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In vitro homing of hemopoietic stem cells is mediated by a recognition system with galactosyl and mannosyl specificities

(colony-forming unit in spleen/long-term bone marrow culture/neoglycoproteins/bone marrow stroma)

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ABSTRACT **We synthesized a number of neoglycoprotein probes by covalently linking three biologically relevant sugars (mannose, galactose, and fucose) to a protein molecule so as to retain the pyranose (ring) form of sugars necessary for their interaction with lectins. In the presence of galactosyl and mannosyl but not fucosyl probes, the production of CFU-S [colony-forming unit(s) in spleen] and total cells was halted in murine long-term marrow cultures. Cobblestone areas disappeared in these cultures, indicating the inhibition of binding of hemopoietic cells to the stroma. Electron microscopy revealed no alterations of the stroma, and the probes did not have direct cytotoxic or inhibitory effects on the growth of CFU-S or CFU-C [colony-forming unit(s) in culture]. Stroma grown for 5 weeks in the presence of the probes could subsequently support the growth of hemopoietic progenitor cells when the probes were removed from the medium. Conversely, the proliferative capacity of CFU-S in the supernate, grown in the presence of the probes, was retained upon grafting to control stroma. Galactosyl and mannosyl but not fucosyl probes differentially agglutinated CFU-S in whole-marrow-cell suspensions, suggesting the presence of membrane lectins with specificity for these sugars on the surface of CFU-S. We conclude that the binding of CFU-S to marrow stroma (homing) is mediated by a recognition system with galactosyl and mannosyl specificities.**

The earliest event in hemopoiesis involves "homing" of hemopoietic stem cells (HSC) to hemopoietic stroma, which sustains their proliferation and differentiation (1–4). Thus, after lethal irradiation and infusion of a source of stem cells in mice, HSC selectively seek the stroma of spleen (5) and marrow (6). The conceptual frame of marrow transplantation also rests on this selective "homing" (7, 8). Here, too, a source of HSC is introduced into the general circulation in anticipation of their "homing" to marrow stroma.

Homing involves the specific association and binding of HSC and other progenitor cells to stromal cells. The nature of this binding at a molecular level is not clear. In recent years, suggestive evidence has been presented to indicate that sugar–protein interactions may be involved in this phenomenon (9, 10). To explore this hypothesis, we synthesized a number of probes by covalently binding three biologically relevant sugars to a large molecule (bovine serum albumin, BSA) (11). We used these probes to inhibit the binding of HSC to adherent stroma in murine long-term bone marrow culture. We now provide evidence that a recognition mechanism involving galactosyl and mannosyl residues of membrane glycoconjugates may be involved in this phenomenon.

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MATERIALS AND METHODS

Cells and Probes. Bone marrow cells were obtained from femurs and tibiae of 12-week-old C57BL mice. Cells were removed aseptically by flushing with phosphate-buffered saline using a 21-gauge needle and a 1-ml syringe. Clumps of cells were then dispersed by repeated passage through a 21-gauge needle. Cells were then washed twice and suspended at the concentration of 10^8 cells per 0.5 ml in phosphate-buffered saline containing 0.02% sodium azide to prevent irreversible cell agglutination (12).

The method for the synthesis of neoglycoprotein probes has been established in our laboratory and described in detail elsewhere (11). These probes are essentially made by covalent binding of activated sugars in the pyranose (ring) form to a larger protein molecule such as serum albumin. The method fulfills 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 pyranose form, necessary for binding to lectins. (*iii*) The sugar-to-protein ratio is high enough (*ca.* 30–40) so that the probability of interaction with membrane lectins will be high.

Long-Term Bone Marrow Culture (LTMC). This was done essentially as described (13, 14). Marrow content of one long bone (femur or tibia) was flushed with a 21-gauge needle into a culture flask (T-25 Falcon 3013) containing 8 ml of Fischer's medium (GIBCO) supplemented with 20% horse serum and 1 μM hydrocortisone. Cells were then gently pipetted several times to suspend them. Cultures were incubated at 33°C under 5% CO_2 in humidified air, and the supernate was demidepopulated at weekly intervals. After 3 weeks, when confluence was reached, all 8 ml of the supernate was removed, and freshly harvested marrow cells were overlaid at a concentration of 5×10^5 cells per ml (total 8 ml) in the absence or presence of the neoglycoprotein probes at 1 mM. Initial dose–response study using several different concentrations of these neoglycoproteins on binding of granulocyte–macrophage progenitors [CFU-C (colony-forming unit in culture)] indicated this concentration to be inhibitory in the case of galactosyl and mannosyl probes. Subsequent to recharging, the cultures again were demidepopulated at weekly intervals, and the total cell count and concentration of CFU-S (colony-forming unit in spleen) in the removed supernate were determined. In parallel cultures, the adherent layer was washed twice with Fischer's medium and then removed by Teflon spatula, and cells were resuspended. The total cell count and CFU-S concentration were determined in this adherent layer every 2 weeks.

