Inhibition of DNA Topoisomerase II Selectively Reduces the Threat of Tumorigenicity Following Induced Pluripotent Stem Cell-Based Myocardial Therapy

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The advent of induced pluripotent stem cell (iPSC) technology creates new opportunities for transplant-based therapeutic strategies. The potential for clinical translation is currently hindered by the risk of dysregulated cell growth. Pluripotent stem cells reprogrammed by three-factor (Sox2, Klf, and Oct4) and four-factor (Sox2, Klf, Oct4, and c-Myc) strategies result in the capacity for teratogenic growth from residual pluripotent progeny upon in vivo transplantation. However, these pluripotent stem cells also have a stage-specific hypersensitivity to DNA-damaging agents that may allow separation of lineage-specific therapeutic subpopulation of cells. We aimed to demonstrate the selective effect of DNA topoisomerase II inhibitor, etoposide, in eliminating pluripotent cells in the early cardiac progenitor population thus decreasing the effect of teratoma formation. Immunodeficient murine hearts were infarcted and received implantation of a therapeutic dose of cardiac progenitors derived from partially differentiated iPSCs. Etoposide-treated cell implantation reduced mass formation in the intracardiac and extracardiac chest cavity compared with the same dose of iPSC-derived cardiac progenitors in the control untreated group. In vivo bioluminescence imaging confirmed the localization and engraftment of transplanted cells in the myocardium postinjection in both groups. Comparatively, the equivalent cell population without etoposide treatment demonstrated a greater incidence and size of teratoma formation. Hence, pretreatment with genotoxic etoposide significantly lowered the threat of teratogenicity by purging the contaminating pluripotent cells, establishing an adjunctive therapy to further harness the clinical value of iPSC-derived cardiac regeneration.

Introduction

THE INDUCED PLURIPOTENT STEM CELL (iPSC) platform offers a novel basis for autologous tissue engineering and has the potential to advance transplant-based therapeutic strategies in regenerative medicine [1,2]. While experimental evidence increasingly supports reparative benefit for heart disease [3–5], iPSC-based clinical translation is currently hindered by the risk of dysregulated cell growth known as tumorigenicity [6–8]. Studies suggest that the therapeutic application of differentiated stem cells carries the potential to form teratomas, which are composed of the three embryonic germ layers, due to residual undifferentiated cells in the

transplanted population [9–11]. Pharmacological strategies to purge pluripotent stem cells offer a possible strategy to eliminate unwanted teratoma formation. Hence, the removal of residual undifferentiated stem cells from the differentiated therapeutic progenitor population has been considered a critical requirement for iPSC-based treatment to progress toward clinical applications.

Human iPSCs generated using multiple reprogramming factors (Sox2, Klf, Oct4, and c-Myc) have tumorigenic potential [12,13]. Mice from germline-competent murine iPSCs have increased tumorigenicity and mortality [14]. Notably, the standard assay that defines *bona fide* pluripotent stem cells is in vivo teratoma formation in immunocompromised

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mice, demonstrating their ideal state to produce any tissue in the body [15,16]. Thus, there exists an inherent paradox in iPSC technology such that their pluripotency represents both the source of their therapeutic advantage and their greatest clinical threat. To efficiently translate the promise of iPSC-derived therapeutic agents, strategies must be developed to reduce the threat of spontaneously occurring teratomas while enabling appropriate tissue-specific differentiation.

The sensitivity of stem cells to different genotoxic drugs is increasingly explored for pharmacological purging of teratoma-forming cells. One such genotoxic drug widely used in clinical practice is etoposide (also known as VP16), which is an inhibitor of DNA topoisomerase II activity. Studies have demonstrated the high sensitivity of human embryonic stem cells [11] and iPSCs to topoisomerase II toxins [17,18]. DNA-damage-induced apoptotic sensitivity of a panel of embryonic stem cell lines has been previously defined [18]. Genomic instability of pluripotent stem cells and their presence in the therapeutically delivered product contributes to unregulated growth [19,20]. The risk for teratoma formation decreases as stem cells undergo differentiation and lose their pluripotency [21]. Hence, to reduce teratoma formation, stem-cell-based therapies could utilize predifferentiated progenitor cells. Yet, the early lineage-specific progenitor population is heterogeneous and could retain undifferentiated pluripotent stem cells. We hypothesized that etoposide treatment of the mixed predifferentiated progenitor population at the administered dose and duration will purge the pluripotent stem cells, thereby increasing the homogeneity of the progenitor population and reducing teratoma formation. The present study is the first to apply this understanding in an acute myocardial infarction model for safe cardiac regeneration due to the purging of residual teratoma-forming progenitors. To address the safety issue of tumorigenicity, our study aimed to demonstrate the effect of *in vitro* etoposide treatment prior to *in vivo* delivery to eliminate the pluripotent cells in the early cardiac progenitor population. This strategy provides the foundation for bioengineering safe cell-based therapies for heart disease.

