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N-linked sialyated sugar receptors support haematopoietic cell-osteoblast adhesions

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Summary

Haematopoietic progenitor cells proliferate and develop predominantly when they adhere to bone marrow stromal cells such as osteoblasts. Therefore, changes in adhesion may be a common mechanism by which stem cells survive, mature and properly traffic between the bone marrow and the circulation. To characterize these adhesion molecules, we reduced the bone marrow cavity to a simple adhesion assay between KG1a (a $CD34^+$ haematopoietic cell line) and osteosarcoma monolayers (MG-63 or SaOS-2). The data demonstrated that adhesion was mediated by cell-to-cell rather than cell-to-matrix contact, was sensitive to trypsin, calcium chelators and glycosylation inhibitors. Selective pretreatment attributed the constitutive binding to N-linked glycans on KG1a. When carboprocessing was inhibited later at the high mannose intermediate (via deoxymannojirimycin), adhesion was retained. Surprisingly, binding of KG1a to SaOS-2 increased past constitutive levels as doses of tunicamycin or deoxymannojirimycin dropped. Selective pretreatment suggested that this 'inducible' binding resides with molecule(s) on SaOS-2. If the terminal sialic acid was digested (via neuraminidase), this induced response was duplicated. These data, verified in primary cells, suggest that the initial tethering between blood and bone cells in this model is probably due to heavily glycosylated, rapidly processed protein(s) on both cell types.

Keywords: osteoblasts, stem cell, bone marrow, microenvironment, adhesion.

In adults the bone marrow is the sole microenvironment that supports all activities of normal haematopoiesis. Haematopoietic stem cells (HSCs) home to bone marrow during development and transplantation where pluripotency is maintained and maturity is facilitated (Nelissen *et al*, 2000). Both of these activities are likely to require different growth factors and cellular contexts. Bone marrow stromal cells are comprised of several different cellular populations including fibroblasts, endothelial and reticular cells, adipocytes and osteoblasts (OB) which serve as a rich source of growth factors (Eaves *et al*, 1991; Wilson *et al*, 1991; Guba *et al*, 1992; Kittler *et al*, 1992; Deryugina *et al*, 1994; Vormoor *et al*, 1994; Muller-Sieburg & Deryugina, 1995; Wineman *et al*, 1996; Lemischka, 1997; Moore *et al*, 1997; Mantel *et al*, 1999; Phillips *et al*, 2000). At present, it is difficult to discern the relative importance of each of these populations in haematopoiesis. However, most evidence points to the importance of a functional dialogue that is established between HSCs and bone stromal cells (Verfaillie, 1992; Satoh *et al*, 1997). HSCs and stromal cells collaborate in the production of the extracellular bone marrow matrix cytokine synthesis, resulting in the formation of the various blood cells (Taichman *et al*, 1997a; Gupta *et al*, 1998; Koller *et al*, 1999).

From the initial engraftment of HSCs in the marrow to the emergence of mature blood phenotypes in the circulation, changes in adhesion correlate with these definitive changes in blood cell maturation and trafficking (Lund-Johansen & Terstappen, 1993). Accordingly, both haematopoietic and

stromal cells express a wide spectrum of adhesion molecules that facilitate these interactions. Relevant cell surface molecules include integrins, cadherins, selectins, mucin-like and members of the immunoglobulin G (IgG) superfamily (Clark *et al*, 1992; Simons *et al*, 1994; Turner *et al*, 1998). The specificity and avidity of any single adhesion is therefore likely to be dependent on multiple adhesion molecules acting transiently, in concert, and within the context of the marrow and the circulation.

In the marrow, HSCs are in close proximity to endosteal bone surfaces rather than being randomly distributed throughout the marrow cavity (Gong, 1978; Islam *et al*, 1990). These observations and recent demonstrations that HSCs lodge near endosteal surfaces during bone marrow engraftment (Lord *et al*, 1975; Cui *et al*, 1996; Nilsson *et al*, 1997; Quesenberry & Becker, 1998), suggest that the resident cells that are localized to these microenvironments are critical regulators of stem cell trafficking. In marrow, mature OBs are found on endosteal surfaces and share several phenotypic characteristics with the haematopoietic-supportive stromal cell lines (Dorheim *et al*, 1993; Kuznetsov *et al*, 1996; Taichman *et al*, 1996; Nelissen *et al*, 2000). Osteoblasts are cells of mesenchymal origin that are primarily responsible for the formation of a calcified extracellular matrix (ECM). In mammals, OB-derived matrices serve several essential functions: a support for locomotion, a reservoir for essential minerals and a housing to protect vital organs from injury.

Previous investigators have shown that to survive *in vitro* in the absence of exogenous cytokines, haematopoietic progenitors and long-term culture initiating cells (LTC-IC) must be co-cultured on stromal cells (Sutherland *et al*, 1990). We have observed that OBs can support progenitor and LTC-IC activities (Taichman *et al*, 1996, 2001; Taichman & Emerson, 1998) and facilitate engraftment across human leucocyte antigen boundaries (El-Badri *et al*, 1998). Moreover, we have demonstrated that the proximity of CD34$^+$ bone marrow cells to OBs can induce the synthesis of several cytokines by OBs (Taichman *et al*, 1997a, 2000a). However, progenitor cell and LTC-IC survival on OBs requires direct cell–cell contact, despite the elaboration of soluble cytokines that are necessary but clearly not sufficient for the maintenance of haematopoietic cells on OBs (R. S. Taichman, unpublished observations). This suggests that direct cell–cell contact of HSCs on OBs is critical to ensure HSC survival.

In the present report, we focused on the initial tethering event that permits stem cell survival on OBs. Using primary and established OB and stem cell lines, we found that the adhesion was mediated by cell-to-cell, rather than cell-to-matrix interactions, which were trypsin sensitive and required the presence of divalent cations. Using glycosylation inhibitors, we determined that a sialogalactose/mannose-specific lectin mediates the initial binding event between these two cell types. Furthermore, we provide evidence that the interruption of normal post-translational processing of complex carbohydrates is sufficient to modulate immediate and profound changes in adhesion following cell–cell contact. These studies lay the groundwork to identify the molecules involved in osteoblastic/haematopoietic cell adhesions. The identification of HSC/OB interactions may have a significant impact on our understanding of the biology of interactions of blood and bone cells in health and disease.

Materials and methods

Cell lines and culture

Human osteosarcoma cell lines MG-63 [CRL1424; American Type Culture Collection (ATCC), Manassas, VA, USA] and SaOS-2 (85-HTB; ATCC) were maintained in minimal essential medium (MEM) with Earle's salts or McCoy's 5A medium, respectively, 1% penicillin/streptomycin antibiotic stock, 1% L-glutamine with 10% heat inactivated fetal bovine serum (FBS; Invitrogen Corp., Carlsbad, CA, USA). Once confluent, cells were detached using 0·05% (w/v) trypsin-EDTA in phosphate-buffered saline (PBS; Invitrogen) and reseeded at $2{\cdot}5–5{\cdot}0 \times 10^5$ cells/75 cm^2. For experiments, cells were replated at an initial density of $1{\cdot}0 \times 10^4$ cells/well in a 96-well flat bottom plate (Becton Dickinson, Franklin, NJ, USA) with complete medium changes on days 5, 7, 10 and 14 in the presence of β-glycerol phosphate (10 mmol/l) and L-ascorbate (50 μg/ml). The human acute myeloid leukaemia cell line KG1a (CCL-246.1; ATCC) was maintained in a suspended culture of Iscove's modified Dulbecco's medium (IMDM; Invitrogen) containing 1% antibiotic stock, 1% L-glutamine, 20% FBS at an initial density of $2–3 \times 10^5$ cells/ml. All cultures were maintained at 37°C, 5% CO_2 and 100% humidity.