In some cultures containing galactosyl and mannosyl probes, after 5 weeks of incubation the supernate was totally

Abbreviations: HSC, hemopoietic stem cell; LTMC, long-term bone marrow culture; CFU-S, colony-forming unit in spleen; CFU-C, colony-forming unit in culture; BSA, bovine serum albumin; CSA, colony-stimulating activity.

removed, and the stroma were overlaid by nonadherent cells obtained from 5-week-old control cultures. This was done to determine if the stroma exposed to these probes still retained the potential for the support of hemopoietic cell proliferation. Conversely, the cells in the supernate, after 1 week of exposure to the probes, were washed and incubated with control stroma to determine the functional integrity of CFU-S in this supernate.

Culture flasks were studied at weekly intervals (before the change of medium) by phase microscopy at low magnification. The entire surface of the culture flask was screened, and the number of "cobblestones" was scored. A "cobblestone" was defined as an accumulation of >30 small round cells bound to the stroma so that they could not be dislodged by tilting the flask. For each time point, 10 flasks were established. In parallel cultures, several round 12-mm coverslips were placed in the flasks and subsequently removed, fixed in modified Karnovsky fixative in cacodylate buffer (pH 7.2) for 2 hr at 4°C, and then postfixed in 1% similarly buffered OsO_4 for 1 hr. The specimens were processed for electron microscopy as described (13).

CFU-S and CFU-C Assay. To assay for CFU-S, an appropriate number of cells were injected into lethally irradiated (8.5 Gy) syngenic mice. Eight recipients were used for each assay; 8 days later spleens were removed and placed in Bouin fixative, and the colonies were counted under a dissecting microscope (5). For CFU-C assay, 5×10^4 cells were incubated in 0.3% semisolid agar in α medium containing 20% horse serum (15). As a source of colony-stimulating activity (CSA), 20% medium conditioned with mouse peritoneal cavity macrophages (16) was incorporated into the agar. Cultures were maintained for 7 days at 37°C in 5% CO_2 in humidified air. Cultures were then examined under a phase microscope, and colonies containing >50 cells were counted as CFU-C.

Differential Agglutination Technique. For differential cell agglutination, marrow cell suspensions (10^8 cells per 0.5 ml of phosphate-buffered saline) were incubated in 0.7 × 10 cm polystyrene tubes with the desired sugar-BSA (final concentration, 1 mM in phosphate-buffered saline) for 60 min at 4°C (17). In this method, those marrow cells that possess membrane receptors capable of binding specific sugars undergo agglutination. This leaves other cells in monodispersed form. The cells were then gently layered with a Pasteur pipette on top of a BSA solution (5% wt/vol; total volume of 7 ml) in a 15-ml conical tube. Cells were then centrifuged for 2 min at 50 × g. Unagglutinated cells remained at the top of the BSA solution. Most agglutinated cells formed clumps (as examined by phase microscopy) that could not be disaggregated easily. These clumps sedimented to the bottom of the tube. Some cells also formed much smaller clumps that remained in the BSA layer. In contrast to the agglutinated fraction, these clumps could easily be disaggregated.

Cells in each fraction were collected and incubated for 15 min at 4°C with 0.2 M of competing sugar solutions in phosphate-buffered saline to disperse the cells and obtain single-cell suspensions again. The dispersed cells were collected by cytocentrifugation, washed thrice with phosphate-buffered saline, and counted in a hemocytometer (representative samples were smeared and stained with Wright–Giemsa for differential counting). CFU-S concentration was then determined, and the results were compared to controls by using Student's t test. To determine the specificity of agglutination, parallel experiments were done in the presence of a 100-fold excess of the appropriate competing sugars to inhibit agglutination.

