Induction of TGF- β by Irradiation or Chemotherapy in Fanconi Anemia (FA) Mouse Bone Marrow Is Modulated by Small Molecule Radiation Mitigators JP4-039 and MMS350

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Abstract. *Background/Aim:* Total-body irradiation and/or administration of chemotherapy drugs in bone marrow transplantation induce cytokines that can suppress engraftment. Fanconi Anemia (FA) patients have a hyperactive responsiveness to the inhibitory cytokine, transforming growth factor-beta (TGF- β). Small molecule radiation mitigator drugs, JP4-039 and MMS350, were evaluated for suppression of irradiation or drug-induced TGF- β . *Materials and Methods:* In vivo induction of TGF- β by total-body ionizing irradiation (TBI), L-phenylalanine mustard (L-PAM), busulfan or fludarabine, was quantified. In parallel, mitigator drug amelioration of TGF- β induction in FA D2^{-/-} (FANCD2^{-/-}) mouse bone marrow, was studied in vitro. Tissue culture medium, cell lysates, and mouse plasma were analyzed for TGF- β levels. *Results:* Induction of TGF- β levels in FANCD2^{-/-} and FANCD2^{+/+} mice and in mouse bone marrow were modulated by both JP4-039 and MMS350. *Conclusion:* Bone marrow transplantation in FA recipients may benefit from administration of small molecule agents that suppress TGF- β induction.

Bone marrow transplantation is an established therapy for Fanconi anemia (FA) patients (1-4) that can result in a significant improvement in survival following donor bone

marrow engraftment (4). Critical to the success of marrow engraftment has been the application of chemotherapeutic agents as a preparatory regimen for marrow transplant that minimize toxicity to the host (3).

FA patients have previously been demonstrated to have a hyperactive TGF- β response pathway (5), which may be a major cause of their initial marrow failure leading to anemia, as well as their sensitivity to the regimens used to prepare for bone marrow transplantation (1-4, 6-9). FA patients are also susceptible to late post-transplant induction of leukemia and solid tumors (7-15). DNA cross-linking agents such as mitomycin-C (14), other chemotherapeutic agents and irradiation induce DNA double strand breaks and must be delivered very cautiously to FA patients. The TGF- β signaling pathway alters both baseline and post-marrow transplant hematopoiesis in FA patients and in FA animal models (16-23). The chemotherapy drug, fludarabine (6, 24-25), has facilitated bone marrow engraftment in FA patients, who are fragile in responsiveness to agents used in preparation for marrow transplantation (1-4, 6-9). We hypothesized that agents, which ameliorate the toxicity of total-body irradiation and/or chemotherapy drugs that may reduce the induction of TGF- β (26-33) might decrease toxicity and improve engraftment in FA patient transplant recipients. Furthermore, improvement in survival of patients with FA (4) might be achieved by reduction in the toxicity of marrow transplant.

In this study, FANCD2^{-/-} mice were used for *in vitro* testing of the effects of two potential modulators (JP4-039 and MMS350) of the toxicity of irradiation or each of three chemotherapeutic drugs on TGF- β induction in hematopoietic progenitor cells. FANCD2^{-/-} mouse marrow hematopoietic progenitors *in vitro* and stromal cell lines derived from the hematopoietic microenvironment (34) were used for TGF- β induction by irradiation and chemotherapy drugs. We also tested the effect of JP4-039 and MMS350 on TGF- β induction in plasma of TBI irradiated or drug-treated FANCD2^{-/-} (C57BL/6 background) mice.

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Key Words: TGF- β , total body irradiation, chemotherapy drugs, Fanconi Anemia, mouse bone marrow, molecular radiation, mitigators, JP4-039, MMS350.

