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Thalidomide promotes transplanted cell engraftment in the rat liver by modulating inflammation and endothelial integrity

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Abstract

Background & Aims—For liver-directed cell therapy, efficient engraftment of transplanted cells is critical. This study delineated whether anti-inflammatory and endothelial disrupting properties of thalidomide could promote transplanted cell engraftment and proliferation in liver.

Methods—We used dipeptidyl peptidase IV-deficient rats for cell transplantation studies, including gene expression analysis, morphological tissue analysis, serological assays, cell culture assays, and assays of transplanted cell engraftment and proliferation.

Results—Thalidomide-pretreatment increased engraftment and proliferation of transplanted hepatocytes due to decreased inflammation. Moreover, thalidomide exacerbated cell transplantation-induced endothelial injury. This combined anti-inflammatory and endothelial injury effect of thalidomide was superior to the anti-inflammatory effect alone of repertaxin or etanercept, which block cytokines/chemokines/receptor-dependent inflammation. In thalidomide-pretreated animals, liver repopulation accelerated, including when cells were primed with bosentan to block endothelin-1 receptors.

Conclusions—Thalidomide improved transplanted cell engraftment and liver repopulation. Therefore, this class of drugs will advance applications of liver cell therapy in people.

Graphical Abstract

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Keywords

cell therapy; inflammation; endothelium; transplantation

Introduction

To replace deficient enzymes or proteins with liver-directed cell therapy, adequately repopulating the liver with healthy transplanted cells is of paramount significance (1). However, this has generally been difficult due to hurdles in the engraftment and proliferation of transplanted cells in the liver. For instance, early clearance of transplanted cells (80–90%) from the liver is a major problem for cell engraftment. Recently, ischemia-related events involving vasoconstrictors, such as endothelin (ET)-1 (2,3), and numerous inflammatory chemokines/cytokines/receptors controlled by cell transplantation-induced expression of TNF- α were determined to play significant roles in clearance of transplanted cells (4). On the other hand, to integrate in the liver parenchyma, transplanted cells must enter the space of Disse by disruption of liver sinusoidal endothelial cells (LSEC), which requires additional interventions and further contributes in clearance of transplanted cells (5). If such cell transplantation-related deleterious events could be controlled especially by drugs that should particularly benefit clinical applications. In preclinical animal models of cell engraftment or liver repopulation, the beneficial potential of multiple discrete drug targets was successfully demonstrated, e.g., vasodilatation of hepatic sinusoids by nitroglycerine, phentolamine, prostacyclin or ET-1 receptor blockers, bosentan (BOS) and darusentan (2,3,6), release of cytoprotective factors from hepatic stellate cells (HSC) by the cyclooxygenase inhibitors, naproxen or celecoxib (7), neutralization of TNF- α expressed by neutrophils (PMN) or Kupffer cells (KC) by etanercept (ETN) (4), and induction of injury to LSEC with cyclophosphamide or doxorubicin (8,9). Similarly, availability of safe and effective drugs with more than one desirable mechanism of action could advance cell therapy strategies.

In searching for suitable candidate drugs, we focused on anti-inflammatory, anti-angiogenic and immunomodulatory effects of the well-known drug, thalidomide (Thal). After gaining notoriety because of its early teratogenic toxicity, Thal has seen a substantial resurgence, and now constitutes a unique class of its own with multiple analogs displaying additional activities, e.g., degradation of substrates by ubiquitination (10,11). It should be relevant that

in regards to the role after cell transplantation of PMN or KC-related expression of cytokines/chemokines/receptors as inflammatory mediators (2–4,12,13), Thal inhibited recruitment of these cell types to sites of inflammation, e.g., skin or liver (14,15), and also downregulated cytokine expression in inflammatory cells, including of TNF- α and interleukins (Il) (10). Moreover, Thal protected hepatocytes from alcohol- or other toxins (15–17). Furthermore, endothelial disrupting effects of Thal or its analogs have been examined (18), including in the clinical setting, e.g., Thal was used to control bleeding from vascular malformations in the gastrointestinal tract (19). These drug effects led us to consider that Thal may be useful for cell transplantation. We examined this possibility by studying engraftment of transplanted cells and kinetics of liver repopulation in mutant dipeptidyl peptidase IV-deficient (DPPIV–) rats, which provide convenient ways for localizing healthy transplanted cells.