RESULTS

Inhibition of CFU-S Binding. In the presence of galactosyl and mannosyl but not fucosyl probes, the concentration of CFU-S in both the supernate and the adherent layer of LTMC declined compared to that in control cultures. The decline was evident as early as 1 week and thereafter gradually approached zero (Table 1). A similar pattern was also noted in total cell production, which approached zero by week 6 [Fig. 1 (see the control in Fig. 3)]. No decline was observed when BSA alone or diffusible sugars alone were used in a similar molar concentration.

Table 1. Number of CFU-S and "cobblestones" in LTMC in the presence of various neoglycoprotein probes

Addition	CFU-S* and cobblestones† in LTMC after 1–7 wk in culture, no.			
	1	3	5	7
	CFU-S in supernate			
Control	486 ± 89	377 ± 178	400 ± 37	242 ± 44
Gal-BSA	132 ± 11	17 ± 4	5 ± 4	0
Man-BSA	110 ± 15	12 ± 3	4 ± 4	0
Fuc-BSA	380 ± 61	359 ± 88	275 ± 69	202 ± 78
	CFU-S in adherent layer			
Control	987 ± 82	407 ± 81	93 ± 44	87 ± 14
Gal-BSA	98 ± 40	13 ± 8	7 ± 5	0
Man-BSA	83 ± 33	10 ± 6	0	0
Fuc-BSA	712 ± 68	402 ± 89	79 ± 22	71 ± 16
	Cobblestones			
Control	477 ± 63	290 ± 28	263 ± 24	239 ± 42
Gal-BSA	138 ± 19	18 ± 4	9 ± 3	4 ± 4
Man-BSA	170 ± 36	23 ± 7	5 ± 5	2 ± 2
Fuc-BSA	395 ± 88	360 ± 44	218 ± 40	180 ± 37

Gal, galactose; Man, mannose; Fuc, fucose.
*CFU-S was expressed as colonies per flask. In this and the subsequent tables, each point is expressed as the mean and standard deviations of 3–10 experiments.
†Cobblestone was defined as an accumulation of >30 small round cells bound to the stroma. The mean number of cobblestones per flask ± standard deviation is shown.

Table 1 also shows the number of cobblestones per flask as quantified by phase microscopy. They initially decreased somewhat in control cultures but stabilized after 3 weeks. In the presence of galactosyl and mannosyl but not fucosyl probes, they declined further, and this was again evident as early as 1 week and then gradually approached zero. Even the few cobblestones that were seen were much smaller and also less tightly bound to the stroma (compared to control) as observed by tilting of the flask. Fig. 2A shows the morphol-

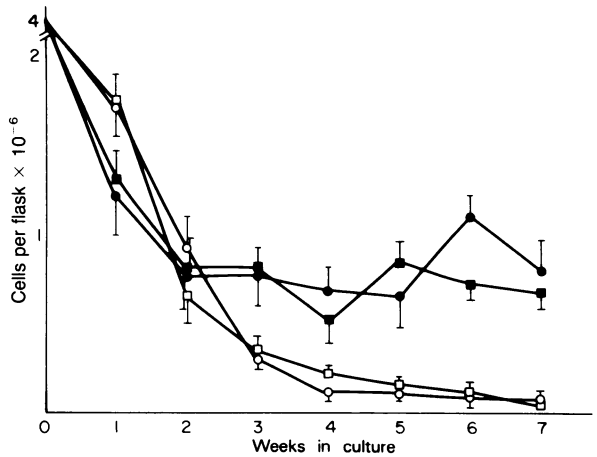

FIG. 1. Weekly total cell count in LTMC. In control cultures the weekly cell count declines initially but stabilizes after 2 weeks. In the presence of galactosyl (○) and mannosyl (□) but not fucosyl (■) probes, the initial decline continues and cell production halts altogether. Each point indicates the mean and standard deviation of 3–10 experiments. ●, Control.