Materials and Methods

Ethical considerations

All protocols were implemented under the National Institutes of Health guidelines with approval obtained from the Institutional Animal Care and Use Committee, and the Biosafety Committee at Mayo Clinic. All procedures on living animals were conducted under general inhalation anesthesia. All animals were sacrificed in accordance with the recommendations of the American Veterinary Medical Association and the Institutional Animal Care and Use Committee.

Stem cell culture

iPSC-4F cell clones were created by transduction with the lentiviral reprogramming vectors expressing Oct4, Sox2, c-Myc, and Klf4 into mouse embryonic fibroblasts (viPS Vector Kit; Open Biosystems) followed by isolation and expansion of individual clones [1,18]. All pluripotent lines were maintained in EmbryoMax Dulbecco's modified Eagle's medium (Millipore, www.millipore.com) containing penicillin

G, streptomycin, L-glutamine (Invitrogen), sodium pyruvate (Lonza, www.lonza.com), nonessential amino acids (Mediatech, www.cellgro.com), 2-mercaptoethanol (Sigma-Aldrich), 10% embryonic-stem-cell-qualified fetal calf serum (FCS; Invitrogen), and leukemia inhibitor factor (LIF; Millipore). Pluripotent authenticity and multilineage profile were validated in individual iPSC clones by established *in vitro* [spontaneous embryoid body (EB) differentiation and stemness marker expression] and *in vivo* (teratoma formation) assays [2,22].

In vitro cardiac differentiation

Murine iPSCs were differentiated into three-layer EBs using the hanging-drop method. Cells were harvested and resuspended in differentiation medium (20% FCS without LIF) to a concentration of 8×10^4 cells per mL. To allow for EB formation, hanging drops were created by depositing 25 μ L of the cell suspension on the lids of 500-cm² square culture plates and incubated for 48 h. To induce spontaneous differentiation, EBs were flushed and transferred to floating suspension for another 48 h. Cells were then transferred to gelatin-coated plate (0.1% EmbryoMax Gelatin; Millipore) and allowed to adhere and further differentiate until day 7. Following differentiation, cells were cultured in Dulbecco's modified Eagle's medium containing penicillin G, streptomycin, L-glutamine, sodium pyruvate, nonessential amino acids, and 20% FCS [18].

Etoposide treatment

For pharmacological treatment, etoposide was purchased from Sigma-Aldrich (www.sigmaaldrich.com). Early cardiac progenitors, day-7 EBs, were treated with 0.01 μ M etoposide in differentiation medium for 24 h. The etoposide concentration was determined based on the IC₅₀ (half-maximal inhibitory concentration) data from earlier studies in mouse iPSC cell lines [18]. Prior to transplantation, the cells were washed once with drug-free media and further incubated in drug-free media for 2 h.

Targeted cell delivery into infarcted regions

Under 1%–2% isoflurane anesthesia, permanent ligation of the left anterior descending coronary artery was performed on athymic nude mice (Harlan Laboratories) as described previously [6,23]. Pain prophylaxis was carried out by an acetaminophen regimen (100–300 mg·kg⁻¹ in drinking water) 2 days prior to and 5 days after surgery. Postligation, infarcted mice ($n=10$ athymic nude) were randomized into etoposide-treated ($n=5$ athymic nude) and untreated ($n=5$ athymic nude) cohorts. Prior to cell transplantation, the number of total viable cells was determined using trypan blue and a Countess Automated Cell Counter (Invitrogen). iPSC-derived day-7 progenitor cell population (2×10^5 cells per heart in 15- μ L propagation media) was delivered into mapped peri-infarcted anterior walls of the left ventricle by epicardial injection (4×10^4 cells per site $\times$ 5 sites per heart) within 30 min following coronary ligation as previously established [1,3]. Safety evaluation included daily observation, check of vital signs, electrocardiography, and cell tracking. Systemic histological evaluation upon autopsy was performed.

