

# EFFECT IN SUPRALETHALLY IRRADIATED RATS OF GRANULOCYTE COLONY-STIMULATING FACTOR AND LISOFYLLINE ON HEMATOPOIETIC RECONSTITUTION BY SYNGENEIC BONE MARROW OR WHOLE ORGAN PASSENGER LEUKOCYTES<sup>1</sup>

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We have previously shown the existence of migratory hematopoietic stem cells in adult solid organs. This study demonstrates that granulocyte colony-stimulating factor (G-CSF) and lisofylline, a phosphatidic acid inhibitor that suppresses hematopoiesis-inhibiting cytokines, can enhance the engraftment of organ-based hematopoietic stem cells. When syngeneic heart grafts or liver nonparenchymal cells were transplanted into lethally irradiated (9.5 Gy) Lewis rats, complete hematopoietic reconstitution and animal survival were significantly improved by treating the recipient with G-CSF or, to a lesser extent, with lisofylline. Pretreatment of hepatic nonparenchymal cell donors with G-CSF, but not lisofylline, also resulted in striking improvement of recipient survival which was associated with an augmented subpopulation of donor stem cells. The results suggest that these drugs can be used to enhance the chimerism that we postulate to be the basis of organ allograft acceptance.

Recent evidence (summarized in [1]) suggests that passenger leukocytes migrate after organ transplantation and produce persistent multilineage chimerism, which is essential for sustained survival of the allografts. Because the reciprocal immune modulation of the coexisting cell populations cancels the risk of graft-versus-host disease in noncytoablated recipients (the two-way paradigm [1]), the donor contribution to the resulting donor-recipient dialogue can be bolstered safely by the perioperative administration of unaltered donor bone marrow (2).

As an alternative to donor leukocyte infusion, or to increase its efficiency, we have suggested that the organ recipient's chimerism might be promoted by posttransplant administration of granulocyte colony-stimulating factor (G-CSF\*), granulocyte-macrophage colony-stimulating factor (GM-CSF), and other hematolymphopoietic growth factors (1, 3) that are already known from clinical experience to facilitate bone marrow engraftment in cytoablated patients (4, 5). This possibility is supported by the results reported herein, comparing the "rescue" efficiency of lisofylline and G-CSF in the same isogenic rat model of supralethal irradiation previously used to demonstrate the presence of pluripotent stem cells in normal rat livers and hearts (6).

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Abbreviations: CFU, colony-forming unit; FBS, fetal bovine serum; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; NPC, nonparenchymal cells.

Lisofylline (a gift from Cell Therapeutics, Inc., Seattle, WA) is a phosphatidic acid inhibitor (7) that has been postulated to facilitate bone marrow engraftment by suppressing hematopoiesis-inhibiting cytokines (e.g., tumor necrosis factor- $\alpha$ , transforming growth factor- $\beta$ , macrophage inhibitory protein 1- $\alpha$ , and platelet factor 4) that are typically released to activation stimuli in the posttransplant period, while not altering levels or activities of the myeloid progenitor cell-promoting cytokines G-CSF and GM-CSF (8).

G-CSF is made principally by endothelial cells, monocytes, and fibroblasts. Although its main biologic consequences have been described as increased proliferation and differentiation of neutrophils from committed progenitor cells (9), it actually has these effects on stem cells and all derivative lineages (10-12). For our experiments, rhG-CSF (Neupogen) was purchased from Amgen (Thousand Oaks, CA).

Eight- to ten-week-old Lewis (RT1<sup>l</sup>, LEW) rats weighing 200-250 g were purchased from Harlan Sprague Dawley (Indianapolis, IN) and kept in a laminar flow, specific-pathogen-free environment. Most of the experiments were performed with male donors and recipients. However, by performing male  $\rightarrow$  female transplantation in separate nonsurvival experiments, we determined (as in our earlier reports [6]) whether the hematopoietic reconstitution was with donor cells as opposed to recovery of the cytoablated recipient stem cells. The tissues and organs of the hematopoietically reconstituted recipient were studied with polymerase chain reaction and Southern hybridization, using rat Y-chromosome (sex-determining region Y)-specific primers (6).