We evaluated the effect of radiation mitigator drugs JP4-039 (29) and MMS350 (32) as well as of the chemotherapeutic agents, L-phenylalanine mustard (L-PAM), busulfan and Fludarabine, on TGF- β induction. The results demonstrated that TGF- β expression was inducible by irradiation and by either of the three chemotherapeutic drugs *in vitro* and *in vivo*. In *FANCD2*^{-/-} mouse marrow *in vitro* and in mouse plasma *in vivo*, induction of TGF- β and modulation of TGF- β induction by both JP4-039 and MMS350, were observed. The data suggest that the agents, which reduce irradiation or chemotherapy-induced TGF- β might be of value in marrow transplant regimen and facilitate better engraftment while reducing toxicity.

Materials and Methods

Mice and animal care. Control 129/Sv, SMAD3^{-/-} (129/Sv), control C57BL/6NTac (34), *FANCD2*^{-/-} (B6) (15), and Double Knockout (DKO) SMAD3^{-/-} (129/Sv) *FANCD2*^{-/-} (C57BL/6) mice (35) were housed, 5 animals per cage, at the University of Pittsburgh Cancer Institute according to the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) protocols. Heterozygote TGF- β ^{+/-} mice (C57BL/6 background) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mice were bred to produce TGF- β ^{-/-} mice. Marrow was removed from TGF- β ^{-/-} mice on the day of birth to establish bone marrow stromal cell lines. All protocols were approved by the University of Pittsburgh IACUC. Animals were fed standard laboratory chow and deionized water.

Bone marrow culture. Fresh bone marrow was explanted from the mouse femur and tibia at serial time points after administration of chemotherapy drugs and plated in 6-well culture plates at 5×10⁴ cells/per well for hematopoietic colony assays according to published methods (34). The medium was supplemented with hematopoietic growth factors as described (29) to stimulate multilineage hematopoietic stem cell growth. Colonies of greater than 50 cells were scored at days 7 and 14 (34).

Chemotherapeutic drugs tested were L-Phenylalanine Mustard (L-PAM), Busulfan, and Fludarabine (Sigma-Aldrich Chemical Company, St. Louis, MO, USA). Each drug was added to bone marrow cultures or injected intravenously into mice using established manufacturer's procedures.

Total body irradiation. Mice were irradiated using a Gammacell Mark IV Cesium-137 gamma cell irradiator at 70 cGy/minute to the lethal dose of 10 Gy (29). Bone marrow was removed from mice at several time points after irradiation and TGF- β levels as well as other biomarkers of the irradiation response were analyzed. Plasma was withdrawn from the cardiac puncture of mice at serial time points after total body irradiation and assayed for TGF- β , as well as other inflammatory cytokines according to established methods (29).

TGF- β assays. Cell conditioned medium, cell lysates, and mouse plasma were tested for TGF- β using TGF β MAG-64K-01 Milliplex Map TGF- β Signaling Pathway Magnetic Bead 6-Plex-Cell Signaling Multiplex Assay (EMD Millipore, Billerica, MA, USA) according to published methods. For plasma studies, blood was

isolated from the mice by cardiac puncture and placed in EDTA collection tubes, centrifuged for 10 min at 1,000 rpm and plasma removed. Plasma was diluted 1:4 with sample diluent from Milliplex kit and 2.0 μ l of 1.0 N HCl per 50 μ l of diluted sample was added and shaken for 15 min at room temperature. The acid-treated samples were diluted 1:6 using Assay Buffer. To the appropriate wells, standard or sample (25 μ l), 25 μ l of matrix solution, and 25 μ l of mixed beads was added. The plates were sealed with a plate sealer, and shaken for 2 h at room temperature or overnight at 4°C. Well contents were removed, washed twice with 200 μ l of Wash Buffer, added 25 μ l of detection antibodies to each well, incubated for one hour at room temperature and added 25 μ l of streptavidin-Phycoerythrin per well. These plates were then incubated for 30 min at room temperature and washed twice with 200 μ l of Wash Buffer. Finally, 100 μ l of Sheath Fluid per well was added and results were read on the Luminex Reader. TGF- β levels were recorded as ng/ml.