treatment as shown in the schematic representation (Fig. 1C). iPSC-based predifferentiation into the cardiac progenitor population facilitates the loss of tumorigenicity associated with lineage specification [26]. Our model shows that mouse iPSCs were differentiated into early cardiac progenitors, day-7 EBs, using the hanging-drop method for cardiac differentiation and treated with and without the established concentration of etoposide for 24 h [18]. We then injected 2×10^5 treated ($n=5$) and untreated ($n=5$) day-8 cells into infarcted athymic mouse hearts (total $n=10$) to evaluate the modulatory effect of etoposide treatment.

Etoposide treatment decreases mass formation in the infarcted murine heart

Prior to cellular transplantation, the viability of the cells with and without the pharmacologic agent was determined. The average percent of live cells at the time of injection was $65.67\% \pm 5.51\%$ (mean $\pm$ std. dev.) for etoposide-treated cells and $80.67\% \pm 4.04\%$ for untreated cells (Fig. 2A), indicating that etoposide induces moderate cellular purging from the starting population. Cell counts were normalized to the control group prior to transplantation, rejecting the potential attribution to a decreased number of delivered cells and ensuring equal number of viable cells being delivered to the intramyocardium. Assessment of the mass formation in the intracardiac and extracardiac cavity, including the chest wall region, revealed reduced growth with etoposide treatment (Fig. 2B). Immunodeficient animals receiving cells treated with etoposide showed 20% reduction in intracardiac and extracardiac mass formation. In contrast, all the animals in the 4F-iPSC and 3F-iPSC groups without etoposide treatment developed intracardiac growth with varying degrees of extracardiac mass in the chest wall region (data not shown).

Hence, in the 4-week postinjection period, we observed an increase in mass formation, either in the myocardium or in the chest wall, in the 4F-iPSC, 3F-iPSC, and untreated implantation groups, compared with the etoposide-treated group.

Among immunodeficient animals with intra- and extracardiac growth in the treated and untreated cohorts, their relative mass diameter significantly varied depending on etoposide treatment (Fig. 2C). In vivo echocardiography of the murine hearts suggested that the mean mass diameter for the control implantation was 9.86 ± 3.13 mm (mean $\pm$ std. dev.). Comparatively, the mean mass diameter for the etoposide-treated implantation was 2.02 ± 2.09 mm, indicating a 4.88-fold reduction in mass growth with the pharmacologic treatment. The total gross mass effect area (length $\times$ width) measured on echocardiography was 63.23 mm² for the control untreated cohort and 9.05 mm² for the etoposide-treated cohort, demonstrating a sevenfold decrease in the total intracardiac mass area (Fig. 2D). Further, the presence and greater size of the intracardiac growth produced by the untreated cellular transplantation resulted in a mass effect and physiological decompression of the murine heart (Fig. 2E). Etoposide-treated transplantation did not demonstrate physiological decompression in the mice as examined by echocardiography during the 4-week follow-up period in this study. Since immunodeficient animals demonstrated deteriorated cardiac function due to tumor development, functional evaluation could not be accurately reported and was not included in the study analysis.

Luciferase-labeled cells localize and engraft in the host heart

In vivo bioluminescence imaging (BLI) [27] measured the luciferase-labeled iPSCs and cardiac progenitors in the

FIG. 2. Etoposide treatment decreased mass formation in infarcted heart. Viability of iPSC and cardiac progenitor culture at day 8, with and without etoposide treatment (A). Implantation of 4F iPSCs postearly cardiac differentiation demonstrates uncontrolled growth in the intra-/extracardiac regions in both treated and untreated groups (assessed from autopsy, B); yet, mass size significantly varies with and without etoposide treatment as measured by diameter (C) and total area (D), which leads to the mass effect and physiological decompression of the LV in the untreated group (E, in vivo echocardiography). M, mass.

