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Molecular basis of the recognition of intravenously transplanted hemopoietic cells by bone marrow

(marrow transplantation/engraftment mechanism/neoglycoproteins/membrane lectins/colony-forming units)

SHIN AIZAWA AND MEHDI TAVASSOLI

Veterans Administration Medical Center and University of Mississippi School of Medicine, Jackson, MS 39216

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ABSTRACT **Bone marrow transplantation is done by introducing a source of hemopoietic stem cells into general circulation in anticipation of their recognition and selective lodging into the bone marrow. We tested the hypothesis that the molecular basis of this recognition is by means of a lectin–carbohydrate interaction. We synthesized a number of neoglycoproteins by covalently binding pyranose derivatives of various monosaccharides to bovine serum albumin (BSA). These reagents and the galactosyl-terminating glycoprotein asialofetuin were used to inhibit engraftment of intravenously transplanted marrow cells into lethally irradiated mice. In splenectomized mice, in whom engraftment occurs only in the bone marrow, galactosyl-BSA, mannosyl-BSA, and asialofetuin but not fucosyl-BSA inhibited homing of transplanted cells. Both the survival and cellular content of marrow, including hemopoietic stem cells and granulocyte-macrophage progenitors, were reduced. In nonsplenectomized mice, in whom engraftment occurs in both spleen and marrow, the reagents did not inhibit splenic homing. Both the survival and cellular content of spleen, including hemopoietic stem cells and granulocyte-macrophage progenitors, were similar to those of the control group. But even in this group, marrow homing of transplanted cells was inhibited by these reagents. We conclude that the recognition and homing of intravenously transplanted hemopoietic cells by marrow, but not by spleen, occurs by means of a recognition system with galactosyl and mannosyl specificities.**

Among the organs whose transplantation has come of age, bone marrow appears to be unique in that its cell suspensions are transplanted intravenously in anticipation of their selective lodging ("homing") to hemopoietic tissues (1, 2). Other organs are transplanted in orthotopic or heterotopic sites by surgical procedures. The molecular mechanism whereby transplanted marrow cells are recognized and selectively taken up by hemopoietic tissues is unclear. Several lines of evidence have suggested that asialoglycoprotein receptors may be involved (3, 4). Recently, we have provided evidence that *in vitro* binding of hemopoietic stem cells [CFU-S (colony-forming unit in spleen)] and granulocyte-macrophage progenitors [CFU-C (colony-forming unit in culture)] to hemopoietic stroma involves an interaction of membrane lectins with specific residues of membrane glycoconjugates (5, 6). The interaction involves galactosyl and mannosyl specificities. These *in vitro* studies suggested that the *in vivo* recognition of intravenously transplanted hemopoietic cells by bone marrow may also occur by means of a similar molecular mechanism. To test this hypothesis, we synthesized a number of neoglycoprotein reagents by covalently linking pyranose (ring) forms of various monosaccharides to a larger protein molecule such as bovine serum albumin (BSA) (7). These reagents were then used to test their inhibition of the grafting of bone marrow cells in lethally irradiated mice.

We now provide evidence that the molecular mechanism of recognition of intravenously transplanted hemopoietic cells by marrow, but not by spleen, also shows galactosyl and mannosyl specificities.

MATERIALS AND METHODS

Marrow Cells and Reagents. Bone marrow cells were obtained from male C57 black mice (10–12 weeks old). At least 10 animals in each group were studied; four long bones from each animal were used. Tibias and femurs were removed and cleaned from the surrounding tissues. The epiphyses were then removed, a needle was inserted into one end of the tubular bone, and the marrow tissue was forced out in a test tube by a syringe containing phosphate-buffered saline (PBS). A cell suspension was made by agitating the tube, and the cell concentration was adjusted as desired.