Cell lines. Bone marrow stromal cell lines (34) and IL-3 dependent hematopoietic progenitor cell lines (34, 36) were established from control C57BL/6NTac, *FANCD2*^{-/-} and *FANCD2*^{+/-} (B6) long-term bone marrow cultures according to published methods (34). Bone marrow stromal cell lines were established from TGF- β ^{-/-} newborn mice on day one after birth according to published methods (34), or from the adherent layer of four week old long-term bone marrow cultures from TGF- β ^{+/-} and TGF- β ^{+/-} control (C57BL/6NTac) mice according to published methods (31). Clonal sublines used were designated TGF- β ^{+/-} 2A6, TGF- β ^{+/-} 2C9, and TGF- β ^{-/-} 2C7. Bone marrow stromal cell lines were passaged weekly in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum.

IL-3 dependent cell lines derived from long term bone marrow cultures that were established from *FANCD2*^{-/-} and *FANCD2*^{+/-} mice have been previously reported (34). Each cell line was passaged in suspension culture in IL-3 supplemented medium according to published methods (34, 36). For TGF- β assays, all cell lines were transferred to serum free medium, left unirradiated as controls or irradiated to 10 Gy or drug treated. Medium was collected 24 h after irradiation or drug treatment. Cell lysates were obtained and TGF- β levels were assayed by Luminex assay as described above.

Western blots. Cell lines were cultured in DMEM medium (Lonza, Cat. #12-604F, Allendale, NJ, USA) with 10% Fetal Bovine serum (Gemini, Cat. #: 100-500, West Sacramento, CA, USA), 1% L-Glutamine (Lonza Cat. #17-605E), and 1% Antibiotics Antimycotic Solution (Corning, Cat. #30-004-C1, Sigma-Aldrich, St. Louis, MO, USA). Total cellular protein was extracted using protein extraction buffer (IP Lysis Buffer, Thermo Scientific, Cat. #87787, Waltham, MA, USA), containing protease inhibitor and phosphatase inhibitor cocktails (Thermo Scientific, Cat. #78442). Protein concentration was determined using the Bio-Rad protein assay system (Bio-Rad Laboratories, Cat. #500-0006, Hercules, CA, USA). The proteins (15 μ g per lane) were separated on denaturing polyacrylamide gels (Bio-Rad Laboratories, Mini-Protein TGX Gels Cat #: 456-1083) and then transferred to PVDF membranes (Bio-Rad Laboratories, Immun-Blot PVDF, Cat. #162-0177) by electrophoresis. Blots were blocked with 5% Fat-free dry milk in TBST for 1 h and then incubated overnight with primary antibodies against p-ERK, p-S6K, p-SMAD3, p-JNK, p21, and RAD51. Membranes were washed with

Table I. Induction of TGF- β in vitro with explanted marrow from *FANCD2*^{-/-} (C57BL/6) and control male mice and in plasma removed from 10 Gy TBI, L-PAM, Busulfan, or Fludarabine treated mice. (Reprinted from: Fanconi Anemia (FA): Genetic Prevalence, Management, and Treatment Outcomes, "Radiotherapy for the patient with Fanconi Anemia: A challenge for the radiation oncologist", 2015, Greenberger, Joel S and Epperly, Michael W with permission from Nova Science Publishers, Inc.)