FIG. 3. Cellular engraftment in host heart. In vivo bioluminescence imaging (BLI) [27] shows transplanted iPSCs and cardiac progenitors into the infarcted myocardium following a 4-week observation period. Initially, 2×10^5 cells were injected into the mice. The BLI signals in the heart indicate the presence of implanted iPSCs at day 8 of cardiac differentiation, without (A) and with (B) etoposide, validating engraftment in both treated and untreated groups.

infarcted myocardium at 4 weeks postimplantation. Initially, 2×10^5 cells were injected into the surrounding area of infarction in athymic mice. The BLI signals in the heart of the immunodeficient mouse model indicate the presence of implanted iPSCs at day 8 of cardiac differentiation, without (Fig. 3A) and with (Fig. 3B) etoposide, validating the engraftment in both treated and untreated groups.

The mean total area of bioluminescence was 183.72 pixels without treatment with a minimum 61.92 pixels and maximum 329.18 pixels in the animals compared with a mean total area of 74.73 pixels with treatment with a minimum 42.12 pixels and maximum 130.08 pixels, suggesting a 2.45-fold reduced presence of cells with etoposide treatment. Although cell counts were normalized, the variation in bioluminescence between the two groups can be attributed to the increased rate of proliferation in the untreated cohort

compared with the etoposide-treated group. This data concurs with the reduced viability or number of live cells following etoposide treatment as it induces cellular purging of dividing cells in the mixed starting population. Despite the variation in the average area of localized cells captured by bioluminescence, unregulated mass formation occurred in both groups with a sevenfold decrease in total mass growth area with etoposide treatment.

Etoposide pretreatment reduces the teratoma formation from iPSC-based cardiac cell therapy

To functionally assess the efficacy of etoposide treatment and to determine the pathology of the mass growth that developed upon transplantation of the heterogeneous cell population, we characterized subsequent tumor or malignant

TABLE 1. SUMMARY OF RESULTS FROM ETOPOSIDE UNTREATED (–) AND ETOPOSIDE-TREATED (+) CELL TRANSPLANTATION

	(–) Etoposide					(+) Etoposide				
Transplanted animal	1	2	3	4	5	6	7	8	9	10
In vivo bioluminescence imaging										
Signal strength (0–3)	3	2	2	3	3	1	1	1	2	1
Perimeter (pixel)	310.89	61.94	103.60	329.18	113.01	91.01	42.14	47.80	130.08	62.63
Area (pixel ²)	6533	166	682	6056	777	414	120	179	1065	199
Mean area ± std. dev. (pixel ²)	2842.80 ± 3164.02					395.40 ± 390.50				
Echocardiography										
Mass length (mm)	12.6	7.2	5.8	12.2	11.5	6.5	2.1	3.1	4.3	4.9
Mass width (mm)	7.9	2.3	3.6	7.9	7.2	2.0	1.0	1.1	3.7	2.2
Mass effect area (mm ²)	99.54	16.56	20.88	96.38	82.80	13.05	2.10	3.41	15.91	10.78
Mean mass effect ± std. dev. (mm ²)	63.23 ± 41.15					9.05 ± 6.04				
Autopsy										
Intracardiac mass (+/–)	+	+	+	+	–	–	+	+	+	–
Extracardiac/chest wall mass (+/–)	+	–	–	+	+	+	–	–	–	+
Inflammatory growth (+/–)	–	+	–	–	–	+	–	–	+	+
Teratoma (+/–)	+	–	+	+	+	–	–	–	–	–

transformation. Herein, we defined the mass growth spectrum as (1) teratogenic with benign characteristics of endoderm, mesoderm, and ectoderm; (2) noninvasive inflammatory growth; or (3) no growth (Table 1). Histological assessment of untreated iPSCs postdifferentiation toward the mesoderm lineage showed intracardiac teratoma formation with a total area of 112.1 mm² (Fig. 4A, B). In the control group without etoposide treatment, gross autopsy indicated two teratomas with corresponding total area of 39.88 and 73.96 mm² in the intracardiac region (Fig. 4C). Under this same condition, teratogenic growth was absent in intracardiac areas (Fig. 4D, E) but extensively present in extracardiac regions, measuring a total area of 94 mm² (Fig. 4F).

