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Modulation of In Vivo Migratory Function of $\alpha 2\beta 1$ Integrin in Mouse Liver

Wai-chi Ho,*† Christine Heinemann,*† Dolores Hangan,*† Shashi Uniyal,*† Vincent L. Morris,*§ and Bosco M.C. Chan*†‖

*Department of Microbiology and Immunology, §Departments of Oncology and Medical Biophysics, †Transplantation and Immunobiology Group, John P. Robarts Research Institute, University of Western Ontario, London, Ontario, Canada

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We report herein that expression of $\alpha 2\beta 1$ integrin increased human erythroleukemia K562 transfectant (KX2C2) cell movement after extravasation into liver parenchyma. In contrast, a previous study demonstrated that $\alpha 2\beta 1$ expression conferred a stationary phenotype to human rhabdomyosarcoma RD transfectant (RDX2C2) cells after extravasation into the liver. We therefore assessed the adhesive and migratory function of $\alpha 2\beta 1$ on KX2C2 and RDX2C2 cells using a $\alpha 2\beta 1$-specific stimulatory monoclonal antibody (mAb), JBS2, and a blocking mAb, BHA2.1. In comparison with RDX2C2 cells, KX2C2 were only weakly adherent to collagen and laminin. JBS2 stimulated $\alpha 2\beta 1$-mediated interaction of KX2C2 cells with both collagen and laminin resulting in increases in cell movement on both matrix proteins. In the presence of Mn^{2+}, JBS2-stimulated adhesion on collagen beyond an optimal level for cell movement. In comparison, an increase in RDX2C2 cell movement on collagen required a reduction in its adhesive strength provided by the blocking mAb BHA2.1. Consistent with these in vitro findings, in vivo videomicroscopy revealed that $\alpha 2\beta 1$-mediated postextravasation cell movement of KX2C2 cells in the liver tissue could also be stimulated by JBS2. Thus, results demonstrate that $\alpha 2\beta 1$ expression can modulate postextravasation cell movement by conferring either a stationary or motile phenotype to different cell types. These findings may be related to the differing metastatic activities of different tumor cell types.

INTRODUCTION

It is well established that $\beta 1$ integrins represent the major receptors for providing the functional linkage between extracellular matrix (ECM) proteins and cytoskeletal components (for review, Hynes, 1992). The expression of $\alpha 2\beta 1$ integrin as receptors for collagen and laminin has been associated with the morphogenesis of mammary epithelial cells (Berdichevsky *et al.*, 1992; Keely *et al.*, 1995a,b) and differentiation of the human erythroleukemia cell line K562 (Burger *et al.*, 1992). In comparison, $\alpha 2\beta 1$ expression was down-regulated on keratinocytes undergoing terminal differentiation (Adams and Watt, 1990). In addition, $\alpha 2\beta 1$ has also been associated with the metastatic activities of tumor cells. Interestingly, there is both a direct correlation (Dedhar and Saulnier, 1990; Chan *et al.*, 1991; Klein *et al.*, 1991; Mortarini *et al.*, 1991; Danen *et al.*, 1993; Chen *et al.*, 1994; Santala *et al.*, 1994) and an inverse correlation (Pignatelli *et al.*, 1990, 1991; Zutter, *et al.*, 1990, 1995) of $\alpha 2\beta 1$ expression with tumor metastasis. The exact mechanisms whereby $\alpha 2\beta 1$ enhances or, in some cases, reduces the metastatic activities of tumor cells are still unclear. One possibility is that $\alpha 2\beta 1$ may confer distinct cell functions among the tumor cells. The present study focuses on $\alpha 2\beta 1$ interaction with ECM proteins and its in vivo effect on cell movement.

Studies in recent years have demonstrated three distinct modes of ligand binding for $\alpha 2\beta 1$: no ligand-binding activity, binding collagen but not laminin,

‖ Corresponding author: Transplantation and Immunobiology Group, John P. Robarts Research Institute, University of Western Ontario, 100 Perth Drive, London, Ontario, Canada, N6A 5K8.

and binding both collagen and laminin. Integrin $\alpha2\beta1$ on human endothelial cells and melanoma cell line LOX bind both collagen and laminin; whereas, on platelets and the fibroblast cell line MRC-5, $\alpha2\beta1$ binds only collagen but not laminin (Elices and Hemler, 1989; Languino *et al.*, 1989; Kirchhofer *et al.*, 1990; Lotz *et al.*, 1990). The observed cell type dependence has also been demonstrated with transfectant cells. Expression of $\alpha2\beta1$ enhanced adhesion of human rhabdomyosarcoma RD transfectant cells (RDX2C2) to collagen and laminin (Chan *et al.*, 1991, 1992). Interestingly, $\alpha2\beta1$ on human erythroleukemia K562 transfectant cells (KX2C2) provided no detectable adhesion to collagen or laminin in static cell adhesion assays (Elices and Hemler, 1989). Certain $\beta1$ subunit-specific monoclonal antibodies (mAbs) stimulate ligand-binding activities. mAbs 8A2 and Ts2/16 have been shown to stimulate high-avidity ligand binding of $\beta1$ integrins on eosinophils, T and B lymphocytes, myelomonocytic cell line U937, and melanoma A375 (Arroyo *et al.*, 1992; Kovach *et al.*, 1992; van de Wiel-van Kemenade *et al.*, 1992; Arroyo *et al.*, 1993; Kuijpers *et al.*, 1993; Sanchez-Mateos *et al.*, 1993; Faull *et al.*, 1994). In the presence of Ts2/16, the $\alpha2\beta1$ on K562 cells was stimulated to bind both collagen and laminin (Chan and Hemler, 1993). Thus, $\alpha2\beta1$ can undergo functional transition with respect to their binding properties for collagen and laminin. However, at present, a direct induction of $\alpha2\beta1$ to binding collagen, but not laminin, has not been demonstrated.

It is well established that $\beta1$ integrins have major roles in cell movement involving the recruitment of cytoskeletal components such as talin and α-actinin, as well as activation of the focal adhesion kinase (for review, Lauffenburger and Horwitz, 1996). The interaction between integrin $\beta1$ subunit and cytoskeletal proteins may be regulated by the cytoplasmic domains of α subunits (Chan *et al.*, 1992; Bauer *et al.*, 1993; Kawaguchi *et al.*, 1994; Briesewitz *et al.*, 1995; Kassner *et al.*, 1995). Therefore, $\beta1$ integrins are critical in conferring a stationary or motile phenotype to cells on ECM substrates. The ability of $\alpha2\beta1$ to confer cell movement appears to be cell type dependent. Integrin $\alpha2\beta1$ mediated migration of human melanoma SK-Mel-2, fibrosarcoma HT1080, and keratinocyte HaCaT cells on collagen but not laminin (Yamada *et al.*, 1990; Scharffetter-Kochanek *et al.*, 1992; Knutson *et al.*, 1996). In comparison, $\alpha2\beta1$ mediated migration of bladder carcinoma 5637 and melanoma EP, AN, and RU cells on both the matrix proteins (Yamada *et al.*, 1990; Etoh *et al.*, 1993). A biphasic dependence of cell motility on fibronectin in vitro has been reported (Wu *et al.*, 1994; Palecek *et al.*, 1997). Furthermore, the inhibiting and enhancing effects of the soluble integrin-binding inhibitor, echistatin, can be predicted from its effect on adhesion (Wu *et al.*, 1994).