The method for the synthesis of neoglycoprotein reagents has been established in our laboratory and has been described in detail elsewhere (7). Briefly *para*-aminophenyl derivatives of various monosaccharides, in pyranose (ring) form, were activated with thiophosgene and then were covalently bound to the amino groups of a larger protein molecule such as BSA. These synthetic neoglycoproteins fulfill several theoretical requirements: (*i*) Binding of the sugar to a larger molecule prevents diffusion of small sugar molecules into the cells. (*ii*) The sugar remains in a pyranose form, which is necessary for binding to lectins or lectin-like substances. (*iii*) The sugar-to-protein ratio is high enough (≈40–50) to permit the use of these neoglycoproteins as inhibiting probes.

In addition, our previous *in vitro* studies (5, 6) have indicated that these reagents are not only completely free of toxicity in several human and murine cell systems but also do not interfere with functional integrity of hemopoietic progenitor and stromal cells. In addition, intravenous injection of probes into normal mice, at a dose calculated to give a 5 mM blood concentration, did not lead to mortality and morbidity in these animals even when injections were repeated at weekly intervals for 5 weeks.

Asialofetuin was purchased from Sigma. In this glycoprotein, terminal sialyl residues are removed to expose penultimate galactosyl residues. The molecule serves as a "natural" counterpart of synthetic galactosyl-BSA.

Splenectomy. Two groups of mice were studied. In the first group, C57 black mice (10 weeks old) were splenectomized through a midline abdominal incision and permitted to recover for 6 weeks before transplantation. Splenectomy was done to prevent homing of transplanted cells to the spleen. Thus, in this group, homing of transplanted hemopoietic cells occurs only to the bone marrow. In a second group, mice were sham-splenectomized by opening and closing the ab-

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Abbreviations: BSA, bovine serum albumin; CFU-S, colony-forming unit(s) in spleen; CFU-C, colony-forming unit(s) in culture.

domen without removing the spleen. In this group transplanted hemopoietic cells "home" to both the spleen and bone marrow.

Transplantation. Each splenectomized and nonsplenectomized mouse was given 8.5 grays (Gy) of whole-body irradiation. This was followed by intravenous injection into the tail vein of 10^5 fresh bone marrow cells (in 0.25 ml of PBS) obtained from syngeneic mice.

In the experimental group, transplanted marrow cells were given simultaneously with each neoglycoprotein (galactosyl-, mannosyl-, or fucosyl-BSA) or asialofetuin in concentrations calculated to give a blood concentration of 10^{-3} M sugar residues. This dose was derived from our previous dose–response studies using an *in vitro* system (5, 6). Marrow cells were preincubated for 15 min before injection at 4°C with each neoglycoprotein or asialofetuin so as to saturate their receptors before transplantation. The cells were then washed with PBS before infusion. Preincubation alone was felt inadequate for inhibition because of the relatively low affinity of the ligands for the receptors. Our previous studies (5) with a cloned hemopoietic stem cell line (B6SUT) have indicated that the lectins are located on the surface of stem cells and the K_d for galactosyl receptors is 3.11×10^{-6} M (receptor number = 2.25×10^8 per cell). Corresponding figures for mannosyl receptors are 6.88×10^{-6} M and 6.0×10^7 per cell (see *Discussion*). This relatively low affinity necessitated infusion of the neoglycoproteins in addition to preincubation.

In the control group, animals were injected with marrow cells in the absence of neoglycoproteins and asialofetuin.

In one set of experiments, marrow-transplanted animals were studied for their survival. In a second set, animals were similarly transplanted, and after 12 days they were sacrificed and the number of spleen colonies were counted. These were designated as primary CFU-S. In a third set of experiments, transplanted animals were killed after 8 days, and marrow and spleen cells were prepared. The concentration of CFU-S in these preparations was determined by injecting the cells into a new set of lethally irradiated mice; this was designated as marrow and secondary splenic CFU-S. The concentration of CFU-C was also determined in all these cell preparations.

Each group was composed of 15 animals, and experiments were done in triplicate. The means and standard deviations were calculated and subjected to Student's *t* test.