Materials and Methods

Drugs and chemicals

Repertaxin (Rep), Ret, Thal and reagents were from Sigma Chemical Co. (St. Louis, MO). Thal was dissolved in dimethylsulfoxide at concentration of 25 mg/ml. A clinical preparation of ETN was purchased (Amgen Inc., Thousand Oaks, CA). Water-soluble BOS sodium salt was from Actelion Pharmaceuticals Ltd. (Allschwil, Switzerland). Drugs were injected into animals in normal saline iv through tail vein (9 mg/kg ETN) or ip (10–30 mg/kg Rep, 5–40 mg/kg Thal) either 2h (Rep, Thal) or 16–20h (ETN) before cells were transplanted.

Animals

The Animal Care and Use Committee at Albert Einstein College of Medicine approved protocols in compliance with NIH regulations. Donor F344 rats were from National Cancer Institute (Bethesda, MD). DPPIV– F344 rats, 8–10 weeks old and weighing 120–180 g, were obtained from Animal Models, Stem Cells and Cell Therapy Core of the Marion Bessin Liver Research Center. For cell engraftment studies, animals were not preconditioned and received only drugs as indicated before cell transplantation. For liver repopulation studies, rats were preconditioned with 30 mg/kg retrorsine (Ret) ip at 6 and 8 weeks of age followed by two-thirds partial hepatectomy (PH) after 4 weeks immediately before cell transplantation. For analysis of KC activity, animals were given 1h pulse of carbon particles before sacrifice, as described previously (12).

Cell isolation

Hepatocytes were isolated from donor rats by two-step collagenase perfusion as described previously (2–4). Cell viability was determined by trypan blue dye exclusion and exceeded 80%.

Cell culture and cytotoxicity assays

Hepatocytes were plated in plastic cell culture dishes at density of $1 \times 10^5/\text{cm}^2$ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics. For elucidating drug cytotoxicity, cells were cultured for 16–18h with 10–100 μM Thal. For

additional cytotoxicity analysis, cultured cells were first incubated with 200 ng/mL actinomycin D for 2h before continued culture with 10 ng/mL TNF- α (Serologicals Corp., Atlanta, GA) for 16–18h. Differences in cell survival as compared with untreated control cells were determined by assays of thiazolyl blue dye (MTT) reduction, as described previously (2–4). Each experimental condition was in triplicate at least.

Cell transplantation

After suspending hepatocytes in 0.2 milliliters of Roswell Park Memorial Institute (RPMI) 1640 culture medium, cells were injected within 2h of isolation into the pulp of spleen over 9 to 12s. For cell engraftment studies, animals received 1×10^7 cells with or without prior drug treatments. For liver repopulation studies, Ret/PH-preconditioned rats received 5×10^6 cells into the pulp of spleen by taking into account smaller size of remnant liver. In some instances, immediately after isolation donor hepatocytes were kept with or without 10 μ M BOS in RPMI medium on wet ice for 1h and then transplanted into recipient rats, according to the procedure described previously (2).

Gene expression analysis

Total RNAs were isolated from 3 rat livers per experimental condition with TRIzol (Life Sciences, Carlsbad, CA) and converted to cDNA with RT² PCR Array First Strand Kit (SABiosciences-Qiagen, Valencia, CA). An array of rat probes for 84 chemokine, cytokine and receptor genes (http://www.sabiosciences.com/rt_pcr_product/HTML/PARN-011A.html) was used with Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The fold differences in gene expression were identified by 2^{-C_t} method in control and experimental samples after normalization with invariantly expressed housekeeping genes. Gene expression differences 2-fold were considered significant. Gene expression pathways were annotated according to Kyoto Encyclopedia of Genes and Genomes (KEGG), as described previously (4).