At present, little is known about $\alpha2\beta1$ function in cell movement in vivo. In a recent study using in vivo videomicroscopy (IVVM), the expression of $\alpha2\beta1$ resulted in an arrest of RD cells (RDX2C2) after extravasation and prevented RD cell migration to the liver subcapsular region (Hangan *et al.*, 1996). This result may be related to a $\alpha2\beta1$-mediated increase in the adhesion of RD cells to matrix proteins in the basement membrane; whether $\alpha2\beta1$ can confer a migratory function in vivo has not been previously determined. In contrast to RDX2C2 (Hangan *et al.*, 1996), we report herein that K562 transfectant cells expressing $\alpha2\beta1$ (KX2C2) were more effective in migration to the subcapsular region of the liver, in comparison with a control transfectant expressing a nonfunctional I-domain deletion variant of $\alpha2\beta1$ (KX2C2[I^-]). Thus, the functional relationship between adhesion and random cell movement upon stimulation of the receptor function of $\alpha2\beta1$ were examined, and the effect of this relationship on the modulation of cell movement in the liver by $\alpha2\beta1$ was then characterized using in vivo videomicroscopy.

MATERIALS AND METHODS

Antibodies and Matrix Proteins

mAbs used in this study for human $\alpha2\beta1$ were BHA2.1 [blocking (Hangan *et al.*, 1996)], HAS4 (Tenchini *et al.*, 1993), and JBS2 [stimulatory, (Stupack *et al.*, 1994)] and for human $\beta1$-subunit was Ts2/16 (Arroyo *et al.*, 1992). mAb P3 (IgG1, κ; Kearney *et al.*, 1979) or normal mouse immunoglobulin (NmIg) was used as control where indicated. Human fibronectin, collagen type I, and mouse Engelbreth-Holm-Swarm (EHS) laminin were obtained from Life Technologies (Gaithersburg, MD).

Flow Cytometry and Immunoprecipitation

Flow cytometry for the determination of $\beta1$ integrin expression was carried out by indirect immunostaining with the $F(ab)'_2$ fragment of fluorescein-conjugated antibody (Cedarlane, Oakville, ON). All antibodies were used at predetermined saturating concentrations. Results were analyzed and compared with isotype-matched control mAbs by using a Becton Dickinson FACScan as described (Hangan *et al.*, 1996). Immunoprecipitation of $\beta1$ integrins was carried out according to established procedures (Chan and Hemler, 1993). Briefly, cells were labeled with ^{125}I using lactoperoxidase and lysed in 0.5% Nonidet P-40 in the presence of protease inhibitors (aprotinin at 1.0 unit/ml, leupeptin at 0.1 M, and phenylmethylsulfonyl fluoride at 2 mM). Precleared cell lysate was then used for immunoprecipitation by using the specific $\beta1$ integrin mAb. The immune complex was isolated using anti-mouse agarose (Sigma, St. Louis, MO). Bound materials were eluted by SDS-PAGE sample buffer under nonreducing condition. Eluted materials from an equivalent of 1×10^6 cells were then analyzed by SDS-PAGE (6% gel). Results were visualized by autoradiography.

Cell Culture and Transfection

Human fibrosarcoma HT1080 and erythroleukemia K562 cell lines were obtained from American Type Culture Collection (Rockville, MD). Transfectant K562 and rhabdomyosarcoma RD cells expressing $\alpha2\beta1$ (KX2C2 and RDX2C2) and mock-transfected K562 cells (KpF) were described in the previous studies (Chan *et al.*, 1991;

Chan and Hemler, 1993). In this study, K562 transfectant cells expressing the nonfunctional $\alpha 2\beta 1$ variant lacking the $\alpha 2$ I-domain (KX2C2[I^-]) were prepared as control of KX2C2. The construction of the I-domain deletion variant of $\alpha 2$ cDNA (X2C2[I^-]) has been described in detail previously (Hangan *et al.*, 1996). Briefly, the cDNA construction involved the creation of *Spe*I restriction enzyme sites at both the 5′ and 3′ ends of $\alpha 2$ I-domain by site-directed mutagenesis. The mutant $\alpha 2$ cDNA was digested by *Spe*I for the removal of I-domain. The I-domain deletion variant of $\alpha 2$ (X2C2[I^-]) was then prepared by religation of the remaining flanking cDNA fragments and cloning into the expression vector pFneo (Ohashi *et al.*, 1985). For preparation of KX2C2(I^-), cDNA of X2C2(I^-) was transfected into K562 cells using the Lipofectin reagent (Life Technologies) as described previously (Chan and Hemler, 1993). The expression of the I-domain deletion variant of $\alpha 2\beta 1$ on KX2C2(I^-) was then characterized by flow cytometry and immunoprecipitation using $\alpha 2\beta 1$ specific mAbs, BHA2.1 and HAS4, as described previously in the preparation of RDX2C2(I^-) cells (Hangan *et al.*, 1996). Thus, as described for RDX2C2(I^-), HAS4 but not BHA2.1 immunostained and immunoprecipitated the I-domain deletion variant of $\alpha 2\beta 1$ on KX2C2(I^-) cells; in comparison, both mAbs were able to stain and immunoprecipitate the complete $\alpha 2\beta 1$ on K562 transfectant cells (KX2C2; our unpublished observation).