Assay for CFU-S and CFU-C. CFU-S was assayed by the method of Magli *et al.* (8). Bone marrow or spleen cells were obtained 1 or 2 weeks after transplantation in each group of splenectomized and nonsplenectomized mice. Cells were suspended in PBS, and the cell count was determined. They were then washed once and resuspended in PBS. Marrow cell suspensions (5×10^5 cells) and spleen cells (5×10^6) were then injected into 8.5-Gy-irradiated syngeneic mice. After 12 days, spleens were removed and placed in Bouin's fixative, and colonies were counted under a dissecting microscope as CFU-S.

For the CFU-C assay, 10^5 bone marrow cells and 10^6 spleen cells were incubated in 0.3% semisolid agar in α medium containing 20% (vol/vol) horse serum (9) and 20% (vol/vol) mouse peritoneal macrophage-conditioned medium as a source of colony-stimulating activity (10). Cultures were maintained for 7 days at 37°C in 5% CO_2 in humidified air and then examined by phase microscopy. Colonies containing >50 cells were counted as CFU-C.

RESULTS

Because in adult mice both marrow and spleen are hemopoietic, data obtained from the splenectomized group represent homing of progenitor cells into the marrow, whereas the differences between nonsplenectomized and splenectomized groups represent an index of homing of transplanted cells into the spleen.

Survival Studies. Table 1 shows the survival of animals in both splenectomized and nonsplenectomized groups. In both groups, all control animals (not injected with neoglycoproteins or asialofetuin) survived, indicating engraftment of progenitor cells in both the spleen and bone marrow and reconstitution of hemopoiesis. When transplanted cells were given with galactosyl- or mannosyl-BSA, the survival was markedly reduced in the splenectomized group but was only marginally reduced in the nonsplenectomized group. This suggests that engraftment of transplanted cells in marrow, but not in spleen, was inhibited by these two reagents. The survival of animals given fucosyl-BSA with transplanted cells was 100% (similar to the control group). When asialofetuin was given with transplanted cells, some reduction in survival was noted in the splenectomized group but not in the nonsplenectomized group. The pattern was similar to that of animals given galactosyl-BSA.

Splenic Homing. Table 2 shows the total number of cells and the concentrations of CFU-C, primary CFU-S, and secondary CFU-S in spleen in nonsplenectomized animals. No difference was noted in any of these parameters in the control group compared to the groups in whom transplanted cells were given with galactosyl-, mannosyl-, or fucosyl-BSA. Asialofetuin, given with transplanted cells, actually appeared to enhance homing of these cells to the spleen as there was a significant difference ($P < 0.05$) between this group and that of the control group.

Marrow Homing. Table 3 shows the total number of cells and the concentrations of CFU-S and CFU-C in the tibias of splenectomized and nonsplenectomized animals. In the splenectomized group, in whom only marrow homing occurred, there was a significant reduction ($P < 0.001$) in all these parameters compared to the control group when transplanted marrow cells were given with galactosyl- and mannosyl-BSA, indicating inhibition of homing by these reagents. No such inhibition was noted when transplanted cells were given with the fucosyl reagent.

In nonsplenectomized animals, in whom homing also occurred in spleen, no differences in total cell numbers and CFU-S concentrations were seen when transplanted cells were given with any of the reagents, including asialofetuin. There was, however, a significant reduction in the CFU-C concentration when transplanted cells were given with galactosyl- or mannosyl-BSA or asialofetuin (but not fucosyl-BSA). These differences were small but statistically signifi-

Table 1. Survival of animals infused with bone marrow cells with and without inhibiting reagents

Reagent added	Spleen status	% survival
None (control)	NX	100
	SX	100
Galactosyl-BSA	NX	83
	SX	47
Mannosyl-BSA	NX	92
	SX	57
Fucosyl-BSA	NX	100
	SX	100
Asialofetuin	NX	100
	SX	71

Animals were given 8.5 Gy of total body irradiation and were then infused with 10^5 fresh syngeneic marrow cells with or without the inhibiting sugar reagents. Reagents were added to give a calculated blood level of 10^{-3} M. In each group, two subgroups were studied: animals were either nonsplenectomized (NX), in whom homing occurs both in the marrow and spleen, or they were splenectomized (SX) six weeks before transplantation. In the latter group, only marrow homing of transplanted cells occurs.