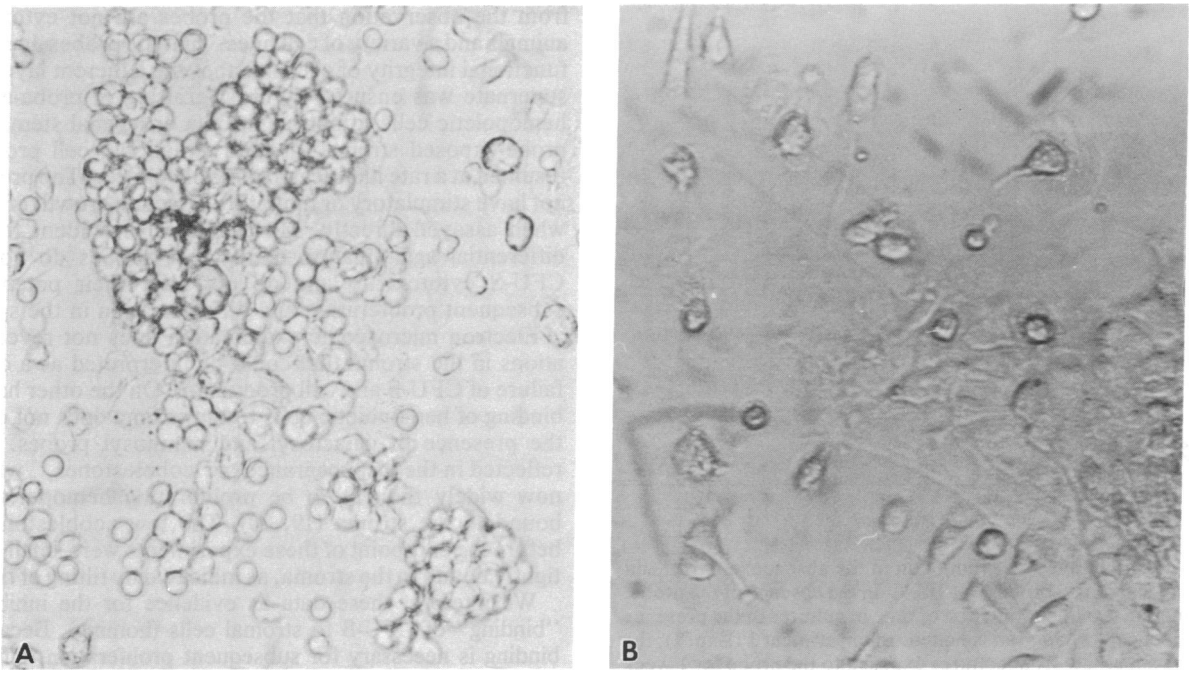

FIG. 2. (*A*) Phase micrograph of a cobblestone in the control culture. (×100.) (*B*) Culture grown in the presence of galactosyl-BSA. Only stromal cells are seen. (×100.)

ogy of cobblestones in the control culture as seen by phase microscopy. In cultures containing galactosyl and mannosyl probes, only the adherent stromal cells were seen (Fig. 2*B*).

Absence of Probe-Induced Cytotoxicity. To ensure that the probes were not cytotoxic, stromal cells were harvested at weeks 1, 3, 5, and 7 from cultures grown in the presence of probes as well as from control cultures. The viability of these cells in all cultures as determined by trypan blue exclusion exceeded 99%. The absence of probe-induced cytotoxicity was also confirmed by growing CFU-C in the presence and absence of these probes, with and without a standard source of CSA (Table 2). In the absence of CSA no growth was obtained, indicating the absence of CSA of the probes. In the presence of a standard source of CSA and 1 mM probe, no inhibition of colony growth was obtained, indicating the absence of cytotoxic or colony inhibitory effect. Moreover, the growth of several human and murine cell lines was examined in the presence of 1 mM probe concentration. No cytotoxicity or growth inhibition was noted. Intravenous injection of probes into normal mice at a dose calculated to give a 5 mM concentration did not lead to mortality or morbidity in these animals even when injections were repeated at weekly intervals.

Table 2. Effect of probes on the clonal growth of CFU-C from normal marrow

Addition	CFU-C per 10^5 marrow cells, no.
Gal-BSA	0
Man-BSA	0
Fuc-BSA	0
CSA	260 ± 16
+ Gal-BSA	264 ± 10
+ Man-BSA	252 ± 14
+ Fuc-BSA	282 ± 12

Gal, galactose; Man, mannose; Fuc, fucose. Bone marrow cells were incubated with neoglycoproteins (1 mM) in the presence or absence of standard CSA (20% vol/vol) in 0.3% agar medium supplemented with 20% horse serum for 7 days. Mouse peritoneal-conditioned medium was used as the standard source of CSA at the maximum stimulatory concentration.