Adhesion Assays and Migration Assays

Cell adhesion assays using fluorescence-labeled cells were carried out as described (Chan *et al.*, 1992; Kawaguchi and Hemler, 1993). Briefly, cells were labeled with (2′,7′)-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF; Sigma) and 5×10^4 labeled cells per 100 μl were allowed to adhere to matrix-coated wells for 45 min at 37°C. Bound fluorescence was measured by a Fluorescence Concentrator Analyser (IDEXX Lab., Westbrook, ME), after the removal of nonadherent cells by gentle washing. For a determination of the adhesive strength, nonadherent cells were removed by centrifugation of the inverted adhesion plate at defined centrifugation *g* force as described (Wu *et al.*, 1994). The level of bound fluorescence was obtained after subtraction of the fluorescence using bovine serum albumin-coated wells as background. Cell adhesion was expressed as number of bound cells per unit area, based on the fluorescence of 5×10^4 labeled cells, after a subtraction of background fluorescence. Random cell migration assays were carried out according to established procedures using the 48-well chemotaxis chamber (Neuro Probe, Cabin John, MD; Hauzenberger *et al.*, 1994; Kassner *et al.*, 1995). Briefly, polyvinylpyrrolidone-free polycarbonate filters of 10-μm pore size were coated on both sides with matrix proteins at 10 μg/ml in 0.1 M $NaHCO_3$ overnight. The filters were then washed with PBS, air-dried, and assembled with the bottom wells filled with 30 μl of RPMI 1640 culture medium containing 1% bovine serum albumin. Cells were added to top chambers at 2×10^4 cells in 50 μl per well and allowed to migrate into the filter for 6 h at 37°C. Cells that remained at the upper side of the filter were removed by mechanical scraping. Cells migrated into the filter were then stained with Harris' hematoxylin. The number of migrated cells was obtained as average of cell counts from five random fields from each of the triplicate wells by light microscopy (400×, high-power field) using a gridded objective. All experiments have been repeated for a minimum of three times.

In Vivo Videomicroscopy

The characterization of in vivo cell movement was done using IVVM. This technique involves real-time observation of cells in the vicinity of the microcirculation of undissected organs in living mice (Morris *et al.*, 1994; Hangan *et al.*, 1996). It permits the quantification of 1) intravascular cells whose presence within blood vessels is made apparent by alterations in the blood flow pattern and 2) extravasation of cells and their migration from the liver sinusoids to the avascular subcapsular region. The movement of cells can be observed over a period of hours rather than as a "snapshot" of time. In addition, "optical slicing" allows a three-dimensional picture of the position of cells in relation to surrounding tissue structures. For this technique cells were washed with Opti-MEM medium (Life Technologies), and 7.5 ml of a 1:50 dilution of microspheres (Fluoresbrite carboxylated beads, 0.059–0.067 μm, YG; Polysciences, Warrington, PA) in Opti-MEM was added. The cells were then incubated for 1 h at 37°C with occasional rocking and the cells spontaneously internalized the microspheres. This labeling procedure does not affect the relative plating efficiency or in vivo behavior of the cells (Morris *et al.*, 1994). Six- to 8-wk-old female *nu/nu* mice (Harlan Sprague Dawley, Indianapolis, IN) were anesthesized with Ketamine (6.7 mg/100 g of body weight) and Xylazine (0.67 mg/100 g of body weight) and the microsphere-labeled cells (3×10^5 cells per mouse) were injected into a mesenteric vein. Mice were given an analgesic (Temgesic, 0.01 mg/100 g of body weight) during their recovery. For a comparison of cell movement between K562 cells expressing the complete $\alpha 2\beta 1$ or $\alpha 2\beta 1$ variant lacking the I-domain, mice were analyzed by IVVM at 24 h after cell injection. At designated times, the injected mice were anesthetized with 6 mg of sodium pentobarbitol/100 g of body weight, and body temperature was kept at 37°C with a heat lamp and adjustable power supply. To examine the liver microcirculation, an incision was made along the midline of the abdomen and transversely beneath the rib cage to expose the intestine and liver. The liver was positioned so that the border of a lobe was placed on a no. 1 coverglass mounted on the viewing platform of an inverted microscope (Zeiss Axiovert 135, Empix Imaging, Mississauga, Ontario) equipped with epifluorescence illumination (excitation wavelength, 450–490 nm) and a fiberoptic light guide positioned at an oblique angle for high-contrast transillumination of the microcirculation. The microcirculation and location of fluorescence-labeled cells were recorded on SVHS videotapes with a videocamera (Hamamatsu C2400, Empix Imaging). For some experiments, mice were injected (intravenously, i.v.) with 100 μg of JBS2, BHA2.1, and/or control mAb P3 12 h after cell injection, the predetermined time when all detectable KX2C2 cells completed extravasation in the liver. Mice were then analyzed by IVVM 3 h after antibody injection. A minimum of 50 cells per mouse and three mice per experimental group was used. Results were compared using Students' *t* test with a level of $p < 0.05$ regarded as statistically significant.

RESULTS

Integrin $\alpha 2\beta 1$ Enhanced K562 Transfectant Cell Movement In Vivo

In the previous study (Hangan *et al.*, 1996), we demonstrated by IVVM that expression of $\alpha 2\beta 1$ resulted in an arrest of human rhabdomyosarcoma RD transfectant cells (RDX2C2) immediately after extravasation and prevented their migration to the subcapsular region of the liver. In contrast to its ability to enhance RD cell adhesion to collagen and laminin in vitro, $\alpha 2\beta 1$ lacked detectable ligand-binding function in a static adhesion assay when expressed on K562 transfectant cells (KX2C2; Chan and Hemler, 1993; Elices and Hemler, 1989). It is therefore likely that KX2C2 would have different in vivo effects on cell movement than RDX2C2 cells. To further explore this idea, we used IVVM to determine the effect of $\alpha 2\beta 1$ expression on K562 cell movement in vivo. Specifically, we determined the percentage of extravasated KX2C2 cells that reached the liver subcapsular region at 24 h after cell injection. The K562 transfectant expressing the non-

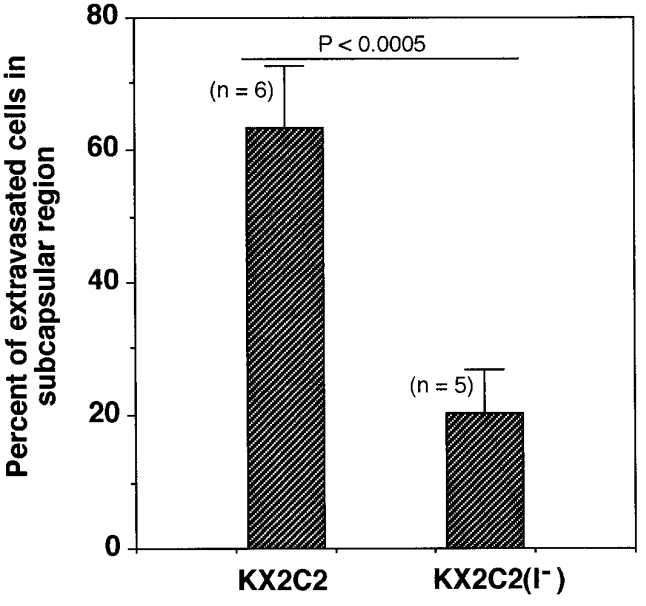

Figure 1. $\alpha 2\beta 1$-mediated migration of K562 transfectant cells to the liver surface. K562 transfectant cells expressing $\alpha 2\beta 1$ (KX2C2) or $\alpha 2\beta 1$ variant lacking the $\alpha 2$ I-domain (KX2C2[I^-]) were compared for their ability to reach the subcapsular region of the liver. In vivo videomicroscopy was carried out at 24 h after injection of 3×10^5 cells into *nu/nu* mice via a mesenteric vein. Results are presented as percentage of extravasated cells at the liver subcapsular region.

functional $\alpha 2\beta 1$ variant lacking the $\alpha 2$ I-domain (KX2C2[I^-]) was used as control. As shown in Figure 1, about 60% of KX2C2 reached the liver subcapsular region at 24 h after cell injection. In comparison, only about 20% of KX2C2(I^-) cells were able to migrate to the subcapsular region. Therefore, in contrast to RDX2C2, which became arrested after extravasation and lacked the ability to move to the liver subcapsular region for up to 8 d (Hangan *et al.*, 1996), results from the present study clearly suggest a functional involvement of $\alpha 2\beta 1$ in KX2C2 cell movement in vivo.