Isolation of human OBs and CD34$^+$ bone marrow cells

Enriched human OB cultures were established using modifications of the methods described by Robey and Termine (1985) (Taichman & Emerson, 1994). Normal human trabecular bone was obtained from orthopedic surgery patients under a protocol approved by the University of Michigan's Investigational Review Board. Bone tissue was incubated in bacterial collagenase as described previously in Dulbecco's MEM (DMEM; Invitrogen) (Taichman & Emerson, 1994). The explants were placed into culture until confluent monolayers were produced in a 1:1 (vol/vol) mixture of DMEM/F12 with low Ca^{+2} and 10% heat inactivated FBS. Thereafter, the cultures were maintained in calcium replete DMEM medium, 10% heat inactivated FBS, antibiotics, 10 mmol/l β-glycerol phosphate and 10 μg/ml L-ascorbate, hereafter referred to as 'experimental medium'. To verify that the cells expressed an OB phenotype, the cultures were screened for the expression of the OB-specific protein osteocalcin (osteocalcin$^+$) by reverse transcription polymerase chain reaction as previously detailed (Taichman & Emerson, 1994, 1996). In addition, the OB cells express several additional features of the OB phenotype. These include expression of mRNA, matrix Gla protein, osteonectin

and type I collagen (Gerstenfeld *et al*, 1987; Robey *et al*, 1987). Moreover, high levels of alkaline phosphatase activity is expressed by the OB cells, which mineralize their ECM, albeit weakly in primary culture and only after extended culture periods (Taichman & Hauschka, 1992). However, the cells do not express mRNA for bone sialoprotein, nor do they appear to express parathyroid hormone (PTH) receptors, as they fail to generate cyclic adenosine monophosphate in response to PTH, nor express mRNA for the receptor (R. S. Taichman unpublished observations). To evaluate the purity of the osteoblastic cells, we utilized stem cell factor (SCF) as a marker of bone marrow fibroblasts to determine the level of contamination that could detect fibroblast populations in our OB preparations. By mixing $SCF^{(-)}$ OB populations with $SCF^{(+)}$ stromal populations, we detected a 1·0% contamination of bone marrow stromal cells in a given 'OB' preparation (Taichman *et al*, 2000b). Therefore, as commonly used, the OB populations were at least 99% pure of contaminating stromal fibroblasts, with a possible maximal contamination of 1% stromal cells. However, until a 'universal' human stromal cell marker is established, which clearly distinguishes immature OBs from stromal cells (i.e. not dependent upon late OBs maturation markers), it is not possible to rule out some level of stromal fibroblastic cell contamination.

Human bone marrow cells were obtained from healthy adult volunteers by iliac crest puncture and aspiration into preservative-free heparin under a protocol approved by the University of Michigan's Investigational Review Board. Mononuclear cells were isolated by density separation on Ficoll-Hypaque (specific gravity 1·077). Following two rounds of plastic adherence at 37°C for 1 h each in IMDM with 10% FBS, 10% equine serum and 1 μmol/l hydrocortisone (Invitrogen), the non-adherent cells were recovered. $CD34^+$ bone marrow cells were isolated by positive immunoselection from the low density non-adherent cell fractions (Miltenyi Biotec Inc., Auburn, CA, USA).

Liquid culture of $CD34^+$ bone marrow cells and LTC-IC assay

Triplicate $CD34^+$ bone marrow cells were seeded directly onto confluent OB monolayers in DMEM/F12 (1:1 vol/vol containing 10% heat inactivated FBS, antibiotics, 10 mmol/l β-glycerol phosphate and 10 μg/ml L-ascorbate) at a final density of 1×10^4 cells/cm^2 for 14 d, either in direct contact or separated by porous 0·4 μmol/l micromembranes (Trans-Well®, Corning Inc. Life Sciences, Acton, MA, USA). At 2 weeks, the cultures were harvested by trypsinization (20 min, 37°C) and prepared for light microscopy, where cell number and morphology was determined, and for progenitor (colony forming units, CFU) or LTC-IC assays as described previously (Taichman *et al*, 1996). For electron microscopic examinations, the cell layers were washed and fixed overnight in 2·5% glutaraldehyde in 0·1 mol/l Sorensen's phosphate buffer, pH 7·4. Thereafter, the samples were postfixed (2 h) in 1% buffered osmium tetroxide, infiltrated with 3:1, 1:1 and 1:3 parts of absolute ethanol, exposed to 2-hydroxypropyl methacrylate (HPMA) for 1–2 h under a vacuum following ethanol exposure, subjected to two changes of HPMA and infiltrated with 3:1, 1:1 and 1:3 parts HPMA:epon epoxy resin for 4 h in a vacuum. Final polymerization was accomplished for 48 h at 55°C under a vacuum. Thin sections were prepared using a diamond knife and double stained with uranyl acetate for examination with a Philips 300 TEM (Philips MD, Eindhoven, the Netherlands).

Adhesion assays

The KG1a or $CD34^+$ bone marrow cells were labelled with 2·5 μg/ml of the lipophilic dye 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM, B-3051; Molecular Bioprobes, Inc., Eugene, OR, USA) in IMDM for 30 min at 37°C, and washed in PBS. Thereafter, the cells were allowed to equilibrate for 30 min to reduce non-specific background, and subsequently resuspended in PBS containing Ca/Mg. MG-63, SaOS-2 or primary OB monolayers were prepared for adhesion assays by washing twice with PBS. In the adhesion assay, haematopoietic cells were introduced to monolayers at a final concentration of 10 cells/100μl/well. Then the plates were spun at 50 ***g*** for 5 min at 4°C and allowed to incubate for an additional 10 min at 4°C. The non-adherent cells were removed in three subsequent washes and the remaining fluorescence was quantified in a 96-well fluorescent plate reader (IDEXX Laboratories, Westbrook, ME, USA). In some cases, the $CD34^+$ primary cells were recovered from the OB monolayers by light trysinization and progenitor (CFU) assays were performed as described previously (Taichman *et al*, 1996). Here progenitor colonies were characterized as either granulocyte/macrophage CFU (CFU-GM), erythroid burst-forming units (BFU-E) or mixed CFU (CFU-GEMM).

In inhibition studies, test reagents were introduced to the haematopoietic cells (KG1a or $CD34^+$ cells) at the second incubation step and the pellet washed with PBS. Likewise, OB or osteosarcoma cultures were washed in PBS, exposed to test reagent and washed again. Both cell types were either reconstituted in PBS (where indicated as a pretreatment) or reconstituted in PBS and the volume adjusted with agent (if a co-incubation). Inhibitors include tunicamycin (*Streptomyces lysosuperficus*; 654380; Cabiochem, La Jolla, CA, USA), 1-deoxymannojirimycin hydrochloride (1,5-dideoxy-1,2-imino-D-mannitol) (D-9160; Sigma, St Louis, MO, USA), neuraminidase (*Clostridium perfringens*; N 2876; Sigma), albumin and coupled glycoproteins (A7638, 5908, 1034, 8303, 5918, 1159; Sigma). For antibody inhibition studies, 1–10 μg/ml were employed as suggested by the manufacturer for function blocking antibodies, which included CD11a (α_L), CD29 (β_1), CD31 (gpIIa), CD44, CD54, CD58 (lymphocyte function-associated antigen 3; LFA-3), intracellular adhesion molecule 1 (ICAM-1), ICAM-3, LFA-1 ($\alpha_L\beta_2$), Mac-1 ($\alpha_M\beta_2$), vascular

adhesion molecule 1 (VCAM-1), very late antigen 4 (VLA-4; $\alpha_4\beta_1$), VLA-5 ($\alpha_5\beta_1$), the vitronectin receptor ($\alpha_V\beta_3$) and L-selectin (Immunotech, Franklin Lakes, NJ, USA).

