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Facilitated engraftment of human hematopoietic cells in severe combined immunodeficient mice following a single injection of Cl_2MDP liposomes

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Transplantation of normal and malignant human hematopoietic cells into severe combined immunodeficient (SCID) mice allows for evaluation of long-term growth abilities of these cells and provides a preclinical model for therapeutic interventions. However, large numbers of cells are required for successful engraftment in preirradiated mice due to residual graft resistance, that may be mediated by cells from the mononuclear phagocytic system. Intravenous (i.v.) injection of liposomes containing dichloromethylene diphosphonate (Cl_2MDP) may eliminate mouse macrophages in spleen and liver. In this study outgrowth of acute myeloid leukemia (AML) cells and umbilical cord blood (UCB) cells in SCID mice conditioned with a single i.v. injection of Cl_2MDP liposomes in addition to sublethal total body irradiation (TBI) was compared to outgrowth of these cells in SCID mice that had received TBI alone. A two- to 10-fold increase in outgrowth of AML cells was observed in four cases of AML. Administration of 10^7 UCB cells reproducibly engrafted SCID mice that had been conditioned with Cl_2MDP liposomes and TBI, whereas human cells were not detected in mice conditioned with TBI alone. As few as 2×10^4 purified $CD34^+$ UCB cells engrafted in all mice treated with Cl_2MDP liposomes. In SCID mice treated with macrophage depletion unexpected graft failures were not observed. Histological examination of the spleen showed that TBI and Cl_2MDP liposomes i.v. resulted in a transient elimination of all macrophage subsets in the spleen, whereas TBI had a minor effect. Cl_2MDP liposomes were easy to use and their application was not associated with appreciable side-effects. Cl_2MDP liposome pretreatment in combination with TBI allows for reproducible outgrowth of high numbers of human hematopoietic cells in SCID mice.

Keywords: SCID mice; macrophages; engraftment; acute myeloid leukemia; umbilical cord blood

Introduction

Severe combined immunodeficient (SCID) mice may be used as a model for the *in vivo* analysis of proliferation of human hematopoietic cells.[1–5] In hematological diseases such as acute myeloid leukemia,[6,7] chronic myeloid leukemia,[8] acute lymphoblastic leukemia[9–11] and lymphoma[12] SCID mice provide a useful model for analysis of long-term growth and therapeutic interventions *in vivo*.[13–15] Conditioning of SCID mice using sublethal TBI and intravenous transplantation allows for outgrowth of these cells in the SCID mouse bone marrow.[1,16]

However, even following sublethal irradiation of SCID mice large numbers of human hematopoietic cells are required for reproducible outgrowth and graft failures are frequently observed.[1–3,5–15] These problems restrict the practical possibilities for analysis of subsets of hematopoietic cells. As SCID mice are T and B cell-deficient, likely explanations for residual graft resistance may be that transplanted human hematopoietic cells are cleared by either recipient mononuclear phagocytes or by recipient NK cells.[17,18]

Macrophage depletion with the purpose of facilitating engraftment of allogeneic and xenogeneic bone marrow grafts has been investigated before, using less specific means such as silica or carageenan. In irradiated mice, carrageenan and silica abrogated or weakened resistance to parental, allogeneic and rat marrow grafts.[19–22] However, both carrageenan and silica are highly toxic agents. Furthermore, they do not completely eliminate macrophages and exert undesired effects on nonphagocytic cells.[19,21,23] These disadvantages clearly compromise their applicability in bone marrow transplantation in general and in the SCID mouse model in particular. *In vivo* macrophage depletion has been achieved with liposomes which contain clodronate (Cl_2MDP). Such liposomes are ingested by macrophages. After intracellular disruption of the liposomes, clodronate effectively kills these cells.[24] Which population of macrophages is eliminated depends on the route of administration of Cl_2MDP liposomes.[25] Intravenous injection of the liposomes mainly eliminates phagocytic cells in liver and spleen, the candidate effector cells in graft resistance, because of their direct contact with circulating hematopoietic cells. Fraser *et al*[26] showed that intravenous injection of Cl_2MDP liposomes into unirradiated SCID mice prolonged the time needed for clearance of intravenously injected human peripheral blood lymphocytes. Similarly, application of these liposomes to SCID mice with an established human thymus/liver graft resulted in increased numbers of circulating human lymphocytes. To determine the effect of Cl_2MDP liposomes on engraftment of normal and leukemic human hematopoietic cells in irradiated SCID mice we compared trans-

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plantations with or without additional Cl_2MDP liposome treatment.