Condition	In Vitro				In Vivo			
	<i>FANCD2</i> ^{+/+}		<i>FANCD2</i> ^{-/-}		<i>FANCD2</i> ^{+/+}		<i>FANCD2</i> ^{-/-}	
	TGF- β (pg/ml)	Fold increase	TGF- β (pg/ml)	Fold increase	TGF- β (pg/ml)	Fold increase	TGF- β (pg/ml)	Fold increase
0 Gy	820 $\pm$ 159		1057 $\pm$ 9		2474 $\pm$ 43		1197 $\pm$ 56	
10 Gy	961 $\pm$ 105	1.2 $\pm$ 0.2	1556 $\pm$ 216	1.1 $\pm$ 0.1	2742 $\pm$ 54 P2=0.0186	1.1 $\pm$ 0.1	1561 $\pm$ 42 P2=0.0067	1.3 0.2
10 Gy + JP4-039	230 $\pm$ 115 P1=0.0152	0.3 $\pm$ 0.1	295 $\pm$ 35 P1=0.0484	0.3 $\pm$ 0.1				
10 Gy + MMS350	435 $\pm$ 45 P1=0.0222	0.5 $\pm$ 0.1	367 $\pm$ 52 P1=0.0435	0.4 $\pm$ 0.1				
L-PAM	5395 $\pm$ 161	6.6 $\pm$ 0.4	2325 $\pm$ 465	2.2 $\pm$ 0.2	2022 $\pm$ 61	0.9 $\pm$ 0.1	2623 $\pm$ 115 P2=0.0004	2.2 $\pm$ 0.2
L-PAM + JP4-309	3116 $\pm$ 324 P1=0.0048	3.8 $\pm$ 0.3	2790 $\pm$ 1116	1.2 $\pm$ 0.5				
L-PAM + MMS350	3690 $\pm$ 1230	4.5 $\pm$ 0.8	1321 $\pm$ 330	1.3 $\pm$ 0.4				
Busulfan	2271 $\pm$ 45	2.8 $\pm$ 0.1	1511 $\pm$ 377	1.4 $\pm$ 0.3	1512 $\pm$ 47 P2=0.0001	0.6 $\pm$ 0.1	1140 $\pm$ 115	0.9 $\pm$ 0.1
Busulfan + JP4-039	2378 $\pm$ 71	2.9 $\pm$ 0.3	528 $\pm$ 176 P1=0.0205	0.5 $\pm$ 0.1				
Busulfan + MMS350	3165 $\pm$ 633	3.9 $\pm$ 0.6	422 $\pm$ 84 P1=0.0214	0.4 $\pm$ 0.1				
Fludarabine	1435 $\pm$ 358	1.7 $\pm$ 0.3	2642 $\pm$ 1321	2.5 $\pm$ 0.5	1994 $\pm$ 82 P2=0.0061	0.8 $\pm$ 0.1	1355 $\pm$ 74	1.1 $\pm$ 0.1
Fludarabine + JP4-039	1025 $\pm$ 256	1.3 $\pm$ 0.3	1717 $\pm$ 214	1.6 $\pm$ 0.1				
Fludarabine + MMS350	1127 $\pm$ 141	1.4 $\pm$ 0.1	1453 $\pm$ 182	1.4 $\pm$ 0.1				

Drugs and irradiation were delivered to freshly explanted whole marrow (triplicate experiments) in culture, and medium collected for analysis of TGF- β . TBI or drugs were delivered to mice (n=10/group) for collection of plasma 24 h later, as described in the methods, and plasma was assayed for TGF- β levels. P1 is comparison of treatment groups treated with JP4-039, or MMS350 to treatment groups only (50 μ M L-PAM, 100 μ M Busulfan, or 2 μ M Fludarabine. P2 is comparison of TGF- β level in plasma from *FANCD2*^{+/+} or *FANCD2*^{-/-} mice to TGF- β level in plasma from *FANCD2*^{+/+} or *FANCD2*^{-/-} mice treated with 10 Gy, L-PAM (15 mg/kg), Busulfan (30 mg/kg) or Fludarabine (30 mg/kg).

TBST and processed with corresponding horseradish peroxidase-conjugated secondary antibodies. The proteins were exposed to x-ray film (5 to 30 sec) using ECL detection reagent (Thermo Scientific SuperSignal West Dura Extended Duration Substrate, Cat. #34075). To ensure equal protein loading, the same blot was subsequently developed for β -actin expression.

Chemotherapeutic drugs and radiation mitigator small molecules. L-Phenylalanine Mustard (L-PAM), Busulfan, and Fludarabine were obtained from the supplier (Sigma-Aldrich, St. Louis, MO, USA). For *in vitro* experiments, L-PAM was used at 50 μ M, Busulfan at 100 μ M, and fludarabine at 2 μ M, while for *in vivo* studies, L-PAM was used at 15 mg/kg, busulfan at 30 mg/kg, and fludarabine at 30 mg/kg. The TGF- β receptor antagonist drug LY36937 was obtained from the supplier (Selleckchem.com, Houston, TX, USA). Drug was added to cultures at 5 μ M. The radiation mitigator drug JP4-039 (30) and MMS350 (32) have been described.