Histochemical stainings

Tissue samples were frozen in cold methylbutane at -80°C . 5 μ m cryostat sections were prepared from 3–6 rats per condition. For KC, sections were fixed in ice-cold ethanol and stained with hematoxylin (2–4). For PMN, ethanol-fixed sections were stained for myeloperoxidase (MPO), as described previously (2–4). For DPPIV or γ -glutamyl transpeptidase (GGT), chloroform-acetone-fixed sections were stained as described previously with toluidine blue counterstain (2–4). Immunostaining for C-C motif receptor (Ccr)-1 and C-X-C motif receptor (Cxcr)-1 was as described previously (13). Grading of carbon-containing KC used multiple areas centered on portal vein radicles under $\times 200$ magnification ($n=3$ per condition) as previously described (2–4). The number of MPO+ PMN and DPPIV+ transplanted cells were counted in 50–100 consecutive areas centered on portal radicles per animal ($n=3$ each) under $\times 100$ magnification. To demonstrate the kinetics of liver repopulation 3 weeks after cell transplantation in Ret/PH-preconditioned rats, multiple tissue sections were stained for DPPIV activity per animal ($n=3$ –6 each). The area of individual transplanted cell foci was graded in multiple serial photomicrographs from individual animals with ImageJ software (NCI, Bethesda, MD). The area of transplanted cells was determined by taking into account image magnifications. The transplanted cell

areas were analyzed in quantile distributions for box plot analyses followed by linear regression for liver repopulation kinetics in experimental groups. The extent of liver repopulation was analyzed as appropriate in consecutive tissue sections from multiple animals per condition.

Serum hyaluronic acid

Serum samples were stored at -20°C and assayed by a kit (Corgenix, Westminster, CO) as described previously (2).

Statistical analysis

Each experimental group used 3–6 rats. Data are shown as means $\pm$ SEM. Significances were analyzed by t-test, Chi-square, or ANOVA with Tukey's test for pairwise comparisons. Polynomial linear regression was used for analyzing trendlines with established methods. $P < 0.05$ was considered significant.

Results

Thal pretreatment improved transplanted cell engraftment and proliferation

In studies to elicit drug toxicity, when rats were given 5 to 40 mg/kg Thal ($n=3$ per dose) we observed no morbidity over seven days based on body condition scoring (20), which was in agreement with the nonlethal nature of this drug despite grams per kg amounts in rodents or people previously (21). In Thal-treated rats 1, 2, 4 and 7d or 1 month after cell transplantation versus drug-untreated controls ($n=3$ each per time-point), the transplanted cell numbers were observed to have increased in a dose-dependent manner. In controls, transplanted cell numbers per 100 liver acini ranged from means of 110 ± 9 to 140 ± 20 after 1, 2, 4 or 7d, whereas in animals receiving 5, 10 or 40 mg/kg Thal, transplanted cell numbers at corresponding times reached differences at the upper distribution levels of 1.9 ± 0.4 -, 2.1 ± 0.3 - and 3.0 ± 0.8 -fold greater, respectively, $p < 0.05$, ANOVA (Fig. 1) (Supplementary Fig. 1 shows numbers of transplanted cells). The mean fold-differences were slightly lower than these levels. We found the fractions of transplanted cells in portal vein radicles and liver parenchyma in control or Thal-pretreated animals were similar, which indicated that lobular distributions of transplanted cells were not altered.