Comparatively, etoposide-treated iPSC implantation following cardiac predifferentiation demonstrated intracardiac regions free of teratoma formation as shown by H&E staining and autopsy (Fig. 4G, H, autopsy I). One animal developed a localized intracardiac region of noninvasive inflammatory

growth with a total area of 10.25 mm² (Fig. 4J, K, autopsy L). This indicates a 10-fold reduction in the total intracardiac growth area with etoposide treatment and an elimination of teratogenic growth as determined by histological measurement. All immunodeficient animals ($n=10$) developed mass growth either in the extracardiac, intracardiac, or both regions. However, the animals receiving cell transplantation postetoposide treatment had lower incidence and size of mass growth without teratoma formation, establishing a significant safety benefit from pharmacologic preconditioning with a single in vitro dose of 0.01 μ M with a 24-h exposure to etoposide.

Discussion

Pluripotent stem cells have the innate capability for self-renewal and unique capacity to differentiate into all somatic lineages, providing an ideal source for patient-specific

FIG. 4. Safety benefit of etoposide pretreatment in reducing teratoma formation from iPSC-based cardiac cell therapy. Histological assessment of untreated iPSCs postdifferentiation toward the mesoderm lineage shows intracardiac teratoma formation (A, 4 $\times$; B, 20 $\times$); also depicted in autopsy (C). Under this condition, uncontrolled growth was absent in some intracardiac areas (D, 4 $\times$) but was extensively present in extracardiac regions (E, autopsy F, 20 $\times$). Etoposide-treated iPSC implantation postearly cardiac differentiation demonstrates intracardiac regions free of teratoma formation as shown by hematoxylin and eosin staining and autopsy (G, 4 $\times$; H, I, 20 $\times$). One animal developed a minor, localized intracardiac region of inflammatory growth (J, 4 $\times$; K, autopsy L, 20 $\times$). I, inflammation; S, suture; T, teratoma.

cell-based therapy that circumvents the ethical issues and immune rejection associated with human embryonic stem cell application [26,28,29]. Yet, the property of tumorigenicity remains a main concern for safe hiPSC transplantation [30]. This study validated that three-factor- and four-factor derived iPSC transplantation yields teratogenic growth. One method for eliminating the problem of teratoma formation is predifferentiating iPSCs into the lineage of choice. Here, we differentiated murine iPSC lines to an early cardiac progenitor stage, day-7 EBs, limiting the starting population of undifferentiated stem cells. Recent developments in iPSC-derived cardiac differentiation protocols have reported higher efficiency and purity using Activin/Nodal and BMP signaling as well as cardiomyocyte inducers including ascorbic acid [31,32]. Our strategies to limit teratoma formation could have been augmented with updated differentiation protocols that yield a greater percentage of cardiac-lineage specification. Previous studies have shown that while intramyocardial transplantation of undifferentiated rat iPSCs resulted in tumorigenesis in the heart [33], terminally differentiated iPSC-derived human cardiomyocytes have significantly improved cardiac function after ischemic cardiomyopathy [33,34]. Additionally, embryonic stem cells have been differentiated to cardiomyocytes and transplanted to form stable intracardiac grafts [35], providing evidence for similar cardiac repair with iPSC-based therapy. Eradicating this intrinsic tumorigenicity by differentiation prior to transplantation contributes to our efforts in minimizing the hazards of iPSC-based therapeutics [8]. Yet, this predifferentiated population is heterogeneous, potentially harboring residual pluripotent stem cells with the ability to form teratomas [18]. Here we coupled the predifferentiation step with pharmacological purging to further advance and optimize the safety of patient-specific cell replacement therapy.