Neuraminidase activity

Neuraminidase activity was detected using the same colorimetric assay as described by the manufacturer to define activity and to evaluate quality control (Sigma, EC 3.2.1.18). Briefly, test substrate (bovine submaxillary mucin at 0·16% w/v) was allowed to incubate at 37°C for 10 min with 0·002–0·004 U neuraminidase to yield N-acetylneuraminic acid (NANA) and asialic acid. Properly buffered at pH 5·0 and extracted, liberated NANA will quantitatively absorb at 550 nm. A standard curve to determine liberated product was generated by plotting micrometre samples of NANA *versus* absorbency at 550 nm.

Statistical analyses

Arithmetic mean values and standard deviations were calculated using Instat 1.14 software (GraphPad, San Diego, CA, USA), and presented as mean cells or fluorescent units/well. Analysis of variance (ANOVA) was utilized to determine statistical significance to a level of $P < 0{\cdot}05$. Tukey's HSD *post hoc* test was utilized for pair wise comparisons.

Results

The binding CD34+ bone marrow progenitors to human OBs in vitro

In vivo, primitive HSCs are closely approximated with the endosteal bone surfaces rather than randomly distributed throughout the marrow cavity (Hermans *et al*, 1989; Lord, 1990). *In vitro*, tight adhesion of human CD34+ bone marrow cells to primary human OBs was observed (Fig 1A and B). These *in vivo* and *in vitro* observations strongly suggest that adhesion plays an important role in haematopoiesis. Therefore, we explored further the role that cell–cell adhesion plays during OB-supported haematopoiesis. For these investigations, human CD34+ haematopoietic bone marrow cells were seeded directly onto OB monolayers in the presence or absence of recombinant cytokines for 2 weeks. In some cases, the haematopoietic cells were separated from the OBs by means of a porous micromembrane. At the conclusion of the culture period the haematopoietic cells were prepared for either electron and light field microscopic examinations or treated with trypsin to recover the cells, and prepared for functional CFU and LTC-IC assays. As shown in Fig 1A and B, haematopoietic progenitor cells were tightly associated with osteoblastic monolayers (Fig 1A), and grew in discrete colonies on the surface of the OBs. Examination of the haematopoietic cells by functional CFU and LTC-IC assay

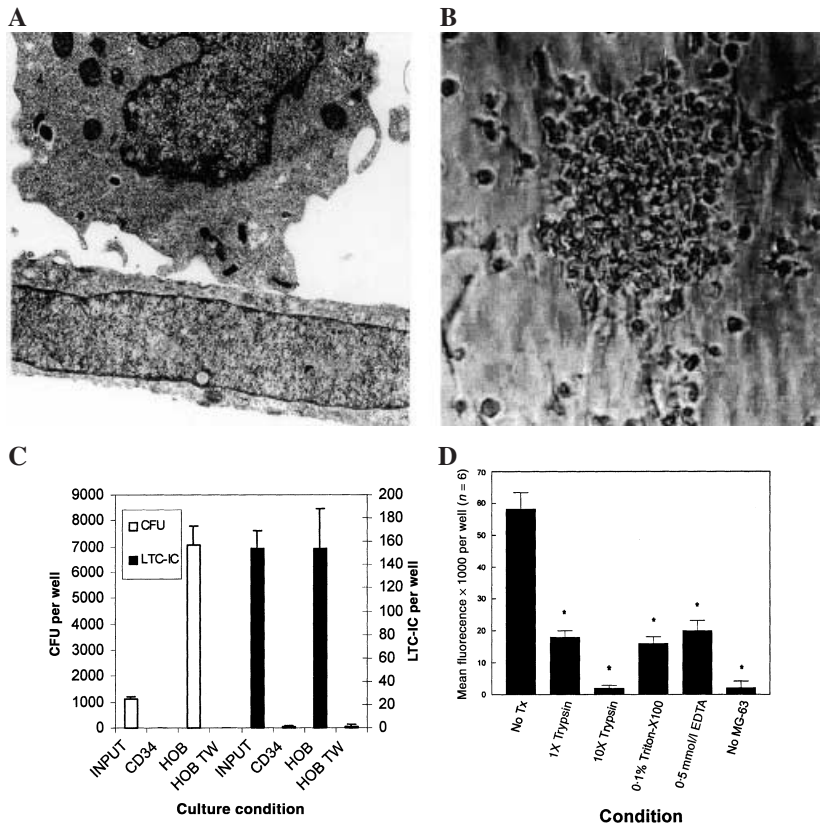

Fig 1. The adherence of haematopoietic cells to osteoblasts. CD34+ were cultured either in the presence or absence of confluent human osteoblasts monolayers in 96-well tissue culture plates at a final density of 1×10^4 cells/well either in direct contact (HOB) or separated by porous micromembrane (HOB-TW) and prepared as described in 'Materials and Methods' for transmission electron microscopic examination (original magnification ×8900) (A) or photographed 20× using light microscopy (B). (C) The haematopoietic cells were recovered by trypsinization and prepared for progenitor (CFU) or LTC-IC assay. (D) KG1a cells were labelled, and adhesion assays were performed on MG-63 osteosarcoma cells. Where indicated, MG-63 cells were exposed to 0·1% Triton X-100, 1 and 10X trypsin for 10 min or the adhesion assays were performed in the presence of 0·5 mmol/l EDTA. *Significantly different ($P < 0{\cdot}05$) from non-treatment control (No Tx).

revealed that, in the presence of OBs, the majority of haematopoietic progenitor cells were maintained or expanded as determined by their ability to form colonies in methylcellulose (Fig 1C). When the recovered cells were examined for LTC-IC activity after culture on OBs, LTC-IC activity was also maintained (Fig 1C). Cultures that were supplied with exogenous granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 3 (IL-3) differentiated along the granulocytic/monocytic pathway into bands/polymorphonuclear neutrophils and initially supported the expansion of progenitor cell populations, but were not able to support LTC-IC activity (data not presented). In the absence of any supportive monolayer or recombinant cytokines, few viable haematopoietic cells were recovered from the cultures, and fewer still were able to support CFU or LTC-IC activity (Fig 1C). Most important to the present investigation, few $CD34^+$ haematopoietic bone marrow cells were recovered from the separated cultures, which were unable to support CFU or LTC-IC activities (Fig 1C). Therefore, direct contact with OBs is necessary for the maintenance of progenitor and LTC-IC activities *in vitro*.

To characterize the adhesion molecule(s) that mediate the binding of haematopoietic cells to OBs, we developed a dual culture system to avoid the limited availability and heterogeneity inherent in primary human haematopoietic cells. We further chose to limit our studies to only those adhesive interactions that should be constitutively expressed. Therefore, our assays were held to a relatively short incubation time (15 min) and under conditions that do not favour transcription (4°C). In this assay system, labelled KGla cells (a $CD34^+$ haematopoietic cell line) were introduced on to MG-63 or SaOS-2 monolayers (human osteosarcoma cell lines). In this system, where as few as 150 cells were detectable, the binding of KG1a cells to MG-63 or SaOS-2 cells represented 55·9 ± 6% and 6·0 ± 1·6%, respectively, of the total input of cells (data not presented). Using this system, adhesion was blocked by the addition of 10 mmol/l EDTA, suggesting that adhesion was dependent upon the presence of divalent cations (Fig 1D). The osteoblastic adhesive determinants were not observed early in culture (days 1–5 of culture) suggesting that either the expression of these molecules are either cell density- or maturation-dependent, or perhaps require the production of a mature ECM (S. M. Crean and R. S. Taichman, unpublished observations). Moreover, based upon Triton X-100 treatment of the OBs, it appeared that the majority of the initial adhesive interactions were not mediated via adhesion to the ECM in our system, but rather by cell–cell interactions (Fig 1D) (Gospodarowicz & Ill, 1980).