Table 2. Total cell number and primary and secondary CFU-S and CFU-C concentrations in the spleen of transplanted animals

Reagent added	Cells per spleen, no. $\times 10^{-6}$	CFU-S		CFU-C
		Primary	Secondary	
None (control)	2.38 ± 0.44	53.5 ± 8.1	59.1 ± 15.6	388 ± 31
Galactosyl-BSA	1.48 ± 0.2	46.1 ± 5.2	49.7 ± 6.8	294 ± 14
Mannosyl-BSA	2.34 ± 0.69	57.2 ± 7.3	70.0 ± 15.3	410 ± 17
Fucosyl-BSA	2.13 ± 0.36	51.8 ± 5.1	62.4 ± 12.0	357 ± 42
Asialofetuin	2.03 ± 0.49	77.6 ± 10.8	102.3 ± 25.5	401 ± 46

Nonsplenectomized animals were radiated and transplanted as described in Table 1. Splenic cell counts were made 12 days after transplantation, and surface colonies were counted as primary CFU-S. Cells were then infused in secondary irradiated recipients to determine its CFU-S concentration (secondary CFU-S) or cultured *in vitro* for the determination of its CFU-C concentration. Significant differences were not noted whether transplanted cells were given with or without various glycoprotein reagents. Only asialofetuin appeared to actually enhance somewhat the homing ($P < 0.05$) of transplanted cells. CFU-S and CFU-C are expressed as colonies per spleen. Values are expressed as the mean ± SD.

cant ($P < 0.05$) when animals were studied 1 week after transplantation. The differences widened in a group of animals studied 2 weeks after transplantation.

The data from the splenectomized group are shown graphically in Fig. 1, which represents the data obtained 1 week after transplantation and again demonstrates significant inhibition of homing to the marrow by galactosyl and mannosyl reagents as well as asialofetuin.

DISCUSSION

These data indicate that the recognition and selective engraftment (homing) of intravenously transplanted hemopoietic cells in the marrow occurs through a molecular mechanism with galactosyl and mannosyl specificities. Thus, in splenectomized mice, in whom homing of transplanted cells is limited to the bone marrow, the survival of animals as well as total cells count and concentrations of CFU-S and CFU-C are reduced when marrow cells are transplanted into lethally irradiated mice in the presence of galactosyl- and mannosyl-BSA but not fucosyl-BSA. A similar reduction is also noted when asialofetuin is used. In this glycoprotein, the glycan moiety terminates in galactosyl residues and, thus, asialofetuin functions similar to galactosyl-BSA. Similarities in the results obtained with asialofetuin and galactosyl-BSA indicate that the inhibition is a function of the galactosyl residues and not other parts of the molecules.

Table 3. Total cell number and CFU-S and CFU-C concentrations in the bone marrow of transplanted animals