Statistical analysis. Data was assayed by Student's modified *t*-test, and significance was determined for differences with *p*-value less than 0.05.

Results

FANCD2^{-/-} Mouse bone marrow stromal cell lines and IL-3-dependent hematopoietic progenitor cell lines produce TGF- β in vitro. TGF- β was secreted by control as well as, 10 Gy irradiated *FANCD2*^{+/+} and *FANCD2*^{-/-} bone marrow stromal cell lines and IL-3 dependent hematopoietic progenitor cell lines (Table I and Figure 1). TGF- β was also detected in the cellular lysates of *FANCD2*^{+/+} and *FANCD2*^{-/-} cell lines (Figure 2). Levels were decreased in lysates from irradiated marrow stroma cells (Figure 2A). TGF- β was also detected in medium from

Figure 1. Quantitation of levels of TGF- β in culture medium from 10 Gy irradiated or control $FANCD2^{-/-}$ and $FANCD2^{+/+}$ marrow stromal cell lines and IL-3 dependent hematopoietic progenitor cell lines. Serum free tissue culture medium was harvested at 24 h after treatment from bone marrow stromal (A) and IL-3 dependent cell lines (B) and tested by Luminex analysis for TGF- β production as described in the methods.

Figure 2. Quantitation of levels of TGF- β in cell lysates from 10-Gy irradiation or control $FANCD2^{-/-}$ or $FANCD2^{+/+}$ marrow stromal cell lines and IL-3 dependent cell lines. Cell lysates were prepared from bone marrow stromal cells (A) and IL-3-dependent cells (B) and tested by Luminex analysis for the production of TGF- β , as described in the methods.

control F1 (129/Sv x C57BL/6), as well as $SMAD3^{-/-}$ (129/Sv), $FANCD2^{-/-}$ (C57BL/6) and double-knockout $SMAD3^{-/-}$ (129/Sv)/ $FANCD2^{-/-}$ (B6) mouse marrow stromal cell lines. $SMAD3^{-/-}$ (129/Sv) mice are known to be defective in TGF- β signaling (20-22). The results confirm and extend prior publications showing TGF- β production with $SMAD3^{-/-}$ bone marrow stromal cell lines (which have a defective canonical TGF- β signal transduction pathway (22)), and the data include

a negative control bone marrow cell line from $TGF-\beta^{-/-}$ mice. There was no detectable TGF- β secreted by $TGF-\beta^{-/-}$ stromal cell lines (data not shown).

All five of the known signaling pathways for TGF- β (37) were intact in $TGF-\beta^{-/-}$ as well as the other cell lines tested (Figure 3). The canonical TGF- β signaling pathway was intact in all cell lines ($TGF-\beta^{-/-}$, $TGF-\beta^{+/-}$, and $TGF-\beta^{+/+}$), as shown by phosphorylated p-SMAD3 (Figure 3). The ERK

Figure 3. Western blot analysis of each of five TGF- β signaling pathways in bone marrow stromal cell lines from TGF- $\beta^{-/-}$ and control mice. TGF- $\beta^{+/+}$ (B6) TGF- $\beta^{+/-}$, and TGF- $\beta^{-/-}$ mouse bone marrow stromal cell lines were tested as described in the methods for protein phosphorylation markers of each of the SMAD-dependent and non-SMAD-dependent TGF- β signaling pathway (37).

non-SMAD pathway was intact, as shown by p-ERK. The JNK/p38 pathway was intact, as shown by p-JNK. Here the TGF- $\beta^{+/+}$ cell line showed increases in p-JNK after 10 Gy or addition of 50 mg/ml TGF- β . The small GTPase (Rho-like) non-SMAD pathway was intact shown by p-PAK. The p13K/Akt non-SMAD pathway was intact as shown by p-S6K (Figure 3). Levels of p21 were more inducible by TGF- β or radiation in TGF- $\beta^{-/-}$ cells.