To further explore the mechanisms involved in the adhesion of haematopoietic cells to OBs, both KG1a and $CD34^+$ cells were first examined by fluorescent-activated cell sorting analysis to confirm the presence of cell surface adhesion molecules, and subsequently adhesion assays were performed following pre-incubation of the KG1a cells with saturating levels of function blocking antibodies (or control). We observed that antibodies directed against CD11a (α_L), CD29 (β_1), CD31 (gpIIa), CD44, CD54, CD58 (LFA-3), ICAM-1, ICAM-3, LFA-1 ($\alpha_L\beta_2$), Mac-1 ($\alpha_M\beta_2$), VCAM-1, VLA-4 ($\alpha_4\beta_1$), VLA-5 ($\alpha_5\beta_1$), the vitronectin receptor ($\alpha_V\beta_3$) and L-selectin failed to alter the binding of KG1a to the osteosarcoma cell lines, or $CD34^+$ bone marrow cells to primary OBs (data not presented). Moreover, peptides directed against the cell adhesion domains of integrins (i.e. arginine–glycine–aspartate, RGD), or short-term exposure to the cytokines granulocyte CSF (G-CSF), GM-CSF, hepatocyte growth factor and SCF failed to influence the adhesive interactions (data not presented).

The binding KG1a to osteosarcoma cells is mediated by N-linked glycoproteins

Antibody blocking failed to identify which receptor was involved in mediating the adhesive interactions between OBs and haematopoietic cells. Therefore, we investigated whether glycosylation affected adhesion in our system. KG1a cells were pre-incubated in increasing concentrations of tunicamycin prior to their placement onto MG-63 or SaOS-2 osteosarcoma cell layers. Tunicamycin (which blocks N-linked transfer of sugars to proteins) (Fig 2A) produced a dose-dependent inhibition of binding of KG1a cells to both osteosarcoma cell lines with inhibitory concentrations (IC_{50}) at 4·625 μmol/l (MG-63) (Fig 2B, co-incubation) and 2·25 μmol/l (SaOS-2) (Fig 2C, co-incubation). The decrease in adhesion was not due to cell death, because tunicamycin-treated cells excluded trypan blue dye and the binding to osteosarcoma cells returned once the drug was removed (% recovery at 15 min; MG-63 = 40%; SaOS-2 = 20%).

Exposure of KG1a cells alone to tunicamycin was sufficient to suppress all binding to SaOS-2 cells and decreased 88% of binding to MG-63 relative to the control (Fig 2B and C). The concentrations that produced 50% inhibition (IC_{50}) when KG1a cells were pretreated with tunicamycin for 15 min (MG-63 *c.* 11·5 μmol/l; SaOS-2 6·8 μmol/l) were comparable with the IC_{50} achieved by pretreatment and co-incubation with the osteosarcomas for 45 min (*c.* 4·6 MG-63; 2·3 SaOS-2 μmol/l). Pretreatment of the osteosarcomas alone failed to inhibit binding, suggesting that the tunicamycin-sensitive adhesive molecules were largely present on KG1a cells.

We hypothesized that tunicamycin may have the capacity to interrupt an established bond between KG1a and osteoblastic monolayers. KG1a cells were allowed to bind for 10 min to triplicate sets of wells containing osteosarcoma monolayers. At this point, the KG1a cells were manipulated to create different scenarios of binding. Set 1 was left unmanipulated to settle for the entire 20 min. We would expect subpopulations of both bound and suspended KG1a in these wells. In a second set of wells, the supernatant was removed and replaced with media. Thus, only KG1a cells bound within the first 10 min should remain in these wells. In the third set of wells, supernatant was again removed but returned to the same wells. We would again expect two subpopulations of KG1a in these wells with some bound KG1a cells resuspended because of the shearing force of

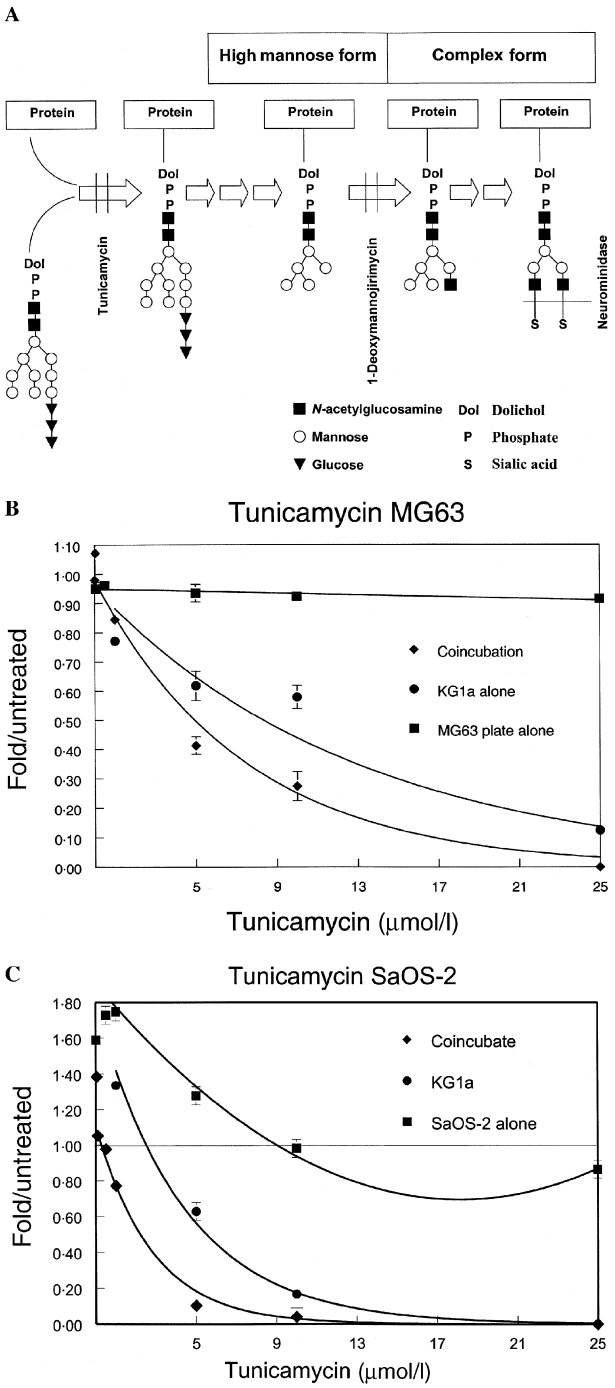

Fig 2. Tunicamycin inhibition of KG1a cell binding to human osteosarcoma cells. (A) Generalized scheme of glycosylation (Humphries *et al*, 1986). Labelled KG1a cells (10^5 cells/well) were directly deposited onto MG-63 (B), or SaOS-2 (C) osteosarcoma cells. Adhesion assays were performed in PBS containing Ca^{+2}/Mg^{+2} for 15 min at 4°C. Where indicated, tunicamycin was introduced (as a pretreatment) to the haematopoietic cells (KG1a), or the osteosarcoma cells prior to co-incubation, and removed, or added during co-incubation. Data are presented as fold increase over control. *Significantly different ($P < 0{\cdot}05$) from non-treatment control (No Tx).

the pipetting. At this midpoint, tunicamycin or vehicle was added and the cells incubated for another 10 min. If tunicamycin acts only at the initial tethering of KG1a to osteosarcoma, we would not expect to see any loss of binding in set 2. As expected, about half of the cells were bound at 10 min when compared with those that were allowed to bind for the entire 20 min (MG-63: 45·5% vs. 83·64%; SaOS-2: 5% vs. 9% control) (Fig 3). Binding of reintroduced KG1a cells onto the osteosarcomas for 20 min was only slightly higher than that achieved by KG1a permitted to incubate for only 10 min (Fig 3). Tunicamycin inhibited some adhesion in all three groups, but proved the most effective in removing KG1a cells that were bound to SaOS-2, suggesting that tunicamycin could 'shed' these established cells from their supports.