Materials and methods

Acute myeloid leukemia cells and umbilical cord blood cells

Peripheral blood (PB) samples were obtained following informed consent from four patients presenting with AML, diagnosed according to the criteria of the French–American–British Committee (FAB).[27] AML cells and umbilical cord blood samples (UCB) were isolated by Isopaque–Ficoll centrifugation (1.077 g/cm^2; Nycomed, Oslo, Norway) and then cryopreserved.[28] After thawing the viability of AML and UCB cells as assessed by trypan blue exclusion was always >70%.

Preparation of CD34$^+$ umbilical cord blood fractions

UCB cells were incubated with an IgG2a antibody against CD34 (MoAb 561) which was noncovalently linked to a rat-anti-mouse IgG2a-conjugated immunomagnetic beads (Dynabeads; Dynal, Oslo, Norway). CD34$^+$ cells were eluted from the beads using a polyclonal antibody preparation directed against the Fab fragment of the CD34 antibody (Detachabead; Dynal).[29]

Immunodeficient mice and transplantation of AML and umbilical cord blood (UCB)

Female-specific pathogen-free CB17 scid/scid mice (5–9 weeks of age) were purchased (Harlan CPB, Austerlitz, The Netherlands). Nonobese diabetic SCID (NOD/SCID), specifically NOD/Lt-SCID/Sz mice (11 weeks of age), were obtained from Jackson Laboratories (Bar Harbour, MA, USA). Housing, total body irradiation (TBI) and transplant procedures have been described.[16] AML1 and AML2 engrafted in SCID mice without support of human hematopoietic growth factors and AML3 and 4 were IL-3-dependent in SCID mice. The latter SCID recipients received 60 μg of human IL-3 (Gist Brocades, Delft, The Netherlands) in 200 μl HBSS and 1% BSA (Sigma, St Louis, MO, USA) intraperitoneally, 5 days a week as described.[16] Mice transplanted with UCB did not receive growth factor treatment.

Liposome preparation

Cl_2MDP liposomes were prepared as reported.[30] Briefly, 75 mg phosphatidyl choline (Lipoid, Ludwigshafen, Germany) and 11 mg cholesterol (Sigma) were dissolved in chloroform in a round bottom flask. After low vacuum rotary evaporation at 37°C the lipids were dispersed by gentle rotation in 10 ml PBS in which 2.5 mg clodronate (a gift of Boehringer Mannheim, Mannheim, Germany) was dissolved. The resulting liposomes were washed twice at 100 000 *g* for 30 min to remove free, non-entrapped diphosphonate. The liposomes were then resuspended in 4 ml phosphate-buffered saline (PBS) (liposome stock solution). SCID mice were injected in a lateral tail vein with 0.2 ml liposome stock solution on the day before transplantation of the hematopoietic cells.

Tissue collections

SCID mice were killed by CO_2 inhalation followed by cervical dislocation in accordance with institutional animal research regulations. Cells or tissues from cohorts of mice transplanted with the same graft were evaluated between days 22 and 47 after transplantation using flow cytometry and cytology.[31] Spleens of selected mice were extirpated and cryostat sections were prepared for pathologic examination.

Flow cytometry

To determine the percentage of human hematopoietic cells in the SCID mouse, bone marrow samples from mice that had been transplanted with UCB grafts were incubated with mouse monoclonal antibodies to human CD33, CD34, CD45 and CD38. The initial leukemias and BM samples from mice that had been transplanted with leukemic grafts were stained or double-stained with the following mouse monoclonal antibodies: CD34-FITC, CD34-PE, CD38-PE, CD34-FITC/IgG_1-PE, CD34-FITC/CD38-PE, CD34-FITC/HLA-DR-PE, CD34-FITC/c-kit-PE, CD34-FITC/CD33-PE and finally CD45-FITC/CD33-PE. Mouse IgG_1-FITC and mouse IgG_1-PE conjugated antibodies and samples from nontransplanted SCID mice were used as controls. c-kit-PE was purchased from Immunotech (Marseille, France) all other antibodies from Becton Dickinson (San Jose, CA, USA). The phenotypes of cells recovered from the SCID mouse bone marrow was compared to those of the grafts. Fluorescence was measured using a FACSCAN flow cytometer and Lysis II software (Becton Dickinson, Immunocytometry Systems). Erythrocytes and dead cells were excluded from analysis by gating on forward and orthogonal light scatter. Cells recovered from SCID mouse bone marrow (BM) with positive staining for two antibodies specific for human hematopoietic cells were considered to be graft derived.[32]