We tested TGF- $\beta^{-/-}$ cell lines for clonogenic radiation survival *in vitro*. TGF- $\beta^{-/-}$ and TGF- $\beta^{+/-}$ cell lines were radiosensitive relative to control TGF- $\beta^{+/+}$ (B6) cells (Figure 4 and Table II).

The data establish that TGF- β was produced by both bone marrow stromal and IL-3 dependent cell lines (Figures 1 and 2) derived from long-term marrow cultures of *FANCD2* $^{-/-}$ mouse marrow (34). The data establish that TGF- β was both accumulated intracellularly, and secreted. Bone marrow stromal cells represent the phenotype of a major component of the hematopoietic microenvironment of recipient bone marrow transplantation. The results also establish that ionizing irradiation induces TGF- β in *FANCD2* $^{-/-}$ mouse bone marrow stromal cells *in vitro* (Figure 1A).

Table II. Analysis of radiosensitivity by clonogenic survival curves of TGF- $\beta^{+/+}$, TGF- $\beta^{+/-}$, and TGF- $\beta^{-/-}$ bone marrow stromal cell lines.

Cell Line	Cloning efficiency (%)	Do (Gy)	n
TGF- $\beta^{+/+}$ 2A6	0.20 $\pm$ 0.01	1.95 $\pm$ 0.03	5.2 $\pm$ 1.3
TGF- $\beta^{+/-}$ 2C9	0.13 $\pm$ 0.06	1.33 $\pm$ 0.03 ($p=0.0003$)	18.0 $\pm$ 3.6
TGF- $\beta^{-/-}$ 2C7	0.05 $\pm$ 0.01	1.31 $\pm$ 0.14 ($p=0.0409$)	6.0 $\pm$ 2.9

Radiation survival curves were carried out in triplicate, as described in the methods. Full curves are shown in Figure 4. p -Values were generating by comparing Do with TGF- $\beta^{+/+}$ cell lines.

*Ionizing irradiation and chemotherapeutic drugs induce TGF- β in *FANCD2* $^{-/-}$ mouse cell lines.* We tested the effect of irradiation, L-phenylalanine mustard (L-PAM), busulfan, and fludarabine on TGF- β induction in bone marrow stromal cells *in vitro* and in IL-3-dependent hematopoietic cells. We compared TGF- β induction levels from *FANCD2* $^{-/-}$ cells with

Figure 4. Clonogenic radiation survival curves of $TGF-\beta^{-/-}$, $TGF-\beta^{+/-}$, and $TGF-\beta^{+/+}$ bone marrow stromal cell lines. Each stromal cell line from $TGF-\beta^{-/-}$, $TGF-\beta^{+/-}$, and $TGF-\beta^{+/+}$ mouse marrow was analyzed for radiation survival. Cells were irradiated to doses of 0-8 Gy and plated in tissue culture plates as published (34). Colonies of ≥ 50 cells were scored at day 7, as described in the methods (results were generated in triplicate experiments).

those from control wild-type C57BL/6J cells. The results are shown in (Figure 5, Table I). Each drug, as well as irradiation, demonstrated significant induction of TGF- β levels *in vitro* (Table I, Figure 5). While the magnitude of induction by each agent varied, there was a uniform increase in levels of TGF- β in $FANCD2^{-/-}$ compared to wild-type C57BL/6NTac mouse bone marrow cells tested *in vitro* (Figure 5, Table I).

These data establish that TGF- β was released into serum-free medium of bone marrow cultures of each stromal cell line including, both $FANCD2^{-/-}$ and wild-type bone marrow stromal cell lines, and was induced by each marrow transplant preparatory agent.

Total-body irradiation and chemotherapy drugs induce TGF- β *in vivo*. We evaluated plasma levels of TGF- β in total body irradiated or chemotherapy treated mice. As shown in Table 1 levels of TGF- β *in vivo* were significantly elevated by treatment with each bone marrow transplant preparatory regimen. The levels of TGF- β were significantly elevated by

irradiation or drug treatment in both $FANCD2^{-/-}$ and control background strain C57BL/6NTac mice, and there was no significant difference between the mouse genotypes in the levels measured at the indicated time point (Table I).