JBS2 Stimulated α2β1-mediated Adhesion to Collagen but Not Laminin of K562 Cells

To determine the functional role of $\alpha 2\beta 1$ in providing cell movement, we used stimulatory and inhibitory mAbs in combination with the KX2C2 and KX2C2(I^-) cell lines. The interaction of $\alpha 2\beta 1$ integrin with collagen and laminin was further characterized by stimulation of its receptor function. mAb Ts2/16 specific for $\beta 1$ subunit has been previously shown to stimulate $\beta 1$ integrin function including the endogenously expressed $\alpha 5\beta 1$ (Chan and Hemler, 1993). In the present study, $\alpha 2\beta 1$-specific mAb JBS2 was used because JBS2 is known to stimulate $\alpha 2\beta 1$ binding of collagen; its effect on adhesion to laminin has not been determined (Stupack *et al.*, 1994; Chou *et al.*, 1996). As shown in Figure 2, A and B, JBS2 (20 μg/ml) stimulated adhesion of KX2C2 to only collagen ($p < 0.05$) but not laminin. When in combination with 10 mM Mn^{2+}, adhesion to collagen was further stimulated; no adhesion to laminin was detectable under these conditions. Although there was an apparent slight increase in KX2C2 adhesion when in the presence of NmIg, results from other experiments revealed no significant difference in comparison with adhesion using cells alone; these findings were also seen in the previous studies (Chan and Hemler, 1993). Results therefore demonstrated progressive activation of $\alpha 2\beta 1$ adhesive function using JBS2 and JBS2 in combination with Mn^{2+}. The blocking $\alpha 2\beta 1$ mAb BHA2.1 abolished the stimulated adhesion by JBS2, with or without Mn^{2+}. In comparison, KX2C2(I^-) did not bind collagen or laminin either constitutively or when stimulated by JBS2 or JBS2 in the presence of Mn^{2+} (Figure 2, D and E). This observation is consistent with studies demonstrating a critical role of I-domains in the ligand-binding properties of integrins (Diamond *et al.*, 1993; Kamata *et al.*, 1994; Lee *et al.*, 1995). The mock-transfectant KpF lacking $\alpha 2\beta 1$ expression yielded results similar to KX2C2(I^-) cells (our unpublished observation). Adhesion of all three transfectants to fibronectin were comparable, and JBS2 did not stimulate increased adhesion to fibronectin (Figure 2, C and F). However, JBS2 did stimulate adhesion of KX2C2 pretreated with cycloheximide (10 μM), indicating that the effect of JBS2 on cell adhesion to collagen did not involve an induction of de novo protein synthesis (our unpublished observation).

Integrin α2β1 Can Mediate K562 Cell Migration on Collagen and Laminin

It has been suggested that integrins mediate cell migration at optimal levels of interaction with ECM proteins (Grzesiak *et al.*, 1992; DiMilla *et al.*, 1993; Keely *et al.*, 1995a). To determine the role of $\alpha 2\beta 1$ in random cell movement upon increases in its adhesive function, migration of KX2C2 cells on collagen and laminin was examined. KX2C2(I^-) cells expressing $\alpha 2\beta 1$ lacking $\alpha 2$ I-domain were used as control. Cell migration was estimated by the average of cell counts from five random high-power fields (400×) of ECM-coated filters under light microscopy. Results presented are the average (±SD) of triplicate wells from a representative experiment that has been repeated for a minimum of three times.

In the presence of normal mouse mAb P3, KX2C2 cells were significantly more migratory on collagen than KX2C2(I^-) cells, which produce a nonfunctional $\alpha 2\beta 1$ molecule ($p < 0.01$; Figure 3, A and C). The blocking mAb BHA2.1 at 20 μg/ml abolished the difference in their migratory activities on collagen. Therefore, although expression of $\alpha 2\beta 1$ did not result in stable adhesion of KX2C2, as detected by static cell

