S phase ($P=0.04$ compared to Ara-C-alone mice), 42% BFU-E in S phase ($P=0.02$), and 55% CFU-GEMM in S phase ($P=0.007$).

Table 1. The absolute hematopoietic progenitor numbers per femur of mice treated with Ara-C or Ara-C plus CCL21. Mice treated with Ara-C and those treated with Ara-C plus CCL21 were compared with simultaneously analyzed saline-treated control mice for each time-point. Each value is the mean from three mice individually analyzed. The analysis was based on the number of progenitors per femur for each mouse obtained using colony formation assays

Treatment	Progenitor	Absolute numbers per femur		
		Day 1	Day 3	Day 5
Saline	CFU-GM	16,710 ± 1380	19,716 ± 313	7,680 ± 860
	BFU-E	1,702 ± 525	985 ± 44	762 ± 45
	CFU-GEMM	1,219 ± 128	650 ± 43	605 ± 40
Ara-C	CFU-GM	4,031 ± 280	5,763 ± 182	5,086 ± 275
	BFU-E	700 ± 164	616 ± 38	344 ± 62
	CFU-GEMM	500 ± 63	257 ± 18	222 ± 45
CCL21/Ara-C	CFU-GM	8,266 ± 1381*	7,354 ± 570	5,703 ± 1156
	BFU-E	1,416 ± 85*	958 ± 93*	308 ± 33
	CFU-GEMM	969 ± 147*	363 ± 14*	207 ± 58

*$P<0.05$ vs Ara-C alone

Table 2. The percent of hematopoietic progenitors actively progressing through the cell cycle after treatment of mice with Ara-C or Ara-C plus CCL21. Mice treated with Ara-C and those treated with Ara-C plus CCL21 were compared with simultaneously analyzed saline-treated control mice for each time-point. Each value is the mean from three mice individually analyzed

Treatment	Progenitor	Progenitors cycling in S phase (%)		
		Day 1	Day 3	Day 5
Saline	CFU-GM	31 ± 3	26 ± 5	27 ± 4
	BFU-E	34 ± 3	20 ± 3	39 ± 2
	CFU-GEMM	29 ± 2	29 ± 13	43 ± 2
Ara-C	CFU-GM	0	37 ± 5	23 ± 2
	BFU-E	3.0 ± 2	38 ± 1	39 ± 7
	CFU-GEMM	0	30 ± 4	37 ± 7
CCL21/Ara-C	CFU-GM	53 ± 4*	46 ± 1*	23 ± 3
	BFU-E	59 ± 2*	52 ± 1*	38 ± 2
	CFU-GEMM	55 ± 7*	55 ± 3*	33 ± 2

*$P < 0.05$ vs Ara-C alone

On day 5 after treatment the Ara-C-alone mice had 23% CFU-GM in cycle as compared to 27% in control mice, 39% BFU-E as compared to 39% in control mice, and 37% CFU-GEMM as compared to 43% in control mice. The Ara-C/CCL21 mice had 23% CFU-GM, 38% BFU-E, and 33% CFU-GEMM in cycle. There were no significant differences on day 5 between Ara-C-alone mice alone and Ara-C/CCL21 mice.

Discussion

These findings indicate that CCL21 had a more protective effect on day 1 than on day 3 and especially day 5 after Ara-C treatment. This could have been because the bioavailability of CCL21 decreases over time after the initial injection, and the progenitors escape the cycle inhibition while Ara-C is still present. However, for future clinical trials, CCL21 dosing could be repeated daily in order to maintain S-phase blockade while Ara-C is still present. Another possibility is that progenitor recovery had progressed sufficiently by day 3 to narrow the difference between the Ara-C- and the Ara-C/CCL21-treated marrow.

CCL21 also protects CFU-GEMM and BFU-E more than CFU-GM, which is a more differentiated progenitor. This may be because CFU-GM are less sensitive to chemotherapy, or because there are so many more CFU-GM progenitors per femur than BFU-E or CFU-GEMM that the kinetics of repopulation are more rapid after Ara-C treatment. It is possible that CCL21 could have less inhibition on acute leukemia cells than in normal marrow because leukemia cells do not express chemokine receptors, or have constitutive activation of growth signaling pathways.

Mip-1α (CCL3) has also been demonstrated to provide some protection against S phase-active cytotoxic drugs [10]. A clinical trial of a variant of Mip-1α called BB10010 has demonstrated mild marrow progenitor protection against cyclophosphamide but no benefit to peripheral blood counts [7]. However, cyclophosphamide is not an S phase-active agent, and therefore might not be as amenable to chemokine hematopoietic protection. Chemokines such as CCL3 and CCL21 inhibit the progression of normal progenitors through S phase of the cell cycle, and therefore would have little therapeutic benefit against a chemotherapeutic agent that was equally active whether or not the progenitor was progressing through the cell cycle [5, 6, 8].

These results are similar to those of Paukovits et al., who found that the tetrapeptide EEDCK protected normal marrow but not leukemia cells from the cytotoxic effects of Ara-C [18]. It is quite possible that this peptide activates a chemokine-like G-protein-coupled receptor, similar to FMLP, producing the same myeloid protection as seen here. While we have previously shown that chemokines inhibit normal human marrow but not acute leukemias from cycling, it was beyond the scope of this study to test this in vivo in these mice. Such a formal demonstration of a therapeutic index in vivo in animals would be the next logical next step in these studies prior to initiation of clinical trials.

Ara-C is one of the most widely used drugs in the treatment of hematologic malignancies, especially AML. The major side effect of Ara-C is myelosuppression from toxicity to cycling normal marrow progenitors. Chemokines such as CCL21 reversibly freeze hematopoietic progenitors from progressing through the cell cycle [5, 6, 8, 15]. Since CCL21 inhibited the damage produced in normal hematopoietic progenitors, it is possible that it might have therapeutic utility in hastening hematopoietic recovery after treatment with Ara-C. Utilization of CCL21 to protect against the toxicity of Ara-C to normal marrow can easily be envisioned for AML induction chemotherapy, where early mortality is often due to the prolonged time for the marrow to recover after exposure to induction agents. Absolute therapeutic benefit can only be demonstrated in randomized clinical trials.

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