Functional Potential of Probe-Exposed CFU-S and Stroma. To ensure that the exposure to galactosyl and mannosyl probes did not interfere with the viability or functional potential of CFU-S, cultures were grown for 1 week in the presence of these probes. The supernate (containing CFU-S) was then removed, washed free of probe, and grafted onto the hemopoietic cell-free adherent layer. Cell production (including CFU-S and CFU-C) was then monitored at weekly intervals. Cumulative cell production is shown in Fig. 3 (experiment 1), demonstrating a curve almost overlapping with control cultures and indicating that, in the absence of probe, the supernate pretreated with probe for 1 week was still capable of cell proliferation at a rate comparable to the control culture.

To ensure that the exposure to the probes did not alter the potential of the adherent stroma to support hemopoiesis, cultures were grown for 5 weeks in the presence of probes. The supernate was then removed, and the adherent layer was washed free of probes and overlaid with stroma-free supernate (containing CFU-S) from control cultures. The results are shown in Fig. 3 (experiment 2). Cumulative cell production, which had been halted in the presence of the probe, now resumed and the slope of the curve was identical to that of control cultures. This indicated that the exposure to these probes did not alter the potential of stromal cells for hemopoietic support.

Electron Microscopy. Electron microscopic features of the stroma in these cultures have been described (14, 18). The stroma essentially consisted of two cell types, the first usually elongated and spreading, forming a "floormat" and containing numerous filamentous structures, and the second with round contours, showing many surface ridges in scanning electron microscopy, some of which appeared to be adherent to the flask surface. These cells contained lysosomes and phagosomes in transmission electron microscopy and were considered to be macrophages. The morphology of stromal cells and the proportion of the two cell types were similar in control and probe-containing cultures.

Differential Agglutination. Both galactosyl and mannosyl probes selectively agglutinated CFU-S so that their concentration rose in the agglutinated fraction and declined in the

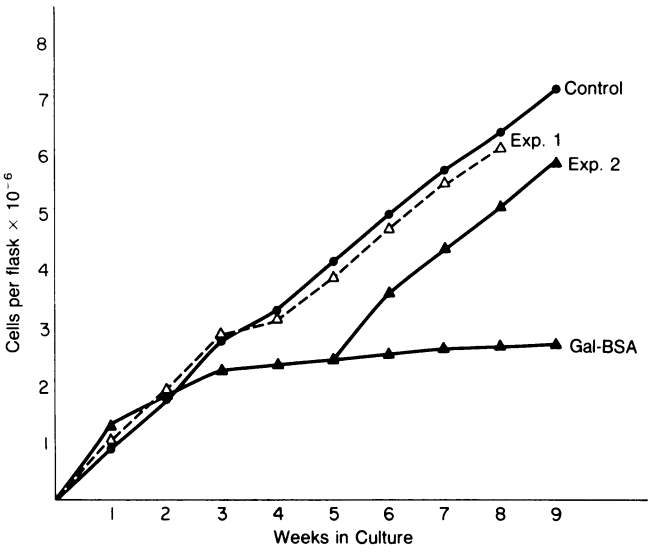

FIG. 3. Cumulative cell production in the absence (control) and presence of galactosyl probe (Gal-BSA). In the absence of the probe, cell production is a linear function of time in culture. In the presence of the probe, cell production is halted. In experiment 1 (Exp. 1), the cells in the supernate from cultures exposed to the probe for 1 week were washed and grafted onto control hemopoietic cell-free stroma. Cell production resumed at a rate identical with that of control cultures. In experiment 2 (Exp. 2) the stroma exposed to the probe for 5 weeks was washed free of the probe, and the supernate from control cultures was grafted onto it. Again, cell production resumed. Note that the slope of the curve is again identical to control cultures. Similar results were obtained with the mannosyl probe. Each point represents the mean of three experiments.

nonagglutinated fraction. The selective agglutination was inhibited in the presence of excess free competing galactose and mannose, indicating the specificity of agglutination (Table 3). A fucosyl probe did not differentially agglutinate CFU-S.