Pharmacological agents provide a viable strategy to selectively target and purge the teratoma-forming cells prior to clinical utilization. Studies have used iPSC-specific antiapoptotic factors, such as survivin or Bcl10, with small-molecule inhibitors to eliminate pluripotent cells with teratoma potential [36]. Recent studies have investigated the inhibition of stearoyl-coA desaturase 1 (SCD1), which induces endoplasmic reticulum (ER) stress and generates reactive oxygen species, as a means to selectively eliminate undifferentiated iPSC derivatives [37]. However, the exact mechanism of SCD1 inhibitor remains unknown. Previous reports have also proposed the targeting of undifferentiated cells using lentivirus-mediated thymidine kinase expression, which converts the prodrug ganciclovir into a toxic metabolite, leading to reduced teratoma formation [38]. Similarly, various approaches using cytotoxic antibodies against undifferentiated pluripotent stem cells have been attempted [39,40]. However, these methods often create concerns with additional steps required for clinical application. Therefore, a targeted pharmacologic strategy in conjunction with stem cell predifferentiation could provide a more feasible method to overcome the challenging safety issue associated with stem-cell-based technologies.

This study exploited the sensitivity of pluripotent stem cells to genotoxic drugs, such as etoposide, an inhibitor of DNA topoisomerase II activity. To address the concern of etoposide-induced genotoxic effects or permanent damage to the genome of tissue-specific progeny, previous studies analyzed untreated and purged-surviving cells to differen-

tiate into a cardiac lineage and demonstrated that both populations equally yield cardiomyocytes without causing genomic instability or loss of daughter cell differentiation potential [18]. We proposed that etoposide treatment of the mixed progenitor population will purge the pluripotent stem cells, thereby increasing its genomic stability and reducing teratoma formation. In this study, we found that the total percentage, area, and location of mass formation were significantly reduced and confined by the pharmacological treatment of etoposide at 0.01 μ M concentration for 24 h as shown by echocardiography and gross autopsy. In accordance, proliferating luciferase-labeled cells in the host murine heart showed a significant decrease in total growth area with etoposide treatment. Although all immunodeficient animals receiving predifferentiated iPSC-derived cardiac cells formed visible mass growth despite normalization of delivered viable cells in this protocol, etoposide-treated implantation produced small noninvasive inflammatory growth compared with the teratogenic growth formed without treatment, indicating that the tumorigenicity of iPSC-derived cells was significantly decreased by the addition of pharmacological pretreatment. Here the spectrum of teratoma development can be classified as no growth to non-invasive inflammatory growth to benign teratogenic growth, suggesting that etoposide treatment curbed the progression of teratoma development. It remains unclear whether the mechanism of modulating baseline toxicity is mediated by reduced pluripotent stem cell participation as a result of initial etoposide purging or by paracrine effects that enhance the ability of native cardiomyocytes to resist unchecked cell growth. The involvement of paracrine mechanisms deserves further investigation. Further, prolonged exposure and/or in vivo dosing with genotoxic drugs may provide added value beyond the transient treatment. Future studies can combine pharmacological purging assays with the starting population of nonvirally derived 3F-iPSC cell clones and optimized differentiation protocols to further reduce the threat of teratoma formation.

It remains to be elucidated whether intra- versus extracardiac mass formation is a measurement of aggressiveness for tumorigenicity. Previous studies have reported variable results about the iPSC-associated tumorigenicity [9,41,42]. We postulated that the transplantation site (beating myocardium vs. subcutaneous regions) could be one factor contributing to this controversy and to the variability observed in intra- versus extracardiac teratoma formation. Inflammatory growth occurring despite etoposide treatment suggests a meaningful yet partial avoidance of dysregulated growth and further implies the need for ongoing pharmacological treatment in the animal throughout engraftment process. Hence, this study recognizes the genotoxic drug, etoposide, as a potential adjunctive therapy to enhance the safety of iPSC-based clinical therapeutics.

Conclusions

While experimental evidence increasingly supports the potential of patient-specific stem-cell-based therapies and its reparative benefit for heart disease, clinical translation is currently hindered by the risk of dysregulated cell growth known as tumorigenicity. Ensuring patient safety prior to utilizing the promise of such transplant-based therapeutics

remains the highest priority. Collectively, our study demonstrates that pretreatment with etoposide significantly reduces the threat of teratogenicity from iPSC-based therapy in the setting of acute myocardial infarction. This strategy establishes a feasible teratoma-purging assay based on natural stem cell sensitivity to genotoxic agents, providing the foundation to further engineer safe cell-based therapies for heart disease.

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Author Disclosure Statement

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