Confirmation that the binding of CD34+ bone marrow progenitors to human OBs in vitro is mediated by N-linked glycoproteins

The survival of $CD34^+$ cells on OBs *in vitro* requires intimate cell-to-cell contact. If the interactions were prevented, few haematopoietic cells were recovered over a 2-week culture period. Using cell lines, we have observed that part of this activity was due to glycoproteins using tuniacmycin. However, while it is clear that osteosarcomas express several phenotypic characteristics of primary human OBs, our previously work suggests that caution should be exercised when evaluating osteoblastic and haematopoietic cell interactions based purely on the use of osteosarcoma cell lines alone (Taichman *et al*, 1997b). Therefore, to verify that observations made observed using cell lines were relevant to primary tissues, $CD34^+$ bone marrow cells were labelled and permitted to adhere to primary OB either in the presence or absence of 25 μmol/l tunicamycin at 4°C for 15 min. The cell layers were then washed and binding enumerated. We observed that during the short course of the investigation, nearly 32 ± 8% of the input cells established functional adhesive interactions that were not disrupted with gentle washing (Fig 4A). In the presence of the N-glycosylation inhibitor, only 7 ± 2% of the progenitors adhered to the OBs (Fig 4A).

As considerable heterogeneity is known to exist in the $CD34^+$ population, we next examined the phenotype of the progenitors that had bound to the OB. For these investigations, adhesion assays were performed in the presence or absence of tunicamycin, whereupon the haematopoietic cells were recovered by light trysinization and progenitor (CFU) assays were subsequently performed after washing. The data demonstrated that *c.* 12·0 ± 1·0% of the total progenitors cells present in the adhesion assays bound to OB (Fig 4A). In the presence of tunicamycin, a significant reduction in the recoverable progenitors was observed during the 15 min incubation at 4°C (Fig 4B). For those cells that bound to the OB no significant alterations in the phenotype of the haematopoietic progenitor cells was observed, relative to the starting populations (Fig 4C). However, there was a slight

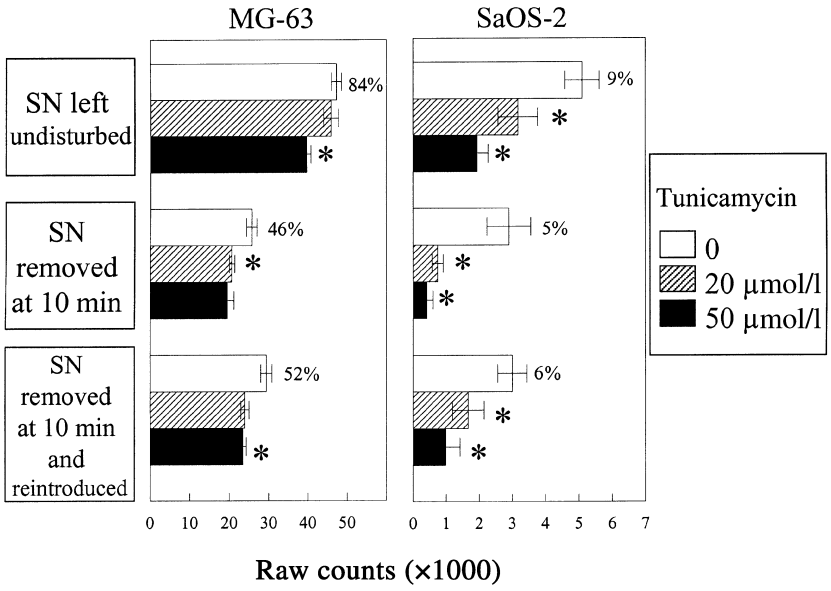

Fig 3. Inhibition of binding by tunicamycin is not due to the prevention of binding or shedding of bound cells. BCECF-AM labelled KG1a cells (10^5 cells/well) were directly deposited onto MG-63 or SaOS-2 osteosarcoma cells in PBS containing Ca^{+2}/Mg^{+2} at 4°C, in the presence or absence of 0, 20 or 50 μmol/l tunicamycin and allowed to bind to replicate wells containing osteosarcoma monolayers for 20 min resulting in populations of KG1a cells that were either bound to the osteosarcomas or not (SN left undisturbed). In parallel, KG1a cells were allowed to bind to a second set of osteosarcoma cells, but the non-adherent KG1a cells were removed after 10 min leaving putatively only bound cells (SN removed at 10 min). To control for the potential of loss of cells due to our experimental manipulations, KG1a cells were also removed from a third set of plates at 10 min, but returned to the same wells (SN removed at 10 min and reintroduced). The non-adherent cells were removed in three subsequent washes and the remaining fluorescence was quantified in an 96-well fluorescent plate reader (IDEXX Laboratories). Data are presented as raw fluorescent counts. *Significantly different ($P < 0{\cdot}05$) from untreated controls (tunicamycin 0 μmol/l).

increase in the CFU-GM, and decrease in the BFU-E fractions recovered from the co-cultures in the presence of tunicamycin, however the relative differences were not significant. Taken together, the data demonstrated that N-linked glycosylation is an essential part of the initial tethering of early haematopoietic progenitor cells to OBs.

Trimming N-linked glycans to a mannose core results in enhanced binding

Surprisingly, when low concentrations of tunicamycin were utilized for 30 min (<1 μmol/l), the binding of the KG1a cells to SaOS-2 increased relative to untreated controls (Fig 2C). By selective pretreatment, we attributed this net 'inducible' binding to N-glycosylated adhesion molecules on the SaOS-2 cell surface (Fig 2C). A similar adhesion molecule may reside on MG-63; however, we were not able to reliably confirm this finding because constitutive binding of KG1a to MG-63 was routinely high.

To further confirm that the binding profiles are due to an interruption of N-linked glycosylation, the investigations were repeated with deoxymannojirimycin. This sugar processing inhibitor disrupted glycoprocessing at the downstream high mannose form (Fig 2A). While deoxymannojirimycin failed to significantly inhibit the binding of KG1a cells to MG-63 cells, we again observed an increase in KG1a/SaOS-2 binding as drug concentrations dropped (Fig 5). These data suggest that complete N-glycosylation of a KG1a cell adhesion molecule (CAM) blocks KG1a/osteosarcoma binding. Partial trimming of an N-glycosylated CAM to its high mannose core retains the observed 'constitutive' binding by MG-63 and imparts additional binding to SaOS-2. Altogether these data suggest that the complex N-glycosylated proteins are responsible for the constitutive binding of KG1a cells to SaOS-2 cells, which, if further restricted to a high mannose core, result in a high-binding activity.