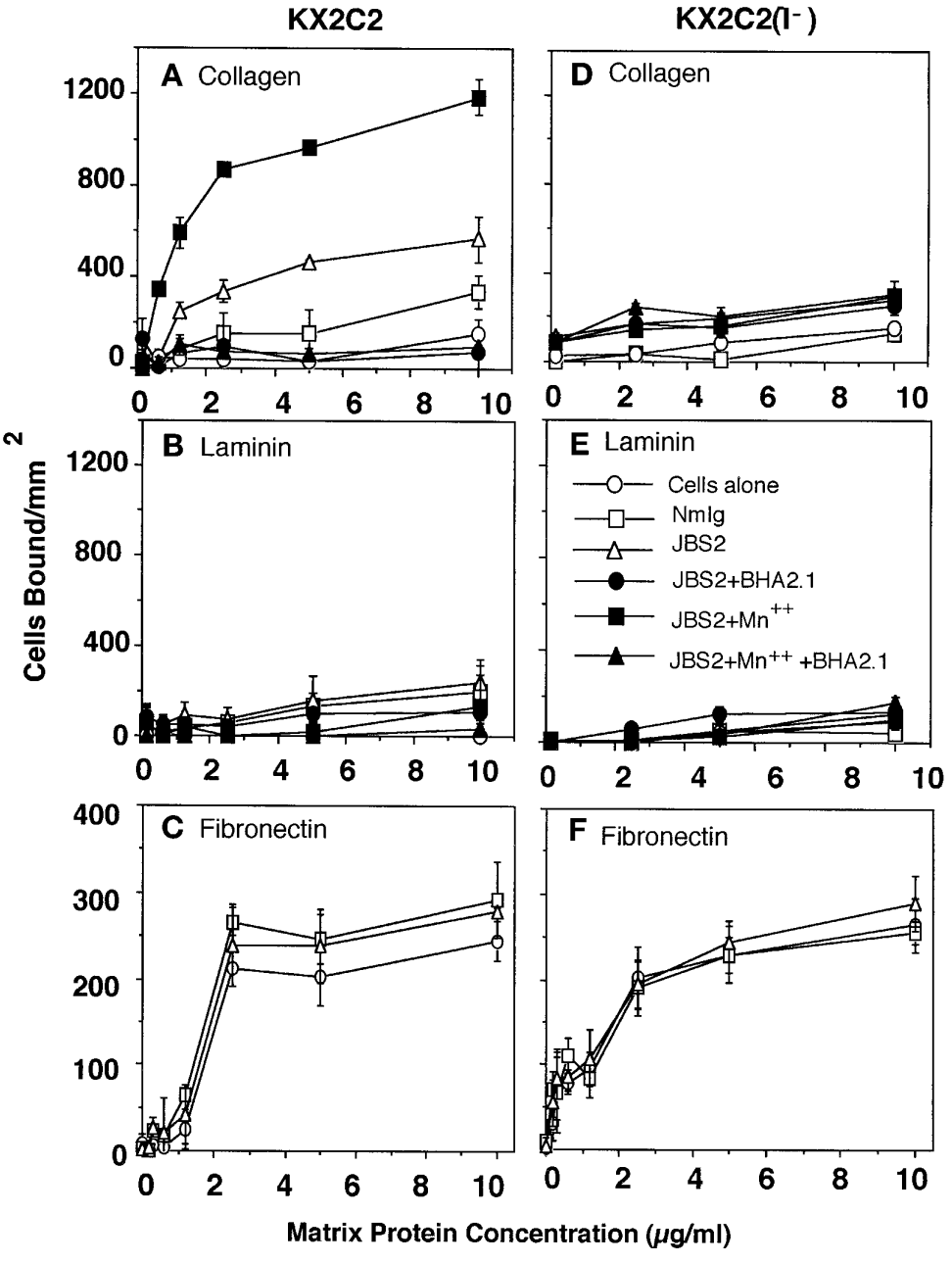

Figure 2. Effect of stimulatory mAb JBS2 on the adhesion of KX2C2 transfectant cells to collagen, laminin, and fibronectin. Adhesion of K562 transfectant cells, KX2C2 (A–C) and KX2C2(I^-) (D–F) to collagen (A and D), laminin (B and E), and fibronectin (C and F) was measured in the presence of stimulatory mAb JBS2 (20 μg/ml) alone or in combination with the blocking mAb BHA2.1 (5 μg/ml), in the presence or absence of 10 mM Mn^{2+}. Results were compared with adhesion in the presence of cells alone or Nmlg.

adhesion assays, it interacted with collagen and allowed increased KX2C2 cell movement. JBS2, which stimulated adhesion to collagen, further stimulated KX2C2 migration on this matrix protein ($p < 0.001$; Figure 3A). Whereas adhesion to collagen was further increased by the combination of JBS2 and 10 mM Mn^{2+}, cell migration was decreased ($p < 0.05$). Blocking mAb BHA2.1 at 20 μg/ml inhibited migration in the presence of P3, JBS2, or JBS2 in combination with Mn^{2+}, which indicated these effects were specifically due to $\alpha 2\beta 1$ expression. KX2C2(I^-), expressing the I-domain deletion variant of $\alpha 2\beta 1$, exhibited only a basal level of migration after treatment of these cells with P3, JBS2 with or without Mn^{2+}, or BHA2.1. Results are therefore consistent with the biphasic relationship between cell adhesion and migration (Grzesiak *et al.*, 1992; Wu *et al.*, 1994; Palecek *et al.*, 1997). Thus, up to a point, there is a progressive increase in migratory function with cell adhesion; however, combined stimulation by JBS2 and Mn^{2+} may go beyond the optimal level of adhesion for migratory function.

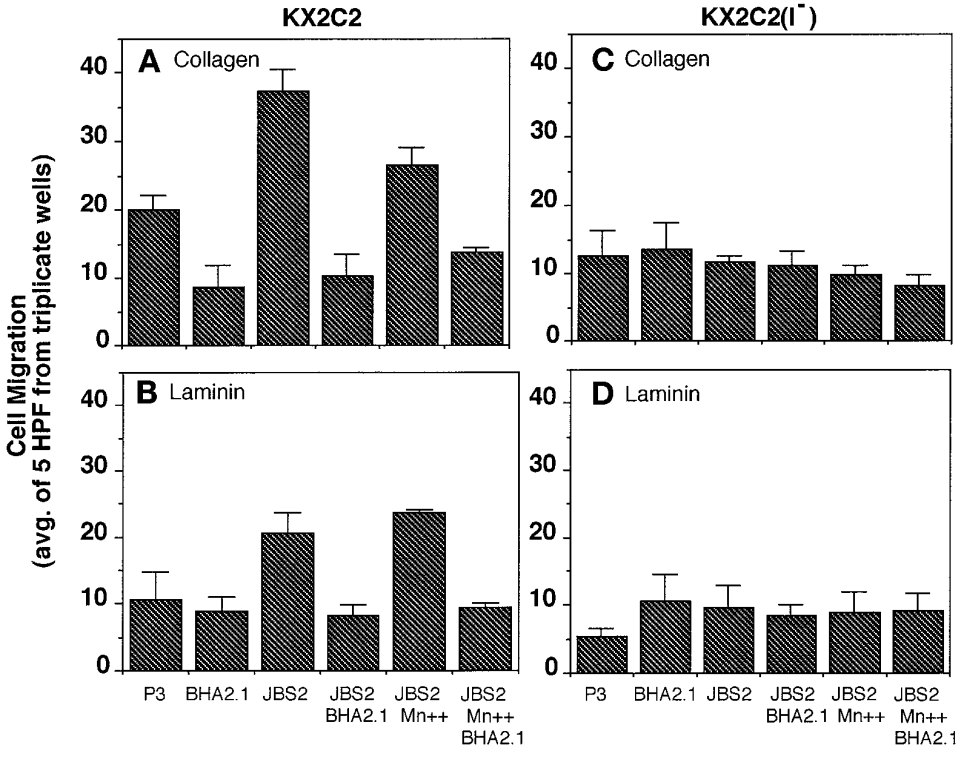

Figure 3. $\alpha 2\beta 1$-mediated K562 transfectant cell migration on collagen and laminin upon stimulation by JBS2. Migration of KX2C2 (A and B) and KX2C2(I^-) (C and D) transfectant cells were carried out on polycarbonate filters (10-μm pore size) coated with collagen (A and C) or laminin (B and D) at 10 μg/ml in the presence of $\alpha 2\beta 1$ stimulatory mAb JBS2 or in combination with 10 mM Mn^{2+}. The role of $\alpha 2\beta 1$ was determined using blocking mAb BHA2.1, with the isotype matched mAb P3 as control. All mAbs were used at 20 μg/ml. Values were enumerated from the average ($\pm$SD) of cell counts from five random high-power fields (400$\times$) from a minimum of three independent experiments.