The transplants used for testing in the irradiated recipients were bone marrow cells, nonparenchymal cells (NPC) isolated from donor livers, and intact hearts. Bone marrow cells with >95% viability (trypan blue) were harvested from the tibias and femurs and processed in RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, and 50  $\mu$ g/ml streptomycin (6). Whole hearts were removed after exsanguination of the normal or pretreated donor under methoxyflurane anesthesia, flushed clean with intra-arterial chilled lactated Ringer's solution, and transplanted heterotopically to the abdominal location (6). Hepatic NPC were purified from donor livers with the collagenase method (13).

The livers were flushed in situ to an asanguinous state through a portal vein cannula with 120 ml of Leibovitz's L-15 medium containing 5 mM EGTA, 10 mM HEPES, and 50  $\mu$ g/ml gentamicin. An additional infusion was then given of 300 ml of L-15 medium enriched with 0.05% collagenase (type B, Boehringer Mannheim, Mannheim, Germany).

0.005% trypsin inhibitor (Sigma, St. Louis, MO), 2.5% fetal bovine serum (FBS), and 50  $\mu\text{g}/\text{ml}$  gentamicin. The liver was removed to a petri dish, and parenchymal cells and NPC were liberated from the connective tissue by careful raking of the liver with scissors and by gentle shaking. The initial cell suspension was filtered through nylon mesh, and a crude fraction of NPC was obtained by differential centrifugation (four times at  $60\times g$  for 3 min).

The number of migratory passenger leukocytes transplanted in the whole hearts was not known. The dose of  $0.5\times 10^6$  bone marrow cells was previously shown to be inadequate for reconstitution. All of the hepatic NPC from single livers were given to individual recipients, with no attempt to compensate for the cell loss incurred during preparation. The yield from the 18 livers used in experimental groups 12–14 was variable, with a mean  $\pm$ SD of  $150.6\pm 57.4$  cells (Table 1) with  $>85\%$  viability (trypan blue). The number of NPC was not significantly different than that in the livers from donors pretreated with lisofylline and G-CSF for use in groups 17 and 18, respectively. The bone marrow and NPC suspensions were injected into the penile vein of the irradiated recipients.

All 18 experimental groups were given 9.5 Gy of total body irradiation from a cesium source (Table 2). No treatment was given to group 1. In group 2, twice daily intraperitoneal lisofylline was given for 9 days (day  $-1$  to day 7), and in group 3, rhG-CSF was injected subcutaneously twice daily (Table 2) at doses previously standardized by Foster et al. (14). Four to eight hours after irradiation, groups 4–14 were given heterotopic hearts or infusions of a cell suspension (bone marrow or NPC), with or without one or the other test drugs (Table 2). In groups 15–18 (Table 2), the effect of pretreating donors for 3 days (day  $-3$  to day  $-1$ ) with either lisofylline or G-CSF was determined; no posttransplant therapy was given (Table 2).

The principal end point in all 18 groups was survival of the irradiated animals. Because no animal died after 40 days in this or in our earlier study (6), survival for this long was considered permanent. As in our earlier study (6), the 9.5-Gy irradiation caused 100% mortality (group 1, Table 2). This was not affected by treatment with lisofylline (group 2). However, half of the animals treated only with G-CSF recovered (group 3). Because they did not receive any kind of transplant, their full reconstitution (see Table 3) obviously was from their own residual stem cells.