Immunohistochemistry of spleen sections

Freshly obtained SCID mouse spleens were embedded in Tissue-tek II (Miles Laboratories, Naperville, MI, USA), frozen and stored at −70°C. Cryostat sections of 5 μm were prepared. Tissue fixation and immunoperoxidase staining of cryostat sections were performed essentially as described by de Jong *et al*.[33] For fixation, a hexazotized pararosaniline solution (0.5 ml, 4%) was added to $NaNO_2$ (0.5 ml, 4%), diluted in 165 ml sterile water and applied to dry tissue sections (2 min) followed by washing in PBS.[33] MAb binding was detected using a modified protocol involving $NiSO_4$-supplemented DAB and counterstaining of the nuclei with nuclear fast red.[34] Sections were incubated with a panel of monoclonal antibodies to determine the effect of TBI and TBI with macrophage depletion by Cl_2MDP on macrophage subsets in the spleen as representatives of phagocytic cells in direct contact with the blood stream. The antibodies used were F4/80[35,36] (identifying especially red pulp macrophages), ER-HR3[37,38] (major subset of red pulp macrophages) ER-TR9[39,40] (marginal zone macrophages), MOMA-1[41] (marginal metallophilic macrophages), Monts-4[42] (marginal metallophilic and white pulp macrophages) and N418 (anti-CD11c)[43,44] (dendritic

Table 1 Growth of human AML cells in SCID mice: effect of pretreatment with Cl_2MDP liposomes

	AML1		*AML1*		*AML2*		*AML3*		*AML4*	
Cell numbers transplanted ($\times 10^6$)	1		10		10		20		30	
Conditioning	MD + TBI	TBI	MD + TBI	TBI	MD + TBI	TBI	MD + TBI	TBI	MD + TBI	TBI
% of AML cells in SCID BM (mean ± s.d.)	76 ± 18	17 ± 16	91 ± 5	41 ± 43	19 ± 18	7 ± 6	63 ± 17	25 ± 22	10 ± 13	1 ± 1
AML tumor load ($\times 10^6$) (mean ± s.d.)	24 ± 8.5	2.0 ± 2.1	58 ± 12	23 ± 27	11 ± 11	3 ± 3	44 ± 10	8 ± 7	2 ± 1	0.3 ± 0.4
Graft failures/transplanted SCID mice	0/8	1/7	0/5	0/5	0/5	1/5	0/4	1/3	0/3	3/5

Irradiated groups of SCID mice (3.5 Gy) were transplanted with cells from four cases of AML with or without Cl_2MDP pretreatment and evaluated on the same day, between days 35 and 47. The tumor load was determined by counting the number of nucleated cells obtained by flushing of two mouse femora, equivalent to 13.5% of the total mouse bone marrow[50] as well as the percentages of human hematopoietic cells determined by flow cytometry.
BM, bone marrow; s.d., standard deviation; MD, macrophage depletion by pretreatment with Cl_2MDP liposomes; TBI, total body irradiation.

cells). To confirm results with other antibodies in addition BM8[45] (red pulp macrophages), SER-4[46] (marginal metallophilic macrophages) and ER-BMDM1[47] (dendritic cells) were applied.

Results

Pretreatment with Cl_2MDP liposomes enhances engraftment of AML

We examined the effect of additional Cl_2MDP liposome treatment on the engraftment abilities of AML cells (from cases 1–4) in SCID mice treated with TBI and liposomes *vs* TBI alone (controls). Liposome treatment in addition to TBI resulted in an increase in the percentages of leukemic cells in the mouse bone marrow (BM) by two- to 10-fold (Table 1). Leukemic tumor load per mouse increased three- to 12-fold (Table 1). Graft failure was defined as less than 0.5% of AML cells in the SCID mouse BM. Among the 25 mice transplanted with AML cells following liposome treatment no graft failures were observed, whereas six graft failures were seen in control recipients ($P = 0.02$, Fisher's exact test) (Table 1). The immunophenotypes of the leukemic cells recovered from the SCID mice (as assessed by flow cytometry) were identical to those of the original grafts.