DISCUSSION

These results indicate that in the presence of galactosyl and mannosyl probes, the production of CFU-S in LTMC is halted. BSA alone and the fucosyl-containing probe do not have this effect, suggesting that the galactosyl and mannosyl residues are responsible for this effect. Halting of CFU-S production leads to the halting of total cell production in cultures. The CFU-S virtually disappears in both the supernate and the adherent layer in the presence of these probes.

That this effect is not due to probe cytotoxicity is evident from the observation that the probes are not cytotoxic to animals and a variety of cell lines. That the probes do not alter functional integrity of cells in either the adherent layer or the supernate was ensured by cross-grafting of probe-exposed hemopoietic cells to normal stroma or normal stem cells to probe-exposed stroma. In both situations, cell production resumed at a rate identical to control cultures. The probes did not have stimulatory or inhibitory effect on growth of CFU-C when assayed directly. Moreover, as is evident from our differential agglutination data, these probes do not show CFU-S cytotoxicity and do not alter their potential for subsequent proliferation and differentiation in the spleen.

Table 3. Differential agglutination (Aggl.) of CFU-S with various neoglycoproteins

Addition	CFU-S, colonies per 10^5 cells		
	Fraction		Ratio
	Aggl.	Non-Aggl.	Aggl./Non-Aggl.
Gal-BSA	66.0 ± 11.8	26.2 ± 4.1	2.52*
+ excess Gal†	51.5 ± 3.1	44.4 + 5.2	1.16
Man-BSA	69.5 ± 5.1	27.8 ± 7.8	2.50*
+ excess Man†	55.6 ± 10.7	40.6 ± 6.4	1.37
Fuc-BSA	42.2 ± 4.7	41.8 ± 6.4	1.01
+ excess Fuc†	ND†	ND†	—
Control‡	—	43.6 ± 6.0	—

*$P < 0.001$.
†Inhibition experiments were done by incubating bone marrow cells with neoglycoprotein probes in the presence of excess galactose (Gal) or mannose (Man), respectively. ND, not determined [in the case of fucose (Fuc)].
‡Sham-treated control.

Electron microscopic examination does not reveal alterations in the stroma that could be interpreted as a cause of failure of CFU-S and cell production. On the other hand, the binding of hemopoietic cells to the stroma does not occur in the presence of galactosyl and mannosyl probes. This is reflected in the disappearance of "cobblestones," which are now widely thought to be proliferating hemopoietic cells bound to the stroma (19, 20). The few cobblestones seen before the end-point of these experiments were small and less tightly bound to the stroma, as indicated by tilting of the flask.

We interpret these data as evidence for the inhibition of "binding" of CFU-S to stromal cells (homing). Because this binding is necessary for subsequent proliferation, differentiation, and maturation of progenitor cells, cell production is halted in these cultures. This binding may involve an interaction between galactosyl and mannosyl residues of membrane glycoconjugates on the one side and, on the other side, membrane lectins capable of recognizing and interacting with galactosyl and mannosyl residues of neoglycoprotein probes. Thus, these probes can differentially agglutinate CFU-S, and this agglutination is inhibited in the presence of excess competing galactose or mannose, indicating the specificity of the reaction. Moreover, using ^{125}I-labeled probes, we have demonstrated the presence of galactosyl and mannosyl but not fucosyl receptors on the membrane of a cloned multipotential hemopoietic stem cell line (B6SUT). Galactosyl binding occurs with a K_d of 3.11×10^{-6} M with the receptor number calculated as 2.25×10^8 per cell. Corresponding figures for mannosyl receptors are 6.88×10^{-6} M and 6.0×10^7.

The specific binding of HSC to stromal cells in LTMC (homing) via a recognition mechanism based on sugar–protein interaction has a precedent. A similar mechanism also operates in the homing of lymphocytes in various lymphoid organs (21, 22). However, lymphocyte-homing receptors have been well characterized owing to the availability of techniques to obtain highly purified preparations of lymphocyte subpopulations. Such techniques are currently difficult to apply to the purification of HSC, although two recent reports have utilized lectins in association with fluorescent-activated cell sorting to obtain highly purified preparations of HSC (23, 24).

Homing of HSC to the stroma is clearly a prerequisite for proliferation and subsequent differentiation of HSC, as its inhibition in the present study inhibited these processes as well. The mechanism whereby homing leads to the subsequent proliferation and differentiation is unclear and requires further study.

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