The numbers of transplanted cells remained unchanged from 7d to 1 month after cell transplantation in rats pretreated with 40 mg/kg Thal, which suggested that this drug did not cause damage in native hepatocytes to produce compensatory proliferation in transplanted cells. Proliferation in transplanted cells represents a superior parameter for identifying losses of native hepatocytes since reliance on liver histology alone was inadequate as noted with toxicity from other drugs previously (22). Also, we excluded adverse effects on cell viability of Thal, including additional TNF- α cytotoxicity, with assays using primary rat hepatocytes (Supplementary Fig. 2). As these studies established that 40 mg/kg Thal improved cell engraftment most effectively, we performed further studies with only this dose.

Next, we determined whether Thal would allow liver repopulation in Ret/PH-preconditioned rats, where hepatic DNA damage inhibits proliferation in native hepatocytes. The extent of

liver repopulation in Thal-pretreated rats 3 weeks after cell transplantation was greater, $24\% \pm 5\%$, versus $12\% \pm 4\%$ in control rats, $p < 0.05$, ANOVA (Fig. 2). This indicated that Thal pretreatment benefited both initial engraftment as well as subsequent proliferation of transplanted cells under liver repopulation conditions.

Thal-treated animals showed differences in liver inflammation and endothelial damage

We examined cell transplantation-induced expression of cytokines/chemokines/receptors 6h after cell transplantation, which represented the peak of these changes in previous studies (4). In datasets normalized against healthy rats, we observed 62 of 84 genes in arrays were expressed differently following cell transplantation with or without Thal pretreatment (Supplementary Fig. 3). In animals after cell transplantation without Thal 45 of 84 (54%) genes were upregulated, whereas fewer genes were upregulated (12 of 62, 19%) and more genes were downregulated (50 of 62, 81%) in animals with Thal pretreatment, $p < 0.05$, Chi-square. Genes that were upregulated in Thal-pretreated rats seemed to be those expressed typically in lymphocytes or other immunocytes (Supplementary Table 1), which had remained unaffected after cell transplantation in syngeneic animals (13). Interestingly, most cases of downregulated genes in Thal-pretreated animals mapped along cytokine/chemokine/receptor groups including ones associated with PMN or KC (Fig. 3). For instance, these included TNF- α and its receptors, Tnfrsf1a and Tnfrsf1b, along with multiple IL (-1, -2, -6) receptors.

We confirmed that expression of cytokine/chemokine/receptor mRNAs was correctly represented at the protein level by immunostaining tissues for Ccr1 and Cxcr1 (Fig. 4). In healthy rat livers, these receptors were not found to be expressed in cells. However, within 6h after cell transplantation, we found 6–8 hepatocytes with Ccr1 or Cxcr1 expression appeared in liver per high power field. By contrast, after cell transplantation in Thal-pretreated rats, hepatocytes expressing Ccr1 or Cxcr1 were not found in the liver. Since Ccr1 and Cxcr1 expression was also abolished in ETN-treated rats after cell transplantation, this verified the central role of TNF- α in this process, and indicated that Thal too had antagonized TNF- α -mediated events after cell transplantation.

In studies of inflammatory cell activations, significant increases were noted in the liver of rats 6h after cell transplantation of MPO PMN + and also of greater carbon uptake in KC, $p < 0.05$, ANOVA (Fig. 5A, 5B). By contrast, such activated PMN and KC were fewer in Thal-treated rats, though not to the numbers observed in healthy rats. Similarly, hepatic GGT expression, which has been another effective marker of cell transplantation-induced liver injury, decreased without returning to normal in Thal-pretreated rats, $p < 0.05$, ANOVA (Fig. 5C). Nonetheless, in Thal-pretreated rats, serum hyaluronic acid levels increased significantly 6h after cell transplantation, 179 ± 22 ng/ml, versus in rats after cell transplantation alone, 82 ± 16 ng/ml, or in healthy rats, 68 ± 25 ng/ml, $p < 0.05$, ANOVA (Fig. 5D). This indicated that cell transplantation in Thal-pretreated rats produced greater endothelial damage, which was in agreement with endothelium disrupting effects of this drug. These effects of Thal on hyaluronic acid levels were transient and short-lived since serum hyaluronic acid levels were similar in rats one week after Thal and in healthy rats.