As shown in Figure 3B, both KX2C2 and KX2C2(I^-) migrated on laminin to similar extents in the presence of P3 or the blocking mAb BHA2.1. Therefore, in contrast to what was observed on collagen, $\alpha 2\beta 1$ did not have a constitutive migratory function on laminin. Although JBS2 did not stimulate detectable adhesion of KX2C2 to laminin (Figure 2B), it stimulated migration on laminin ($p < 0.001$; Figure 3B). While JBS2 stimulation in the presence of 10 mM Mn^{2+} resulted in a decrease in the migration of KX2C2 on collagen, Mn^{2+} had no effect on the stimulated cell movement on laminin. BHA2.1 abolished the migration stimulated by JBS2 alone or in combination with Mn^{2+}. The basal levels of KX2C2(I^-) cell movement on collagen and laminin were not affected by JBS2 alone, JBS2 in combination with Mn^{2+}, or with blocking mAb BHA2.1 (Figure 3, C and D).

To determine whether the observed modulation of KX2C2 cell adhesion and migration on collagen (Figures 2 and 3) was due to an increase in the adhesive strength by JBS2 stimulation, adherent cells were removed at defined centrifugation force (g; Figure 4). At both $17 \times g$ and $30 \times g$, the presence of JBS2 increased the percentage of KX2C2 cells adherent on collagen, in comparison with control mAb. The percentage of adherent cells increased further when Mn^{2+} was added. For both JBS2-treated and JBS2- plus Mn^{2+}-treated cells, more cells remained adherent at $17 \times g$ than at $30 \times g$.

Modulation of $\alpha 2\beta 1$ Adhesive Function Can Affect RD Cell Movement

In a previous study, $\alpha 2\beta 1$ expression was shown to enhance RD cell adhesion and did not have a detectable effect on cell movement when compared with control RDpF transfectant (Chan *et al.*, 1992). This may be related to the biphasic cell movement with respect to adhesive function. Thus, RDpF exhibited basal adhesion and migration on collagen. An expression of $\alpha 2\beta 1$ resulted in increases in adhesion to the point that interaction was already beyond the optimal level, resulting in a reduction in cell movement to the same basal level of movement observed with RDpF cells. To determine whether RDX2C2 cell movement on collagen can be regulated by modulation of its adhesive function using blocking mAb BHA2.1, RDX2C2 cell movement was characterized in the presence of various concentrations of BHA2.1. As shown in Figure 5A, results from a static adhesion assay showed that the total number of adherent RDX2C2 remained relatively constant between 0 and 0.0002 μg/ml BHA2.1. Within this range of concentration of BHA2.1, RDX2C2 cell movement increased with increasing concentration of BHA2.1 (Figure 4B). At concentra-

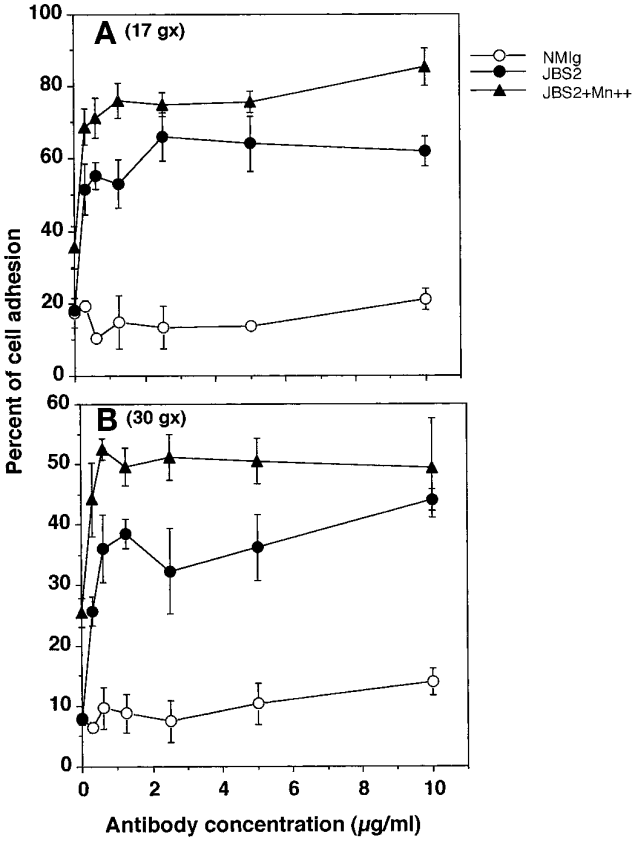

Figure 4. Determination of adhesive strength of KX2C2 cells bound onto collagen. KX2C2 cells were labeled with BCECF fluorescence label, treated with various concentrations of NmIg, JBS2, or JBS2 with Mn^{2+} at 20 μg/ml, and allowed to adhere to collagen-coated wells for 45 min at 37°C. Nonadherent cells were removed by centrifugation at 17 $\times$ g (A) or 30 $\times$ g (B). Adherent cells were expressed as the percentage of bound cells based on the fluorescence of 5 $\times$ 10^4 labeled cells after a subtraction of background fluorescence.