Radiation mitigator drugs suppress induction of TGF- β . The radiation mitigator drugs JP4-039 (30) and MMS350 (32) were each tested for mechanism of action *in vitro*, specifically for effects of 5-Gy irradiation on autophagy compared to apoptosis *in vitro* using 32D cl 3 cells (36). *In vitro* JP4-039 was dissolved in DMSO (26), and MMS350 (32) dissolved in water. Each drug had effects on radiation suppressed autophagy and radiation induced apoptosis (Figure 6). Irradiation suppressed autophagy *in vitro* and the suppression was corrected by each drug delivered before or after irradiation (Figure 6). JP4-039 and MMS350 each reduced levels of TGF- β that was induced *in vitro* in $FANCD2^{-/-}$ and control mouse cell lines treated with 10-Gy irradiation (Figure 5A), L-PAM (melphalan) (Figure 5B), busulfan (with the exception of the MMS350 effect on $Fancd2^{+/+}$ cells) (Figure 5C) and fludarabine (Figure 5D).

The small molecule inhibitor of TGF- β signaling (LY364947) ameliorates the TGF- β growth-suppression of fresh marrow colony forming cells. We tested the effect of a small molecule TGF- β signal transduction inhibitor (LY364947 delivered at 5 μ M) on freshly-explanted bone marrow hematopoietic cell colony formation cells. Freshly explanted marrow cells from $FANCD2^{-/-}$ or $FANCD2^{+/+}$ mice were treated with 50 ng/ml TGF- β or received 5-Gy irradiation. Subgroups received 5 μ M LY364947. As shown in Figure 7, small molecule TGF- β inhibitor LY364947 delivered alone increased colony formation by both $FANCD2^{-/-}$ and $FANCD2^{+/+}$ bone marrow cells. The drug also increased colony growth in both genotypes cell treated with TGF- β . $FANCD2^{+/+}$ marrow irradiated to 5 Gy also shows stimulation of colony formation by LY364947. The dose of 5 Gy was toxic to $FANCD2^{-/-}$ marrow such that LY364947 showed no detectable stimulation of recovery.

Discussion

Bone marrow transplantation is a standard treatment option for FA patients, as well as other patients with genetic or acquired failure of hematopoiesis (1-4). Fanconi Anemia patients now show significant improvement in survival and quality of life by addition of bone marrow transplantation to their therapeutic regimen (14-15). The success of bone marrow transplantation includes preparation of the recipient for engraftment of donor bone marrow stem cells. Donor hematopoietic stem cells home to the recipient microenvironment niche *in vivo*, where they return to quiescence, and are called upon by competing proliferative demands to display self-renewal or differentiation to multiple lymphopoietic and hematopoietic cell lineages (17).

Figure 5. Effect of JP4-039 (10 μ M) or MMS350 (100 μ M) on TGF- β levels in FANCD2^{+/+} and FANCD2^{-/-} bone marrow stromal cells. (A) 10-Gy irradiation; (B) melphalan (L-phenylalanine mustard) (50 μ M); (C) busulfan (100 μ M), or (D) fludarabine (2 μ M). Medium was harvested 24 hrs later and assayed for TGF- β using Luminex assay (triplicate experiments).

Central to successful marrow stem cell engraftment is clearing the recipient bone marrow niche for donor stem cell transplantation. Preparation of the transplant recipient has

included the use of total body irradiation and/or cytotoxic chemotherapeutic agents including: L-Phenylalanine Mustard (L-PAM or Melphalan), Busulfan, and Fludarabine (24-25).

Figure 6. Mechanism of action of JP4-039 and MMS350. Assays for autophagy (40) and apoptosis (36) were carried out with JP4-039 or MMS350 treated cells using 5 Gy irradiated or control non-irradiated 32D cl 3 cells. Cells from triplicate experiments were treated with each drug 10 min before or immediately after 5-Gy irradiation and tested in each assay. Medium was changed to serum free medium and cell cultures treated with cytotoxic agents.