	Cells per tibia, no. $\times 10^{-5}$	CFU-S	CFU-C
	Splenectomized		
1 week			
Control	2.62 ± 0.87	11.2 ± 3.3	242 ± 14
Galactosyl-BSA	1.54 ± 0.24	4.7 ± 2.7	85 ± 17
Mannosyl-BSA	1.47 ± 0.42	4.5 ± 1.3	77 ± 15
Fucosyl-BSA	2.16 ± 0.03	10.6 ± 3.3	219 ± 17
Asialofetuin	2.04 ± 0.67	3.3 ± 2.1	21 ± 3
2 week			
Control	4.81 ± 1.26	67.8 ± 11.3	1192 ± 101
Galactosyl-BSA	4.50 ± 1.50	5.3 ± 3.6	163 ± 14
Mannosyl-BSA	3.34 ± 0.82	17.5 ± 6.0	312 ± 20
Fucosyl-BSA	5.36 ± 1.08	57.5 ± 15.1	909 ± 83
Asialofetuin	ND	ND	ND
	Nonsplenectomized		
1 week			
Control	1.87 ± 0.44	6.2 ± 3.4	138 ± 3
Galactosyl-BSA	0.92 ± 0.15	5.0 ± 2.3	39 ± 10
Mannosyl-BSA	1.04 ± 0.20	3.0 ± 1.8	27 ± 6
Fucosyl-BSA	1.94 ± 0.19	4.5 ± 3.0	106 ± 18
Asialofetuin	1.38 ± 0.18	5.2 ± 3.0	13 ± 4

Splenectomized and nonsplenectomized animals were irradiated and transplanted as described in Table 1. After 1 or 2 weeks, tibias were removed and total cell count and CFU-S and CFU-C concentrations were determined. In the splenectomized group, in whom only marrow homing occurs, there was significant reduction ($P < 0.001$) in all parameters when transplanted marrow cells were given with galactosyl- or mannosyl-BSA or asialofetuin. In the nonsplenectomized group (in whom transplanted cells also home in spleen), the only statistically significant ($P < 0.05$) difference was in the CFU-C concentration. ND, not done. CFU-S and CFU-C are expressed as colonies per tibia. Values are expressed as the mean ± SD.

This is consistent with our previous finding that the *in vitro* binding of CFU-S and CFU-C to the marrow stroma also involves membrane lectins with similar specificities (5, 6). Our results, therefore, assign a potential function to membrane lectins that we have found on the membrane of a cloned multipotential hemopoietic stem cell. These lectins show similar specificities, with a K_d of 3.11×10^{-6} M (2.25×10^8 receptors per cell) for galactosyl receptors and a K_d of 6.88×10^{-6} M (6×10^7 receptors per cell) for mannosyl receptors (5). It is probable that similar lectins with similar specificities on the membrane of progenitor cells in the intravenously transplanted marrow recognize specific domains in the glycan moiety of membrane glycoconjugates of marrow stromal cells. The recognition leads to the binding (homing) of transplanted progenitor cells.

By contrast, the homing of transplanted cells to spleen is not affected by these synthetic neoglycoproteins, indicating that different mechanisms are involved in the recognition and homing of transplanted cells by the two hemopoietic tissues. Thus, in nonsplenectomized animals the survival, the total cell count, and the concentrations of primary and secondary CFU-S as well as CFU-C in spleen are not different when transplanted cells are given with any of these reagents.

The finding that in nonsplenectomized animals the concentration of marrow CFU-S in the control group is similar to that in the group to whom transplanted cells were given with inhibiting reagents may reflect primary homing of CFU-S to spleen, with secondary seeding to the marrow when the blood concentration of these reagents was no longer inhibitory. It is of interest that this experimental group shows a difference, compared to control, in the concentration of CFU-C in the marrow. This may reflect a delay in the secondary seeding of CFU-C from spleen so that by one week the proliferation and differentiation of CFU-S into CFU-C has not yet been able to compensate and elevate CFU-C concentration to normal levels. The fact that in the murine system marrow is primarily involved in granulopoiesis whereas spleen is primarily ery-