Comparison of growth of AML in SCID mice and in NOD/SCID mice

The outgrowth of graded cell doses of AML2 in SCID mice pretreated with Cl_2MDP liposomes was comparable with that in NOD/SCID mice (Table 2).

Macrophage depletion enhances outgrowth of UCB cells in SCID mice

Enhancement of engraftment of normal human hematopoietic cells was assessed in cell dose titration experiments of UCB cells. Transplantation of 10×10^6 UCB cells after TBI did not allow for reproducible engraftment (Table 3). By comparison grafts of only 1×10^6 UCB cells engrafted in five of six SCID mice prepared with additional macrophage depletion. Thus an approximately 10-fold reduction of the minimal cell numbers required for engraftment was seen as a consequence of additional conditioning with Cl_2MDP liposomes (Table 3). Cell dose titrations with $CD34^+$ selected UCB cells were performed in mice conditioned with TBI and macrophage depletion. As few as 10×10^3 $CD34^+$ UCB cells reproducibly engrafted SCID mice (data not shown).

Extensive depletion of all spleen macrophage subsets by the combination of Cl_2MDP liposomes and TBI, limited effect of TBI alone

To evaluate the effect of Cl_2MDP liposomes on SCID mouse macrophages, which are possibly involved in scavenging of transplanted human hematopoietic cells, cryostat spleen sections were incubated with a panel of antibodies identifying distinct mononuclear phagocyte subpopulations. TBI at 3.5 Gy without administration of Cl_2MDP liposomes had negligible effects on macrophage subpopulations in the spleen at day 4 after conditioning. Red pulp, white pulp and marginal zone macrophage populations identified by immunophenotypic analysis had not changed significantly (Table 4). In contrast, dendritic cells (identified by the monoclonal antibodies

Table 2 Cell dose titration of AML2 in SCID mice with and without Cl_2MDP liposome pretreatment and in NOD/SCID mice

Conditioning	*SCID mice*		*NOD/SCID mice*
	MD + TBI	*TBI*	*TBI*
Cell numbers transplanted ($\times 10^6$)			
30		77	
		93	
10	81	19	45
	56	38	36
	69		66
3.3	83	3	34
	7	1	34
	32	3	33
	31		
1	1	0	7
	4	0	2
	5	0	13
	2		
0.3	0.2		5
			4

NOD/SCID mice were transplanted in the same experiment as the SCID mice. All mice were evaluated on days 28 and 29. Data represent percentage of infiltration of human cells in the bone marrow of individual mice.
BM, bone marrow; MD, macrophage depletion by pretreatment with Cl_2MDP liposomes; TBI, total body irradiation.

Table 3 Cell dose titration of two umbilical cord blood samples in SCID mice with and without Cl_2MDP pretreatment

Conditioning	*UCB1*		*UCB2*	
	MD + TBI	*TBI*	*MD + TBI*	*TBI*
Cell numbers transplanted ($\times 10^6$)				
30	71		75	
			56	
10	24	1	11	0
	6	0	18	0
	11	0	12	1
	6	0	6	0
3.3	9	0	3	0
	3	0	0	0
	9	0	1	0
			0	
1	1		1	
	1		3	
			11	
			0	

Irradiated SCID mice were transplanted with graded doses of umbilical cord blood cells with or without macrophage depletion. Data represent percentage of infiltration of human cells in the bone marrow of individual mice. All mice were evaluated on days 41 and 42.
ND, not done; UCB, umbilical cord blood graft; MD, macrophage depletion by pretreatment with Cl_2MDP liposomes; TBI, total body irradiation.