To separate the effects on cell engraftment of inflammatory cytokines/chemokines/receptors and endothelial injury, we used a small molecule antagonist of Cxcr1 and Cxcr2, Rep, that blocks activation via Cxcl1 or Cxcl2 of PMN (23), and ETN, which blocks virtually all cytokines/chemokines/receptors produced additionally by KC after cell transplantation (4). These drugs do not affect angiogenesis. In rats treated with up to 30 mg/kg Rep- or 9 mg/kg ETN, cell engraftment increased by 1.8 ± 0.1 and 1.7 ± 0.1 -fold above controls. However, cell engraftment improved more in Thal-treated rats, 2.4 ± 0.1 -fold above controls, $p < 0.05$, ANOVA (Fig. 6, Supplementary Fig. 4). After cell transplantation in Rep- or ETN-treated animals, activation of PMN or KC was lower, similar to that in Thal-treated rats (not shown). Therefore, the gains in cell engraftment with Thal that were beyond those yielded by interference in inflammation-specific processes with either Rep or ETN further substantiated that endothelium disrupting effects of this drug contributed additionally.

Role of Thal for liver repopulation with combined cell transplantation approaches

Previously, liver repopulation improved substantially when Ret/PH-preconditioned rats had been pretreated by darusentan and donor cells had been primed in suspension conditions for 1h with BOS before transplantation to block deleterious activity of ET-1 receptors under conditions both in vitro and in vivo (2,3). Now, we tested whether liver repopulation could improve by such brief priming of cells in suspension with BOS for one hour in vitro followed by transplantation in Thal- or ETN-treated Ret/PH-preconditioned rats (Fig. 7A). Incubation of cells with BOS did not decrease cell viability. However, compared with controls receiving cells without drug priming, in recipients of cells primed with BOS, the numbers and sizes of transplanted cell foci were grossly larger in both ETN or Thal-pretreated and Ret/PH-preconditioned animals (Fig. 7B). Moreover, in animals pretreated with either ETN or Thal followed by transplantation of BOS-primed cells, the sizes of transplanted cell foci increased further, which indicated that liver repopulation kinetics had been accelerated. Examination of the areas of transplanted cell foci in multiple animals indicated these were nonparametrically distributed, as was anticipated, since transplanted cells constituted foci of 2–10 cells and also far more cells, which was likely due to variabilities in the replication potential of individual donor hepatocytes. Consequently, we organized our grading of the sizes of transplanted cell foci into medians (range), and found that the sizes of transplanted cell foci in Thal- or ETN-pretreated animals were significantly larger than those in drug-untreated controls, $p < 0.0000$, ANOVA (Supplementary Table 2). The representation of these data in boxplots showed that transplanted cell foci in Thal-pretreated animals with or without BOS priming tended to be the largest, including in comparison with ETN-treated animals (Fig. 7C). To elucidate the extent of transplanted cell proliferation, we then analyzed the quantile distributions of the areas occupied by transplanted cell foci (Supplementary Table 3). In ETN- or Thal-treated animals, transplanted cell foci were distributed disproportionately along larger quantiles (Fig. 7D). Upon linear regression of these quantile distributions, trendlines emerged to indicate that the kinetics of transplanted cell proliferation diverged in ETN- or Thal-pretreated animals versus in drug-untreated controls (Fig. 7E). Also, these trendlines diverged in ETN- versus Thal-pretreated animals with or without transplantation of BOS-primed cells. Although these differences did not reach statistical significances, nominal comparisons of these data versus controls established that the maximal sizes of transplanted cell foci were 2.9-, 3.5-, 6- and 7-

fold larger in animals that had received ETN, Thal, ETN plus BOS-primed cells, and Thal plus BOS-primed cells, respectively. Taken together, these findings indicated that pretreatment of animals with Thal was highly conducive for liver repopulation in the Ret/PH setting.