N-linked glycans, if trimmed of terminal sialic acid, yields higher binding

To further explore the role of complex sugar structure on binding, KG1a cells were treated with neuraminidase H1, which cleaves terminal sialic residues. The resulting cleavage of sialic residues from complex sugars on the membranes of KG1a or SaOS-2 cells increased the binding of KG1a to SaOS-2 cells by fourfold to 11-fold above the levels of untreated cells. This observation was made regardless of whether the KG1a cells or the osteosarcoma cells were first exposed to the enzyme (Fig 6A and B). Not surprisingly however, the binding of KG1a cells to MG-63 was not reproducibly increased as the binding of these two cell types to one another initially approached 80% of capacity.

To verify that the induction of additional binding was not an artifact of transformed cell lines, the neuraminidase

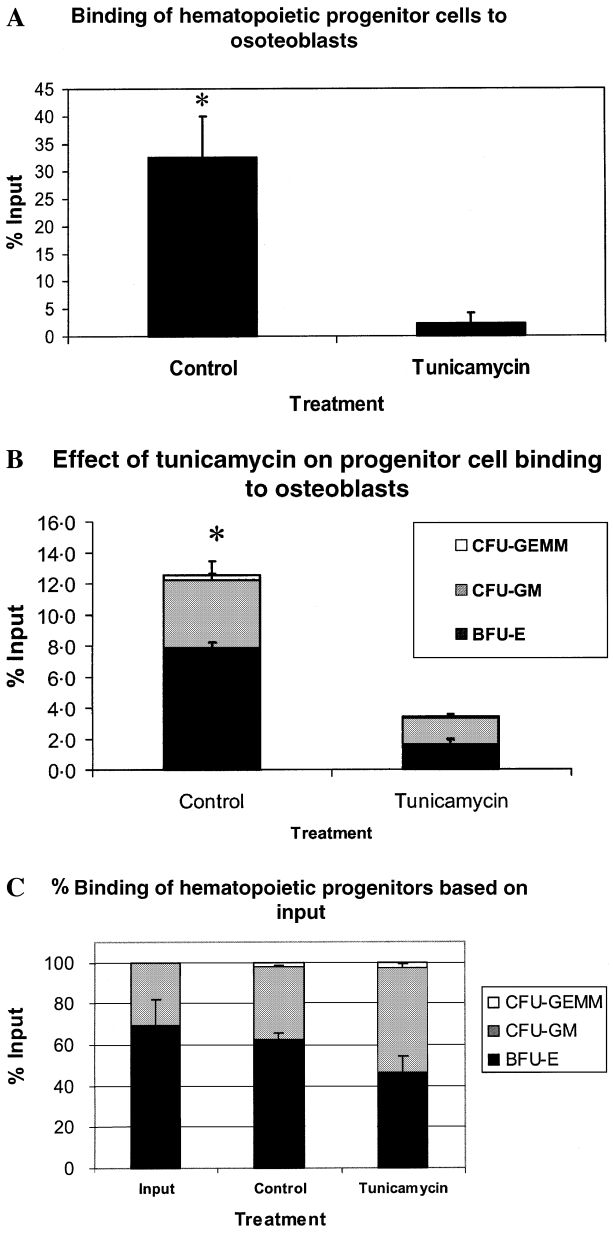

Fig 4. Tunicamycin inhibits the binding of primary human haematopoietic progenitor $CD34^+$ bone marrow cells to primary human OB. Adhesion assays were performed in PBS containing Ca^{+2}/Mg^{+2} in which the labelled primary human bone marrow $CD34^+$ cells (10^4 cells/well) were added to a final reaction volume of 100 μl for 15 min at 4°C in the presence of 25 μmol/l of tunicamycin or vehicle. In (A) the non-adherent cells were removed, and the remaining fluorescence was quantified in a 96-well fluorescent plate reader (IDEXX Laboratories). In some cases unlabelled cells were recovered from the OB monolayers by light trysinization and progenitor (colony forming units, CFU) assays were performed as described previously (Taichman *et al*, 1996) and presented as per cent total binding (B), or per cent relative to the starting population (C). Progenitor colonies were characterized as either granulocyte/macrophage CFU (CFU-GM), erythroid burst-forming units (BFU-E) or mixed CFU (CFU-GEMM).

treatments were repeated with primary human $CD34^+$ haematopoietic cell and primary bone isolates. Baseline $CD34^+$ cells to MG-63 represented 40% of the total cells available, whereas negligible adhesion was detected between the haematopoietic cells and the SaOS-2 osteosarcomas was observed (Fig 6C). Exposure of the haematopoietic cells ($CD34^+$ or KG1a cells) to neuraminidase yielded a threefold induction of binding to all osteoblastic target cells tested. Induction of additional binding by the treatment of KG1a with neuraminidase was twofold to 10-fold greater in binding experiments on primary human OBs relative to the osteosarcoma cell lines.

To ensure that the removal of terminal sialic acid was the mechanism responsible for the increased binding we had observed, sialic acid levels were assayed from the supernatants of neuraminidase-treated KG1a cells and osteosarcoma cultures. When treated with neuraminidase, sialic acid was detected from KG1a cells and both of the osteosarcoma cells, which was not observed in the absence of the enzyme. Unexpectedly, significant increases in the sialic acid levels of the culture supernatants were noted when the KG1a cells were incubated with SaOS-2 cells. This increase in sialic acid was greater than the sum of the sialic acid produced by either cell type alone (Fig 6D). Altogether these data indicate that the first tethering between early blood cells and OBs in this model is probably due to heavily glycosylated, rapidly processed protein(s) on both cell types. While continuous N-glycosylation of the KG1a glycocalyx is necessary for constitutive binding; a minimal galactosyl and mannosyl core lectin is sufficient. In contrast, when exposed on SaOS-2, these same residues impart profound additional binding. Therefore, the extent and the complexity of post-translational N-glycosylation elicits a dramatic biphasic pattern of binding between bone marrow cell types.

Discussion

Adhesive events next to endosteal surfaces seem to be crucial for the full functioning of early haematopoietic cells as well as for the pathophysiology of myeloproliferative disorders and tumour metastasis. Surface studies show that multiple CAMs are likely to engage in even the most rudimentary encounters between early HSCs and OBs. Furthermore, final affinity and avidity would extend beyond the identification of a candidate molecule, as many adhesion molecules exist in multiple isoforms and glycosylation states and can be displayed in different conformations and cell surface clusters. Therefore, we have begun to explore this unique tissue compartment with several reduced models of endosteal haematopoiesis (Lord *et al*, 1975; Gong, 1978; Islam *et al*, 1990; Cui *et al*, 1996; Nilsson *et al*, 1997; Quesenberry & Becker, 1998). We elected to consider the impact of post-translational modifications on the first tethering between blood cells and OBs. Glycosylation seemed an attractive strategy, as carbohydrates can act as adhesion structures directly or indirectly by modifying a lectin, a receptor or a cytokine target (Pahlsson *et al*, 1995). In