G-CSF strikingly potentiated the rescue effect of heart transplantation, allowing 83.3% reconstitution and survival (group 8) compared with only occasional survival (14.3%) with heart transplantation alone (group 4). Similarly, survival after heart transplantation was increased to 62.5% and 75% with the administration of 200 and 150 mg/kg/day lisofylline (groups 5 and 6), respectively, but not with doses of 100 mg/kg/day (group 7). Two additional female animals were studied 30 days after rescue with a male heart plus

lisofylline (as in group 6). Only about 1% of the DNA in spleen was of male origin, indicating ultimate recovery of cytoablated recipient stem cells.

Both test drugs also increased survival when given to the irradiated recipient after transplantation of a suboptimal dose of bone marrow. Survival was increased from 0 (group 9) to 16.7% with lisofylline (group 10) and to 80% with G-CSF (group 11).

Similar results were obtained when the test agents were combined with transplantation of hepatic NPC: from 0% using NPC only (group 12) to 50% with the addition of lisofylline (group 13) and to 66.7% with G-CSF (group 14). In one special male  $\rightarrow$  female NPC experiment under the lisofylline treatment conditions of group 13, approximately 1% of the splenic DNA was male at 100 days. This indicated self-reconstitution, in contrast to our previous finding after transplantation of the whole liver without drug treatment, following which fully reconstitution was primarily by donor stem cells (6).

The superior therapeutic qualities of G-CSF versus lisofylline were evident in the lineage characteristics in peripheral blood 45–55 days after rescue with liver NPC (Table 3). Monoclonal antibodies were used against R7.3 ( $\alpha\beta$ -T cell receptor), W3/25 (CD4), OX8 (CD8), OX33 (B cells), 3.2.3 (NKR-P1), and ED1 (macrophages) (Sera-Lab, Crawley Down, UK). Animals given G-CSF plus NPC (group 14) had a profile similar to that in normal LEW rats and rats that self-reconstituted with the aid of G-CSF alone (group 3). In contrast, one survivor with lisofylline plus NPC (group 13) had evidence of delayed or incomplete hematolymphopoietic reconstitution.

The inability of NPC isolated from single livers to rescue any animal (group 12, Table 2) unless either lisofylline or G-CSF was added may have been due simply to loss of, or damage to, the donor leukocytes during their purification. This was suggested by the ability to restore the rescue capability of the hepatic NPC to 100% survival by donor pretreatment with G-CSF (Table 2, group 18) but not with lisofylline (group 17), a difference that also was present but not statistically significant in hearts procured from similarly pretreated donors (Table 2, group 15 vs. group 16). Because the number of hepatic NPC was not dramatically altered by donor pretreatment, the relative effect of lisofylline and G-CSF on hematopoietic precursor cells in the liver was estimated by determining colony-forming unit(s) (CFU), using the fibrin clot culture system with modifications (Table 4).

The crude extract of liver NPC was passed through a nylon wool column at  $37^\circ\text{C}$ ; nonadherent cells were left for the assay. These cells were resuspended in complete Iscove's modified Dulbecco's medium supplemented with 10 mM HEPES, 50  $\mu\text{g}/\text{ml}$  gentamicin, 8 mM L-glutamine, and  $5\times 10^{-5}$  M 2-mercaptoethanol. They were further mixed with 2% pokeweed mitogen (Sigma)-stimulated, LEW splenocyte-conditioned medium, 20% FBS, 1% bovine serum albumin, 1.0 mg/ml bovine fibrinogen solution (Sigma), 1.0 U/ml bovine thrombin solution (Sigma), and 0.5 mM  $\text{N}^G$ -monomethyl-L-arginine-HOAC (Cyclo,ps Biochemical Co., Salt Lake City, UT) in a final concentration of  $1\times 10^6/\text{ml}$ .