Discussion

These studies established that Thal pretreatment decreased cell transplantation-induced hepatic recruitment of PMN and KC along with dampening of inflammatory cytokine/chemokine/receptor responses with simultaneously inducing endothelial injury to increase engraftment of transplanted cells and yield superior liver repopulation. These benefits of pretreating recipients with Thal synergized with priming of cells with BOS in vitro to provide further gains in transplanted cell engraftment and liver repopulation. In these ways, the anti-inflammatory and endothelial disrupting effects of Thal offer excellent paradigms for cell therapy strategies.

The Thal class of drugs acts through multiple mechanisms that are as yet incompletely understood. Whereas anti-inflammatory and anti-angiogenic properties of Thal interested us most, this drug has other properties, that could also be relevant for cell transplantation. Our observations of decreases in the recruitment of PMN and KC after cell transplantation in Thal-pretreated animals recapitulated these effects of the drug in other settings, e.g., erythema nodosum lesions in people with leprosy or alcoholic liver injury in animals (14,15). Similarly, our findings indicating transcriptional downregulation of multiple cytokine/chemokine/receptors in Thal-pretreated animals were in agreement with previous studies showing such effects in other inflammation-related settings of Thal and its analogs on cytokine expression, including that of TNF- α , ILs, etc. (10). In some cases of chemokines and cytokines, notably interleukins, that are well known to be associated with the activation of lymphocyte subsets, we observed increased expression levels in Thal-pretreated animals following cell transplantation. These gene expression differences likely represented the effects of Thal on dendritic, NK or T-cell populations in the liver, which were unaffected after transplants of syngeneic cells, as was established previously (13). However, these cell types would be expected to become engaged after transplants of allogeneic cells, where immunomodulatory effects of Thal should be of interest, judging from the similarity of benefits of Thal in the setting of solid organ transplants (24). Therefore, it will be reasonable to consider that immunomodulation via regulated expression of cytokine/chemokine/receptors for preventing or treating allograft rejection will be helpful for transplantation medicine since additional interventions are currently needed for this purpose.

Previously, cell transplantation-induced differences in cytokine/chemokine/receptor expression were found to be under local neural controls in the liver itself that regulated PMN and KC activation (4). This mechanism differed from the regulation of monocyte/macrophage activation by central neural controls in other situations, e.g., septic shock (25). The local inflammatory processes were amenable to Rep, ETN and Thal since cell engraftment improved in animals pretreated with each of these drugs. This effect on superior cell engraftment was thus consistent with drug-induced interferences in ligand-receptor engagements of chemokines/cytokines promoting activation and/or recruitment of PMN and

KC, e.g., decreased expression of Ccl3 (ligand) and its receptor, Ccr1, as well as of Cxcl1 and Cxcl2 (ligands) and their receptors, Cxcr1 and Cxcr2, respectively, in Thal-pretreated animals, which had been documented in case of ETN previously (4). On the other hand, our results of superior cell engraftment in Rep-treated rats indicated that Cxcr1 and Cxcr2 will be additional potential drug targets for anti-inflammatory interventions in the cell transplantation setting.

Further gains in cell engraftment in Thal-treated animals despite the anti-inflammatory effects of Rep or ETN indicated additional endothelial disrupting properties of this drug served a role, which was verified by increased levels of serum hyaluronic acid, a marker of LSEC damage (22), after cell transplantation in Thal-treated animals. As the entry of transplanted cells in liver parenchyma requires disruption of LSEC (5), prospective disruption of the hepatic endothelial barrier in Thal-treated rats will have benefited cell engraftment. This would be similar to superior cell engraftment in previous studies when hepatic endothelium was prospectively damaged by the chemical, monocrotaline, or the drugs, cyclophosphamide or doxorubicin (8,9,22). The mechanisms by which Thal likely exerted endothelial disrupting effects should have included alterations in endothelial cell survival, proliferation or angiogenesis through regulation of intracellular nitric oxide production, transcription factor expression, or alterations in signaling pathways that have been demonstrated previously (18,26–28).