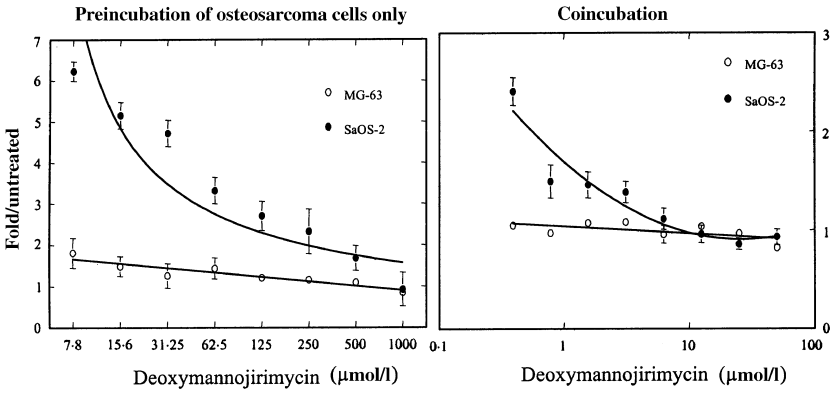

Fig 5. Deoxymannojirimycin inhibition of KG1a cell binding to human osteosarcoma cells. Labelled KG1a cells (10^5 cells/well) were directly deposited onto MG-63 or SaOS-2 osteosarcoma cells respectively. Adhesion assays were performed in PBS containing Ca^{+2}/Mg^{+2} for 15 min at 4°C. Where indicated, deoxymannojirimycin was introduced first to the osteosarcoma cells and removed (left) or remained during the co-incubation period (right). The non-adherent cells were removed in three subsequent washes and the remaining fluorescence was quantified in a 96-well fluorescent plate reader (IDEXX Laboratories). Data are presented as fold increase over control.

addition, a cell surface molecule that is fully extended with bulky oligosaccharide chains would constitute the first and exquisitely specific contact between cells (Arkwright *et al*, 1994).

Proper N-linked glycosylation globally influences the folding, transport, activity, stability, antigenicity and transport of surface proteins (Asosingh *et al*, 1998). Using specific, independently derived inhibitors of this pathway, we observed that KG1a/osteosarcoma tethering was affected by the extent and the complexity of N-linked glycosylation. Our model proposes that the net de-N-glycosylation of the KG1a glycocalyx results in a loss of all constitutive binding to both OB lines. In contrast, partial disruption of this process imparts a net increase in binding attributable to molecules on SaOS-2 cell

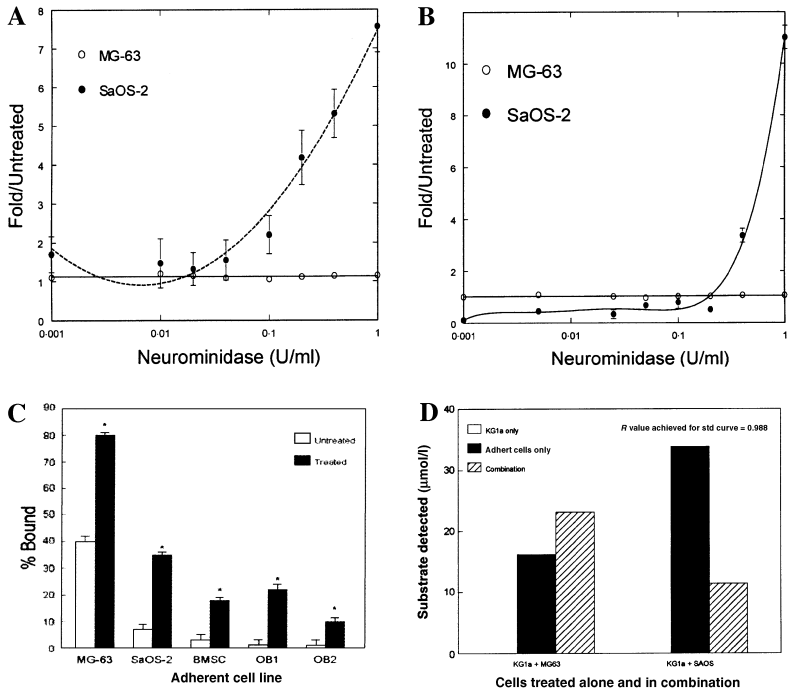

Fig 6. Binding of KG1a and human $CD34^+$ cells to human osteosarcoma cells or primary human osteoblasts *in vitro* in the presence of neuraminidase. (A) Labelled KG1a cells were treated with 0·5 U/ml neuraminidase H1 (which cleaves terminal sialic residues), washed, and 10^5 cells/well were deposited onto MG-63 and SaOS-2 human osteosarcoma cell lines. (B) Adhesion was allowed to proceed in the presence of neuraminidase during the co-culture. (C) To verify that the induction of additional binding was not an artifact of transformed cell lines, the neuraminidase treatments (0·5 U/ml) were repeated with primary human $CD34^+$ bone marrow haematopoietic cells (BMSC) and two primary human osteoblast isolates (OB1 and OB2). In all cases, adhesion assays were performed in PBS containing Ca^{+2}/Mg^{+2} for 15 min at 4°C. (D) 0·1 U/ml neuraminidase was added for 5 min to individual cell lines and co-cultures, and the resulting media was assayed for sialic acid. Data are presented as fold increase over control (A and B), % bound (C) or μmol/l of substrate (D). *Significantly different from non-treated control ($P < 0·05$).

surfaces. This induced binding is reproduced when the nascent surface molecules of SaOS-2 are partially deglycosylated, reduced to high mannose forms, or trimmed of terminal sialic acids by tunicamycin, deoxymannojirimycin and neuraminidase treatment respectively.

Tunicamycin is a native antibiotic of several *Streptomyces* spp. It is a reversible, highly specific and competitive inhibitor of the polyprenol-phosphate, N-acetylhexosamine-1-phosphate translocase, that governs the initial transfer of a high-mannose oligosaccharide with the composition (Glc)3(Man)9(GlcNAc)2 from a lipid carrier to the nascent polypeptide chain. Using a continuous fluorescence-based enzyme assay, Brandish *et al* (1996) reported the kinetic activity of tunicamycin on solubilized *Escherichia coli* phospho-N-acetylmuramyl-pentapeptide translocase. They found that the IC_{50} for tunicamycin was 2 μmol/l in assay conditions that were allowed to proceed to equilibrium from 3 to 5 min. The K_m for the donor and the acceptor substrates were 13 and 19 μmol/l respectively. Further, tunicamycin was estimated to bind target enzyme at 50-fold greater avidity than either substrate. Cell cultures used for adhesion assays have been traditionally incubated in tunicamycin for 1–3 d with the intent of stripping the glycocalyx by a relatively non-toxic method (Brandish *et al*, 1996). However, other investigators have shown that a change in glycosylation (e.g. a shift in electrophoretic mobility of an immunoprecipitate) that parallels a change in binding phenotype is possible with significantly shorter assays (Pahlsson *et al*, 1995; Salmi & Jalkanen, 1995). These data suggest that tunicamycin is a robust inhibitor that should interrupt global N-linked glycan biosynthesis with a short incubation at low doses.