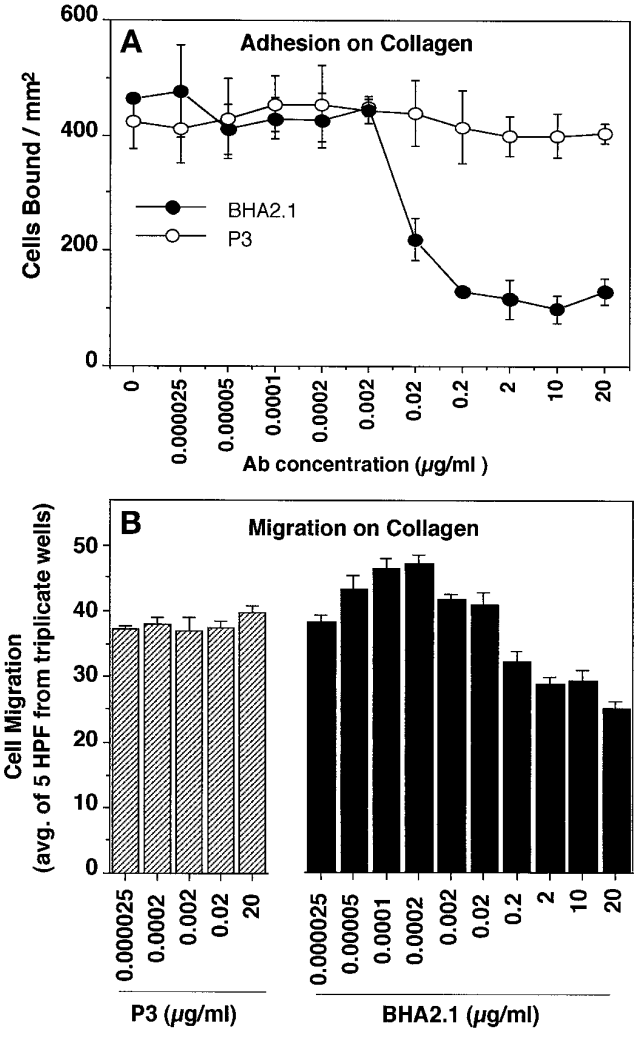

Figure 5. Adhesion and migration of RD transfectant cells at various concentrations of blocking $\alpha 2\beta 1$ mAb BHA2.1. Adhesion (A) and migration (B) of RD transfectant cells expressing $\alpha 2\beta 1$ (RDX2C2) on collagen substrate (10 μg/ml) in the presence of BHA2.1 were compared using isotype-matched control mAb P3 at the indicated concentrations.

tions greater than 0.002 μg/ml, RDX2C2 were inhibited from adhering and migrating on collagen. Control mAb P3 at the corresponding concentrations had no effect on adhesion and migration. A similar modulation of cell movement by various concentrations of BHA2.1 was also observed when cell migration was characterized by the method of wounding assay using collagen-coated glass slips (our unpublished observation). To determine whether the observed increases in RDX2C2 cell movement on collagen was due to a reduction in the adhesive strength by BHA2.1, adherent cells were removed at defined centrifugation force (g; Figure 6). The percentage of RDX2C2 remaining adherent decreased with increasing BHA2.1; as expected, a greater percentage of cells were removed at 30 $\times$ g than when at 17 $\times$ g ($p < 0.05$). In addition, at 17 $\times$ g, JBS2 (20 μg/ml) increased the adhesive strength and neutralized the effect of BHA2.1 blocking; the percentages of RDX2C2 remained adherent were similar to those observed in the presence of control mAb P3. At 30 $\times$ g, JBS2 was no longer able to compensate for the reduction in the adhesive strength by BHA2.1. In the presence of control mAb P3, the percentage of adherent RDX2C2 cells remained relatively constant at 17 $\times$ g and 30 $\times$ g. When the concentration of BHA2.1 was at zero, the percentage of RDX2C2 cells adherent on collagen was not significantly different after treatment with control antibody or JBS2 alone. This result indicated that $\alpha 2\beta 1$ expressed on RDX2C2 cells was already fully functional, and JBS2 stimulatory effect could be

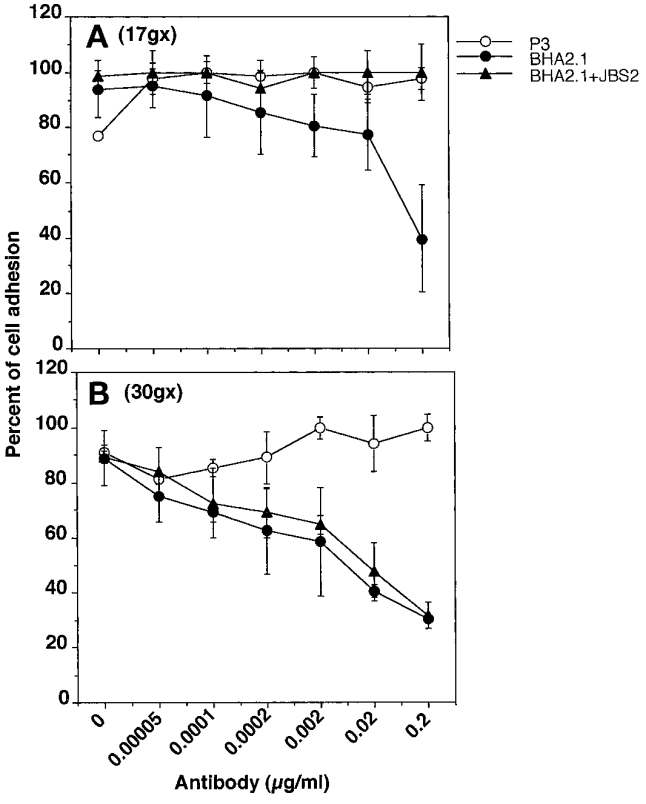

Figure 6. Determination of adhesive strength of RDX2C2 cells bound onto collagen. RDX2C2 cells were labeled with BCECF fluorescence label, treated with various concentrations of mAb P3, BHA2.1, or BHA2.1 with JBS2, at a constant concentration of 20 μg/ml, and allowed to adhere to collagen-coated wells for 45 min at 37°C. Nonadherent cells were removed by centrifugation at 17 $\times$ *g* (A) or 30 $\times$ *g* (B). Cell adhesion was expressed as the percentage of bound cells based on the fluorescence of 5×10^4 labeled cells after a subtraction of background fluorescence.

detected only when $\alpha 2\beta 1$ function was not fully active or partially blocked.

Integrin α2β1-mediated Cell Movement Can Be Modulated In Vivo

To determine whether the modulation of $\alpha 2\beta 1$ adhesion and migratory function observed in vitro can be demonstrated in vivo, KX2C2 and KX2C2(I$^-$) cell movement was further examined by IVVM. KX2C2 cells completed extravasation within 12 h after cell injection (our unpublished observation). Thus, to determine whether the $\alpha 2\beta 1$ migratory function in postextravasation can be modulated in vivo, combinations of JBS2, BHA2.1, and P3 were injected (i.v.) at 100 μg per mouse 12 h after cell injection. The percentage of extravasated KX2C2 cells that reached the liver subcapsular region was then determined by IVVM at 15 h after cell injection. As shown in Figure 7A, an injection of JBS2 with or without the control P3 increased the percentage of KX2C2 cells that reached the liver subcapsular space in comparison with either the cells alone or treated with the control mAb P3 ($p < 0.01$). In addition, injection of BHA2.1 abolished the migratory function of $\alpha 2\beta 1$ in vivo. Therefore, results suggest that the modulation of $\alpha 2\beta 1$ function by JBS2 in vitro can also be demonstrated in vivo. In addition, the ability of KX2C2(I$^-$) cells, expressing the nonfunc-