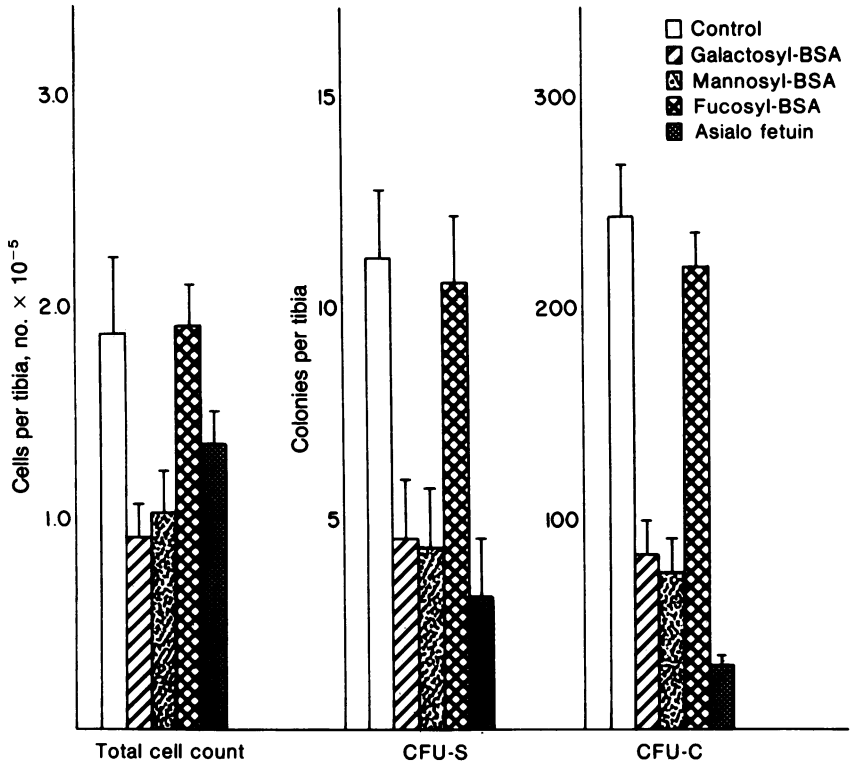

FIG. 1. Total cell count and the concentrations of CFU-S and CFU-C per tibia 7 days after intravenous transplantation of 10^5 bone marrow cells from syngeneic animals into lethally irradiated splenectomized mice. Transplanted cells were preincubated and infused with reagents of known carbohydrate specificities. Galactosyl- and mannosyl-BSA as well as asialofetuin (but not fucosyl-BSA) inhibited engraftment of hemopoietic cells to the marrow.

thropoietic (9) may also be a contributing factor. The enhancement of splenic homing when transplanted cells are preincubated and given with asialofetuin (compared to controls) is consistent with the existent literature (3), which indicates that asialoglycoproteins improve the efficiency of engraftment in the spleen.

The results indicating that the molecular mechanisms of homing in spleen and marrow are different deserve a comment. Anatomically there is a major difference between the two tissues (11). In bone marrow the hemopoietic compartment is separated from the circulating space by a thin layer of continuous endothelium (12). Transplanted cells must then be recognized by the luminal surface of the sinal endothelium and transported to the extravascular space (2). A galactosyl-specific lectin has been identified on the luminal surface of marrow sinus endothelium (13) and may be involved in the recognition and the transport of transplanted stem cells. By contrast, endothelial transport is not a precondition in spleen, where the circulation is largely open (14). Splenic arterioles open directly into splenic cords where blood must traverse a fine-meshed three-dimensional web to reach vascular sinuses. It is in these splenic cords that transplanted cells home. Thus, direct opening of vessels into splenic cords may preclude the requirement for recognition and tissue transport of transplanted cells by endothelium. Transplanted cells can directly enter the parenchyma. This anatomical difference between the two tissues may explain the difference in the mechanism of homing of transplanted cells. Alternatively, the difference in the homing of transplanted cells to the two tissues may reflect a difference in the surface characteristics of their stromal cells and different molecular bases for the binding of progenitor cells to stromal cells.

It is of interest that similar membrane lectins with specificity for mannosyl phosphate have been identified on the lymphocyte surface and have been shown to be responsible for lymphocyte homing in lymph nodes by means of an interaction with the high endothelium of postcapillary venules (15–19).

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