Independently, other investigators have noted that, in addition to blocking mannose incorporation, tunicamycin treatment significantly depressed protein synthesis. For example, Humphries *et al* (1986) showed a 45% inhibition of leucine incorporation when murine melanoma cells were incubated over 18 h in 2 μg/ml tunicamycin. To counter the argument that our observations were not due to the possible secondary effects of tunicamycin on the components of protein synthesis, we performed our assay in conditions inhospitable to translation (15 min at 4°C). The experiments that others have performed to validate the mechanism of action for tunicamycin appeared either impractical or impossible, given that the present data do not suggest a candidate molecule to evaluate (e.g. tritiated mannose incorporation, electron microscopy, changes in gel migration or an affinity for a lectin). Instead, we repeated our assays using a second, independently derived inhibitor of post-translational sugar processing. Deoxymannojirimycin, a mannose analogue of 1-deoxynojirimycin, is also highly specific to its target enzyme mannosidase I. Finally, rather than employ another downstream enzyme inhibitor to interrupt the same pathway, we used a cell surface enzyme neuraminidase to alter the final presented product. Our observations from these three different experimental approaches are consistent with a model that, in this haematopoietic/osteoblastic bone assay, adhesion can be biphasically modulated with dynamic, reversible, postcell contact changes in glycosylation. The simplest interpretation of the data is that tunicamycin, deoxymannojirimycin and neuraminidase are indeed working as suggested and not along secondary, but poorly understood, collaborating lines. Using inhibitors, glycan digestion and glycoconjugate probes and supports, other investigators have likewise demonstrated that a minimal galactosyl and mannosyl core lectin on progenitor cells is necessary for constitutive binding to stroma or a stroma-derived ECM component (Hass *et al*, 1990; Hardy, 1995; Hardy & Megason, 1996; Lemischka, 1997). Sugar receptors are also found on the cell surfaces of progenitor and myeloid leukaemia cell lines (Gabius *et al*, 1994; Oxley & Sackstein, 1994; Hardy, 1995; Sackstein, 1997). Our binding results are consistent with the kinetics of the putative haematopoietic homing receptor characterized by Hardy (1995). Found on stem cells but not on marrow cell lines, this sugar receptor is densely distributed, avid and can be saturated with galactosyl and mannosyl probes.

Other cell assays confirm that complex N-linked sugars are not necessary for binding and indeed that binding proportions may increase if the penultimate galactosyl residues on stroma are exposed. A pre-B and a fibroblast cell line, sorted as low or inducible binders to hyaluronic acid, converted to high constitutive binding when cultured with low levels of tunicamycin (Katoh *et al*, 1995). Treatment with deoxymannojirimycin retained binding (Bartolazzi *et al*, 1996). Likewise, stringent tunicamycin treatment abrogated constitutive binding of lymphoma and melanoma cell lines to hyaluronic acid; deoxymannojirimycin retained binding or increased the observed phenotype (Bartolazzi *et al*, 1996).

Other investigators have shown that a decrease in sialyated glycoproteins correlates with new or enhanced ligand recognition (Katoh *et al*, 1995, 1999). However, this property has been ascribed to monocytic primary and glycosylation defective Chinese hamster ovary cells and tested against single ECM components such as collagen-4, -5, fibronectin and laminin (Katoh *et al*, 1995, 1999). As in our model, Hardy (1995) demonstrated that selective treatment of stroma alone with neuraminidase is sufficient to increase KG1a binding. While sialic acid residues are a necessary component for complex sugar molecules on KG1a (Oxley & Sackstein, 1994), we propose that the net induction in adhesion is due to the unmasking of sialyated complex sugars on SaOS-2 and stroma. Lineage and bone-specific molecules, such as haemonectin, and major ECM components (e.g. CD44) may remodel in response to haematopoetic demands (Sullenbarger *et al*, 1995; Katoh *et al*, 1999; Dimitroff *et al*, 2000). A temporal and spatial shift in affinity as a result of glycosylation may promote the recruitment of immature blood cells back into the bone marrow.

Based upon our results, the initial tethering between bone marrow CD34+ cells and OBs is a rapid and dynamic event. The molecules that mediate these interactions are likely to be constitutively expressed, as the relatively short periods of time

(15 min) and conditions (4°C) used for our investigations do not favour transcriptional control. These molecules must rapidly process and cycle to the cell surface, as glycosylation inhibitors would be expected to affect only nascent molecules. This is apparent as the adhesion returned after tunicamycin was removed, suggesting the rapid re-expression of receptors. Our model suggests that a renewal of N-linked glycans is necessary to maintain the initial adhesion to OBs because the added tunicamycin released progenitor cells, which was already bound to OB supports. Moreover, our data suggest that the molecules responsible for these adhesive events are modulated after cell contact. A rapid increase in free sialic acid was detected when KG1a and SaOS-2 cells were co-incubated in the presence of neuraminidase, and this increase was much larger than the sum of either cell line alone. In order for this to occur, receptor turnover, even at 4°C, must have been extremely rapid from intracellular pools.

The developmental mechanisms of bone and blood formation have traditionally been viewed as distinct, unrelated processes, but there are compelling reasons to suspect that they are functionally intertwined. Both cell populations originate in the bone marrow and come into direct physical contact with each other. Indeed, *in situ* observations confirm that haematopoietic precursors reside in close proximity to endosteal surfaces in human bone marrow (Gong, 1978; Deldar *et al*, 1985; Hermans *et al*, 1989; Lord, 1990; Cui *et al*, 1996). Moreover, *in vitro* studies demonstrated that tight adhesions between these cells are critical for long-term viability and development of primitive human haematopoietic cells (Sutherland *et al*, 1990; Taichman *et al*, 1996, 1997b; Taichman & Emerson, 1998). The data presented here suggest that early haematopoietic cells all have CAMs that are sensitive to neuraminidase, presumably sialic acid. To characterize these adhesion molecules, we reduced the bone marrow cavity to a simple adhesion assay between KG1a (a $CD34^+$ haematopoetic cell line) and an osteosarcoma monolayer (MG-63 or SaOS-2). We showed that adhesion was mediated by cell-to-cell rather than cell-to-matrix contact, and that these interactions were sensitive to trypsin, chelators and glycosylation inhibitors. Specifically, if nascent proteins are stripped of carbohydrate (via tunicamycin), adhesion was steeply abrogated. Selective pretreatment attributed this net constitutive binding to N-linked glycans on KG1a. When carboprocessing was inhibited later as a high mannose intermediate (via deoxymannojirimyicin), adhesion was retained. In contrast, binding of KG1a to SaOS-2 increased past constitutive levels when doses of tunicamycin or deoxymannojirimycin dropped. Selective pretreatment suggested that this 'inducible' binding resided with molecule(s) on SaOS-2. When terminal sialic acid residues were digested from all high mannose forms present on cell surfaces (via neuraminidase), this induced response was duplicated. Altogether these data suggest that the first tethering between blood and bone in this model was probably due to heavily glycosylated, rapidly processed protein(s) on both cell types. While continuous N-glycosylation of the KG1a glycocalyx is necessary for constitutive binding, a minimal galactosyl and mannosyl core lectin is sufficient. In contrast, these same penultimate residues, when exposed on SaOS-2 cells, impart profound additional binding. Therefore, the extent and the complexity of post-translational N-glycosylation elicits a dramatic biphasic pattern of binding between bone marrow cell types.

In vivo, HSCs and their maturing progeny are in close proximity to bone marrow stromal cells. While the mechanisms that stromal cells use to support haematopoiesis remain unclear, CAMs, including the cadherins, immunoglobins, integrins and selectins, are likely to be critical determinants in the process. The data reported here represent the first analysis of haematopoietic cell adhesions, and demonstrates that close cell-to-cell contacts between these cells are critical for the survival of haematopoietic cells on OBs. We observed that haematopoietic progenitor cells' survival on OB is dependent upon direct cell–cell contact, and that the initial binding of haematopoietic cells to OBs is probably mediated by sialyated N-linked glycoproteins present on HSCs. Moreover, immediately after the establishment of initial contact, OBs are likely to up regulate those CAM(s) that are rich in sialic acid, which limits KG1a adhesion to its normal low levels. When these charged terminal ends are lost from the cell surface, either by digestion or the loss or complex sugar supports, the core CAM on the cell surface of the OBs converts from a low binding form to one that binds $CD34^+$ haematopoietic progenitors to much higher levels. Further investigation will be required to identify the CAMs that determine the biological basis of OB-supported haematopoiesis, its regulation and its precise role in stem cell survival, maintenance and proliferation.

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