A total suspension volume of 1.0 ml was plated in the middle of a 60-mm Parmanox dish (Nunc, Naperville, IL), and 1 ml of complete Iscove's modified Dulbecco's medium with 20% FBS and 1% bovine serum albumin was added around the clot. The cultures were kept for 6 days in a fully

TABLE 1. Total number of NPC in normal and in lisofylline- and G-CSF-treated livers

Donor (groups)	n	Total NPC/liver ( $\times 10^6$ )
Untreated (12, 13, 14)	18	$150.6 \pm 57.4$
Lisofylline pre-treated (17)	7	$104.4 \pm 19.3$
G-CSF pre-treated (18)	7	$210.6 \pm 36.7$

TABLE 2. Improved survival of lethally irradiated (9.5 Gy) animals of syngeneic heart graft, liver NPC, or bone marrow infusion with lisofylline and G-CSF treatment<sup>a</sup>

Group	Organ/cells	Treatment	n	Survival (days)	Median (days)	40-Day survival rate (%)
Recipient treatment						
1	None	None	10	10, 10, 11, 11, 12, 12, 13, 13, 13, 13	12.0	0
2	None	Lisofylline	7	11, 11, 12, 13, 13, 15, 16	13.0	0
3	None	G-CSF	6	9, 9, 9, >100 × 3	>54.5	50
4	Heart	None	7	12, 12, 13, 14, 17, 25, >100	14.0	14.3
5	Heart	Lisofylline	8	12, 18, 30, >100 × 5	>100	62.5
6	Heart	Lisofylline (75)	8	12, 18, >100 × 6	>100	75.0
7	Heart	Lisofylline (50)	7	12, 13, 13, 13, 17, 17, >100	13.0	14.3
8	Heart	G-CSF	6	13, >100 × 5	>100	83.3
9	BM	None	5	10, 10, 10, 12, 14	10.0	0
10	BM	Lisofylline	6	12, 13, 13, 14, 38, >100	13.5	16.7
11	BM	G-CSF	5	16, >100 × 4	>100	80.0
12	Liver NPC	None	6	10, 13, 13, 13, 15, 15	13.0	0
13	Liver NPC	Lisofylline	6	15, 17, 19, >100 × 3	>59.5	50
14	Liver NPC	G-CSF	6	13, 18, >100 × 4	>100	66.7
Donor treatment						
15	Heart	Lisofylline	6	10, 11, 11, 13, 13, 18	12.0	0
16	Heart	G-CSF	6	12, 15, 17, 17, >100 × 2	17.0	33.3
17	Liver NPC	Lisofylline	7	11, 12, 12, 12, 12, 12, 13	12.0	0
18	Liver NPC	G-CSF	7	>100 × 7	>100	100

<sup>a</sup> Recipient doses were 100 mg/kg lisofylline intraperitoneally every 12 hr on days -1 to 7 except in group 6 (75 mg/kg) and group 7 (50 mg/kg), and 100 µg/kg rhG-CSF subcutaneously every 12 hr on days -1 to 7. Donor pretreatment was 100 mg/kg lisofylline intraperitoneally every 12 hr on days -3 to -1, and 100 µg/kg rhG-CSF subcutaneously every 12 hr on days -3 to -1. Group 3 versus groups 8, 11, or 14: no significant difference (Mann-Whitney *U* test).

TABLE 3. Hematopoietic recovery in lethally irradiated (9.5 Gy) animals of syngeneic liver NPC infusion with lisofylline and G-CSF treatment (45-55 days after radiation and transplantation)

Group	Organ/cells	Treatment <sup>a</sup>	n	αβ-TCR+ (R7.3) (%)	CD4 (W3/25) (%)	CD8 (OX8) (%)	B cells (OX33) (%)	NK (3.2.3) (%)	Monocytes/macrophage (ED1) (%)
Recipient treatment									
3	Normal LEW	G-CSF	5	71.7 ± 8.3	62.7 ± 4.0	25.1 ± 3.5	14.0 ± 4.9	3.4 ± 0.6	10.4 ± 4.2
	None		2	78.2	65.6	30.0	16.5	4.9	16.5
13	Liver NPC	Lisofylline	1	66.8	69.8	27.4	14.6	3.1	11.7
14	Liver NPC	G-CSF	1	46.5	80.2	12.8	1.3	4.6	NT
			2	65.0	63.2	29.2	18.8	2.1	17.6
				68.3	64.2	28.0	14.8	2.5	12.7
Donor treatment									
18	Liver NPC	G-CSF	2	69.4	67.5	21.9	4.5	3.3	NT
				71.9	67.2	20.0	10.8	3.2	NT