Figure 7. Effect of TGF- β receptor antagonist, LY364947 (5 μ M), on TGF- β levels in 5 Gy irradiated or non-irradiated control FANCD2^{-/-} or FANCD2^{+/+} fresh explanted mouse bone marrow hematopoietic progenitor cells. FANCD2^{-/-} and FANCD2^{+/+} marrow was tested for colony formation in semisolid hematopoietic colony supportive medium as described (29).

Total body irradiation (TBI), or subtotal body irradiation is still used to facilitate engraftment (6).

The irradiated or drug treated recipient marrow niche produces negative regulators of hematopoiesis including TGF- β (16, 19-22). A hyperactive TGF- β response pathway has recently been demonstrated in both FA patients and in animal models of FA (5). The data have suggested the potential value of utilizing a TGF- β signal transduction inhibitor as a therapeutic agent to improve marrow stem cell engraftment in FA patients.

We tested the hypothesis that TGF- β was elevated in FA marrow following application of the agents used in the preparative regimen for transplant. We found that chemotherapy drugs L-PAM, busulfan, and fludarabine, and

irradiation increased levels of TGF- β both in FANCD2^{-/-} mouse bone marrow cells *in vitro* and *in vivo* in FANCD2^{-/-} mice. The results demonstrate that both FANCD2^{-/-} and background control C57BL/6NTac mouse bone marrow show increased TGF- β in response to cytotoxic agents used in the bone marrow pre-transplant regimen.

The present results also demonstrate that TGF- β is induced by each agent in whole bone marrow, and by cells of the hematopoietic microenvironment (bone marrow stromal cells) in both FANCD2^{-/-} and control mice. Previous studies have shown the hyper-responsiveness to TGF- β of hematopoietic cells from FA mice and patients with FA (5) suggesting that TGF- β elevation following the transplant preparatory regimen may suppress both surviving FA hematopoietic cells and

donor stem cells. Elevation of TGF- β by cytotoxic agents in *FANCD2*^{-/-} marrow may facilitate more efficient clearing of the recipient marrow since those cell populations are more sensitive to TGF- β . Thus, engrafting donor bone marrow stem cells might have an advantage for homing and proliferation in the TGF- β rich microenvironment due to their relative resistance to inhibition by TGF- β .

In addition to TGF- β , there is a broad range of ionizing irradiation-induced inflammatory cytokines and stress response gene products in the tissues of wild type, as well as, *FANCD2*^{-/-} mice (38-39). Inflammatory cytokines including: TNF- α , and IL-1 are induced by irradiation of the oral cavity/oropharyngeal tissue and the irradiated lung (32, 38-39). Other categories of biomarkers that are induced by irradiation include genes for proteins that bind to DNA and act as gene transcription promoters, such as NF κ B, Nrf1, AP-1, and SP-1 (32). Whether some of these other induced gene products neutralize, counteract, or synergize with elevated TGF- β in FA patients to either suppress or exacerbate the hyperactive TGF- β responses and how these other gene products relate to the specific categories of toxicity in bone marrow transplant recipients is unknown.

The present data establish that the radiation mitigator drugs, JP4-039 (27-31) and MMS350 (32-33), each modulate the induction of TGF- β by cytotoxic agents in cell lines *in vitro*. The TGF- β inhibitor, LY364947 also modulated irradiation or drug induced TGF- β *in vitro* with fresh marrow hematopoietic colony forming cells. Production of TGF- β by irradiated or cytotoxic drug treated bone marrow stromal cells from *FANCD2*^{-/-} mice demonstrates that the bone marrow in the post-transplant FA patient may continue to be a source of inhibition of hematopoietic stem cells. This phenomenon may decrease stable donor bone marrow engraftment in FA patients, and limit survival of residual recipient marrow. Continued elevation of TGF- β may contribute to late effects in post-transplant FA patients including: organ failure (9), carcinogenesis (10-12), and susceptibility to infectious agents (13). Further studies will be required to establish the need for adding to FA marrow transplant recipient's therapeutic agents, which abrogate the induction of TGF- β .

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