Table 4 Macrophage populations in the spleen of SCID mice after TBI or TBI and macrophage depletion

Antibodies		*Control*	*Day 4*		*Day 45*	
			TBI	*MD + TBI*	*TBI*	*MD + TBI*
F4/80	wp	2+	4+	0*	1+	2+
	rp	4+	4+	0*	4+	4+
ER-HR3	wp	1+	1+	0	1+	2+
	rp	3+	3+	0	3+	3+
ER-TR9	mz	2+	1+	0	0	0
MOMA-1	pwp	3+	2+	0	3+	2+
	rp	2+	3+	0	1+	1+
Monts-4	wp	3+	3+	0	3+	2+
	rp	1+	2+dim	0	0	1+
N418	wp	4+	0	1+	3+	4+
	mz	2+	1+	0	2+	3+
	rp	1+	0	0	1+	1+

To determine the effects of TBI and macrophage depletion by Cl_2MDP liposomes on spleen macrophage populations SCID mice were evaluated on days 4 and 45 after TBI, and compared to untreated SCID mouse controls. The data obtained with these antibodies were similar to data obtained with the use of independent antibodies BM-8, SER-4, and ER-BMDM1 (data not shown). Two or three mice per group were evaluated.
TBI, total body irradiation; MD, macrophage depletion by pretreatment with Cl_2MDP liposomes; wp, white pulp; rp, red pulp; pwp, peripheral white pulp; mz, marginal zone. The number of macrophages staining with a specific antibody within these anatomically defined regions were scored semiquantitatively: 4+, >50% positivity-confluent; 3+, 50%; 2+, 10–50%; 1+, scarce; *, only remnants.

N418 and ER-BMDM1) had disappeared from the red pulp and from the white pulp as a consequence of the sublethal dose of TBI alone. Combined treatment with TBI and Cl_2MDP liposomes depleted all identifiable macrophages from the white pulp, the red pulp and the marginal zone (Table 4). At day 45 after transplantation, all mononuclear phagocyte subsets had reappeared except ER-TR9 positive marginal zone macrophages. (Table 4).

Toxicity of Cl_2MDP liposomes

The injection of 0.2 ml of Cl_2MDP liposomes on the day before TBI and transplantation did not result in appreciable side-effects during the 45 day observation interval. Among the 56 mice conditioned with TBI alone six animals died before evaluation (11%). In comparison six deaths were noted among 91 mice conditioned with TBI and additional macrophage depletion (8%).

Discussion

Macrophage depletion by intravenous administration of Cl_2MDP liposomes prior to sublethal TBI enhances the engraftment of human hematopoietic cells in the SCID mouse bone marrow. This treatment is easy to apply and without significant toxicity. Outgrowth of primary human AML increased two- to 10-fold and engraftment of umbilical cord blood cells was achieved with at least 10-fold smaller grafts (Tables 1 and 3). Probabilities of graft failure were significantly reduced (Table 1). Thus, conditioning with TBI and Cl_2MDP liposomes in combination permits the establishment of relatively small human hematopoietic cell grafts, eg 1×10^6 unseparated UCB cells or 1×10^4 CD34-positive UCB cells and may facilitate the use of the SCID mouse model for the study of normal and malignant human hematopoietic cells.

The observations suggest a functional role of murine phagocytic cells in the clearance of human hematopoietic cells that engraft SCID mouse bone marrow, similar to the delayed clearance of human lymphocytes from the SCID mouse circulation as observed by Fraser *et al*.[26] Other more indirect immunological mechanisms cannot be excluded. One of these mechanisms relates to the modulation of NK cell function by macrophages. Depletion of macrophages in the liver (Kupffer cells) with Cl_2MDP liposomes was associated with a parallel decrease of the number of NK cells.[48] Furthermore, Kupffer cell-conditioned media appeared to enhance NK cell viability and function *in vitro*.[48] In the study by Fraser *et al*[26] complete depletion of macrophages in the red pulp of the spleen was observed while many white pulp macrophages remained. Our data show that white pulp macrophages are eliminated by Cl_2MDP liposomes in sublethally irradiated mice.

NOD/SCID mice have multiple immunological defects. In addition to B and T cell deficiency, NK function is absent and these mice may also have a macrophage maturation defect.[49] It has been shown that the tumor load in the spleen of SCID/NOD mice transplanted with human CEM T lymphoblasts was increased four-fold as compared to similarly transplanted SCID mice.[49] Our results indicate that the outgrowth of AML and UCB grafts in irradiated SCID mice conditioned with additional Cl_2MDP liposomes may be as effective as that in sublethally irradiated NOD/SCID mice.

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