<sup>a</sup> Recipient doses were 100 mg/kg lisofylline intraperitoneally every 12 hr on days -1 to 7, and 100 µg/kg rhG-CSF subcutaneously every 12 hr on days -1 to 7. Donor pretreatment was 100 µg/kg rhG-CSF subcutaneously every 12 hr on days -3 to -1.

humidified atmosphere with 5% CO<sub>2</sub> in air at 37°C. Clusters (10-50 cells/aggregate) and colonies (>50 cells/aggregate) were quantitated as CFU-C counts. The striking augmentation of CFU by G-CSF and the surprising absence of a lisofylline effect are evident in Table 4.

It might be argued that the foregoing differences in the reconstitution by transplantation of the whole liver versus its NPC could be due to removal of a collaborative engraftment function of the hepatocytes or parenchymal cells of other organs in the second instance, e.g., lymphopoietic growth factors known to be secreted by hepatocytes, as discussed by Murase et al. (3). Our study does not exclude this possibility.

The administration of G-CSF to the hepatic NPC recipient (group 14, Table 2) and especially to the donor (group 18, Table 2) allowed survival of irradiated animals at the same rate as reported previously with the whole liver (6). Reconstitution with a suboptimal supply of donor pluripotent cells apparently was predominantly by recovery of the recipient stem cells, which was made possible by the "bridge" variably provided by transplanted stem cells.

If, as with bone marrow transplantation, the quantity of stem cell engraftment is critical to successful transplantation of organs, the findings reported herein suggest that recipient treatment with recombinant G-CSF, GM-CSF, c-kit ligand,

TABLE 4. Frequency of CFU in liver NPC obtained from normal and lisofylline- or G-CSF treated-donors

Donor	n	CFU-C/10 <sup>6</sup> cells <sup>a</sup>
Untreated	4	7.9 ± 3.9
Lisofylline treated	2	4.5 ± 3.1
G-CSF treated	4	50.3 ± 33.5 <sup>b</sup>

<sup>a</sup> Nylon wool nonadherent liver NPC.

<sup>b</sup>  $P < 0.001$  vs. untreated (unpaired Student's *t* test).

the flt3 ligand (15), and other hematolymphopoietic growth factors may constitute a therapeutic tool at least as powerful as (and not mutually exclusive of) the donor leukocyte augmentation procedures currently under evaluation in organ recipients. Administration of G-CSF to liver transplant recipients was recently reported by Foster et al. (16), who attributed improved patient and graft survival to buttressed neutrophil-dependent infectious defenses. However, we postulate that the benefits noted in patients (16), as well as the significant prolongation of heart allograft survival in otherwise untreated rat recipients (14), was due primarily to augmentation of spontaneous chimerism.

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## BIOPHYSICAL ASPECTS OF LIVER AERATION BY VASCULAR PERSUFFLATION WITH GASEOUS OXYGEN

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**Background.** Venous systemic oxygen persufflation of the liver (i.e., gaseous insufflation of oxygen via the venous vascular system) has proven to be an effective tool for preventing anoxic tissue injury during extended time periods of ischemic preservation. It also

allows for an improved recovery of the persufflated organ after orthotopic transplantation.

**Methods.** Biophysical aspects of the persufflation technique with regard to persufflation pressure (9 mmHg versus 18 mmHg) and oxygen concentration (pure oxygen versus air) in the persufflation gas were investigated in rat livers, using epi-illumination microscopic detection of autofluorescence of NADH, which accumulates in anoxic tissue.

**Results.** We demonstrated that a low-pressure persufflation (9 mmHg) is as sufficient as a higher pres-

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