Another potential mechanism of interest in cell engraftment for Thal and analogs may concern microenvironment modifications involving cell adhesion. For instance, these drugs were previously found to decrease expression of cell adhesion molecules, such as integrin or intercellular adhesion molecule-1, including signaling pathways activated by cell adhesion (29–31). Although these properties will be relevant for inflammatory cell recruitment or tumor invasion in diseases, this should also be relevant for engraftment of transplanted hepatocytes in liver sinusoids. For instance, interactions during cell engraftment of integrins expressed in transplanted cells and extracellular matrix receptors expressed in LSEC was established previously (32). Whether Thal enhanced beneficial cell-cell interactions in liver sinusoids to improve transplanted cell engraftment through cell adhesion mechanisms is not excluded. Thal retained its efficacy in the Ret/PH model with clear trends for acceleration of the liver repopulation kinetics. The multi-fold increases in liver repopulation kinetics within the short period of three weeks after cell transplantation should bode particularly well since with more time this should lead to major gains for liver repopulation.

The drug-based approach to increase cell engraftment should be of considerable translational value for cell therapy applications in people. On the one hand, drug priming of donor cells to improve their survival under adverse microenvironmental conditions of hypoxia and inflammation immediately after transplantation, as with ET-1 receptor blocker, BOS, was helpful for cell engraftment, similar to previous studies (2). On the other hand, cell engraftment improved by pre-treating recipients with some vasodilators (nitroglycerine, phentolamine, prostacyclin, BOS or darusentan), although other vasodilators (calcitonin gene-related peptide, glucagon, nifedipine or labetalol) were not effective for this purpose (2,3,6), modifiers of extracellular matrix components (fibronectin-like engineered polymer) (31), disruptors of LSEC (cyclophosphamide, doxorubicin) (8,9), inhibitors of

cyclooxygenases releasing cytoprotective substances (naproxen, celecoxib) (7), or regulators of inflammation with TNF- α antagonism (ETN) (4), and now Thal. The anti-inflammatory and endothelium disrupting effects of Thal without affecting priming of cells with other drugs will be helpful for cell transplantation. The extensive safety margin of Thal compared with cytotoxic drugs, e.g., cyclophosphamide or doxorubicin should simplify anti-angiogenic applications for cell therapy. The combination of additional drugs and strategies to avoid various deleterious events and processes that are not well controlled by individual drugs should be similarly helpful. In this respect, studies to elicit benefits on cell engraftment of Thal along with vasodilators, extracellular matrix components, etc., should seem appropriate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ET-1	endothelin-1
TNF-α	tumor necrosis factor, alpha
LSEC	liver sinusoidal endothelial cells
BOS	bosentan
HSC	hepatic stellate cells
PMN	neutrophils
KC	Kupffer cells
ETN	etanercept
Thal	thalidomide
IL	interleukin
DPPIV	dipeptidyl peptidase IV
F344	Fischer 344
DMEM	Dulbecco's minimal essential medium
RPMI	Roswell Park Memorial Institute
Rep	repertaxin

Ret	retrorsine
iv	intravenous
ip	intraperitoneal
MTT	thiazolyl blue
PH	partial hepatectomy
cDNA	complementary DNA
RT-PCR	reverse-transcription and polymerase chain reaction
KEGG	Kyoto encyclopedia of genes and genomes
MPO	myeloperoxidase
GGT	gamma glutamyltranspeptidase
Ccr1	C-C motif receptor-1
Cxcr1	C-X-C motif receptor-1
NCI	national cancer institute
ANOVA	analysis of variance
Tnfrsf	tumor necrosis factor receptor
Ccl	C-C motif ligand
Cxcl	C-X-C motif ligand

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Figure 1. Effect of Thal on transplanted cell engraftment

(A) Protocol and time-line for gene expression analysis, tissue changes and cell engraftment analysis. The numbers of animals analyzed were 3 each 6h, 1d and 7d after cell transplantation while 6 animals each were analyzed 2–4d and 1 mo after cell transplantation. (B) Representative examples of DPPIV+ transplanted hepatocytes (red color) 7d after cell transplantation in control and Thal-treated rats. Original magnification $\times 200$. (D) Morphometry showing cumulative results of cell engraftment in comparison with control animals in Thal-treated rats. Asterisks indicate $p < 0.05$ versus controls.

Figure 2. Thal and liver repopulation

(A) Protocol to precondition rats with retrorsine and PH for liver repopulation analysis 3 weeks after cell transplantation and tissue analysis. (B) Liver repopulation with clusters of proliferating DPPIV+ transplanted cells after 3 weeks in control and Thal-treated rats. (C) Morphometry indicating greater liver repopulation after 3 weeks in Thal-treated rats. Asterisk indicates $p < 0.05$ versus controls.

Figure 4. Effect of Thal on cell transplantation-induced expression of chemokine receptors

Shows immunostaining of liver samples for Ccr1 (A) and Cxcr1 (B) in healthy control rats, rats 6h after cell transplantation, and rats pretreated with either Thal or ETN followed by cell transplantation 6h before tissue collection. The cells with Ccr1 or Cxcr1 expression are shown with diaminobenzidine substrate color (arrows) in panels next to the panels at the top. In healthy control rats and rats pretreated with Thal or ETN followed by cell transplantation, Ccr1 or Cxcr1 were not expressed. Original magnification $\times 400$. Hematoxylin counterstain.

Figure 5. Effect of Thal on cell transplantation-induced tissue changes

Shown is cumulative morphometric analysis of various parameters in healthy control rats and rats 6h after cell transplantation with or without Thal-treatment. Compared with healthy control rats, MPO+ PMN numbers (A), number of carbon-containing KC (B), and liver area with GGT+ hepatocytes (C) were greater after cell transplantation, whereas all of these changes were less pronounced in Thal-treated rats. Panel D shows serum hyaluronic acid levels, which increased significantly after cell transplantation in Thal-treated rats. Asterisks indicate $p < 0.05$ versus healthy control rats.

Figure 6. Comparative effects of Rep, ETN and Thal on transplanted cell engraftment
Morphometric analysis of cell engraftment after 7d in rats with or without drug treatments indicated (n=3–4 each). The improvement in cell engraftment in Thal-treated rats was more pronounced than either Rep-treated or ETN-treated rats. Asterisks indicate $p < 0.05$ versus controls. The difference in cell engraftment in Thal-treated and Rep- or ETN-treated rats was also significant with $p < 0.05$.

Figure 7. Liver repopulation kinetics in Ret/PH-conditioned rats

(A) Protocols to precondition rats with Ret/PH followed by administration of ETN or Thal and liver repopulation analysis 3 weeks after cell transplantation. In other animal groups, cells were primed with BOS for 1h in vitro. (B) Representative examples of transplanted DPPIV+ cells arranged in discrete foci of various sizes in animal groups after 3 weeks. (C) Box plots showing cumulative medians and dispersions in quantiles of transplanted cell areas determined by morphometric analysis of multiple foci per animal. The data indicated that transplanted cell foci were significantly larger than controls in animals pretreated with ETN or Thal and were bigger still when BOS-primed cells were transplanted in animals treated with these drugs. The median (range) of these areas is in Supplementary Table 2. (D) Sizes of transplanted cell foci distributed according to quantiles in various conditions shown. The differences in ETN vs ETN+ BOS and Thal vs Thal + BOS were statistically significant, $p < 0.05$ although ETN vs Thal and ETN + BOS vs Thal + BOS did not achieve statistical significance. (E) Linea regressions showing trendlines for liver repopulation kinetics, which accelerated most in Thal-treated animals with or without BOS-primed cells.