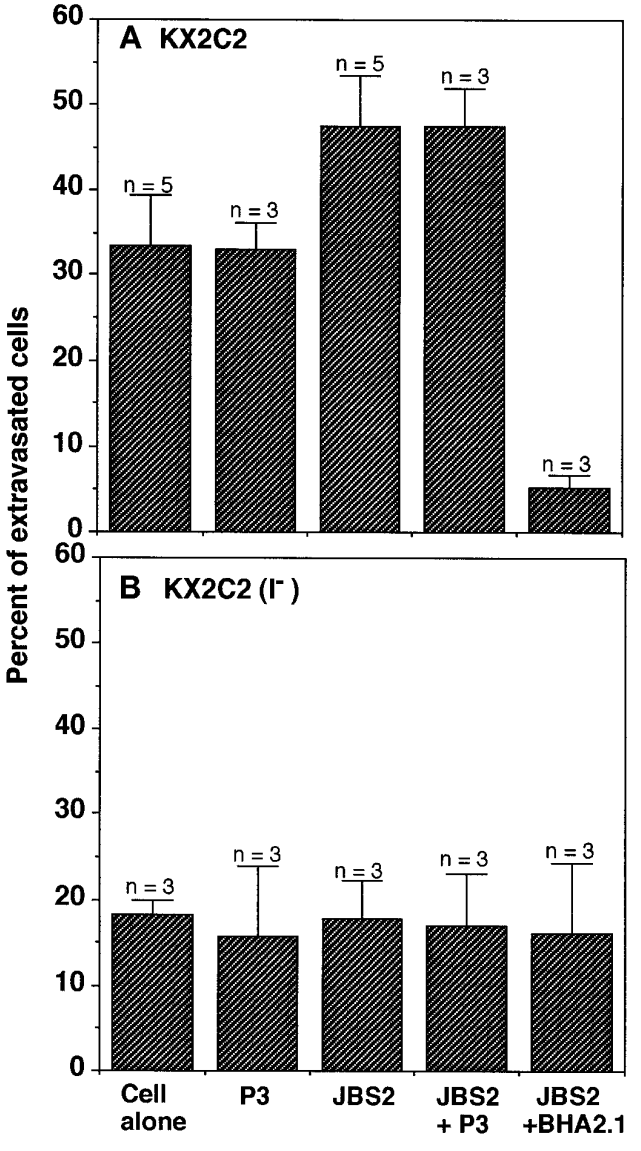

Figure 7. Modulation of migratory $\alpha 2\beta 1$ function in vivo. At 12 h after mesenteric vein injection of K562 transfectant cells KX2C2 (A) or KX2C2(I$^-$) (B) into *nu/nu* mice, the mice received an injection of mAb JBS2 alone (100 μg/mouse) or in combination with either blocking $\alpha 2\beta 1$ mAb BHA2.1 or control mAb P3 (i.v.) at 100 μg/mouse. Control mice were not injected or injected with control P3 mAb alone (200 μg/mouse). IVVM was then carried out 3 h after mAb injection to determine the percentages of extravasated cells that reached the subcapsular region of the liver.

Table 1. Functional relationship between $\alpha 2\beta 1$-mediated adhesion and migration of KX2C2 cells on collagen and laminin

	Collagen		Laminin	
	Adhesion	Migration	Adhesion	Migration
Control Ab	Not detectable[a]	+	Not detectable	Not detectable
JBS2	+	+++	Not detectable	+
JBS2 + Mn^{2+}	+++	++	Not detectable	+

Ab, antibody.

[a]Not detectable as determined by static cell adhesion assay involving simple washing steps.

tional $\alpha 2\beta 1$, to migrate to the liver subcapsular region was also examined. KX2C2(I^-) cells alone or treated with control mAb, JBS2, JBS2 and control mAb, or JBS2 and BHA2.1 had approximately the same low basal percentage of cells reaching the subcapsular region of the liver (Figure 7B). Thus, expression of a functional $\alpha 2\beta 1$ on KX2C2 was required for JBS2 modulation of KX2C2 cell migration to the liver subcapsular region.

DISCUSSION

It is well established that $\beta 1$ integrins have major roles in the process of extravasation (Lawrence and Springer, 1991; von Andrian *et al.*, 1991; Springer, 1994; Alon *et al.*, 1995; Berlin *et al.*, 1995). However, at present, little is known regarding their involvement in the movement of cells after extravasation into tissues. To reach the nonvascularized subcapsular region of the liver, cells in the circulation are required to extravasate and migrate through the parenchyma. In a recent study, we have demonstrated that expression of $\alpha 2\beta 1$ reduced the ability of RD cells to migrate through liver parenchyma, and RDX2C2 cells became arrested after extravasation with the appearance of wrapping around the sinusoids (Hangan *et al.*, 1996). Moreover, an injection of blocking mAb BHA2.1 restored RDX2C2 cell movement in liver parenchyma. Although this observation indicated a critical role of $\alpha 2\beta 1$ in affecting cell movement, it was not clear whether $\alpha 2\beta 1$ expression could in fact directly cause cell movement in vivo. We report herein that $\alpha 2\beta 1$ integrin can provide migratory function to human erythroleukemia K562 cells after extravasation into the liver. Thus, significantly more KX2C2 cells, expressing wild-type $\alpha 2\beta 1$, migrated through the parenchyma and entered the subcapsular region than did KX2C2(I^-), which expressed the nonfunctional $\alpha 2\beta 1$ variant lacking the I-domain. Therefore, the observed $\alpha 2\beta 1$ integrin migratory function in vivo could be stimulated or inhibited by $\alpha 2\beta 1$-specific mAbs JBS2 and BHA2.1, respectively.

Thus, with results from the previous study (Hangan *et al.*, 1996), it is apparent that $\alpha 2\beta 1$ expression enhanced K562 but reduced RD cells movement into the subcapsular region of the liver after extravasation. These in vivo observations correlated with in vitro results showing differential $\alpha 2\beta 1$ function between K562 and RD transfectant cells. Thus, expression of $\alpha 2\beta 1$ enhanced RD cell adhesion to collagen and laminin; however, on K562 cells $\alpha 2\beta 1$-mediated adhesion to either matrix protein was not detectable in static cell adhesion assays involving simple washing steps. These results are also consistent with studies demonstrating that $\alpha 2\beta 1$ function may vary among cell types (Elices and Hemler, 1989; Languino *et al.*, 1989; Kirchhofer *et al.*, 1990; Lotz *et al.*, 1990). Thus, on platelets and fibroblast cell lines, $\alpha 2\beta 1$ bound only collagen but not laminin, whereas on endothelial cells, $\alpha 2\beta 1$ bound both collagen and laminin. Although integrin function in cell movement may be modulated by local production of cytokines and chemokines (Luscinskas *et al.*, 1994; Issekutz, 1995; Campbell *et al.*, 1996; Carr *et al.*, 1996; Lloyd *et al.*, 1996; Weber *et al.*, 1996), it is apparent that the cell type dependence of integrin function initially demonstrated in vitro may also have a major role in determining cell movement in vivo.

At present, the exact mechanism that explains the different ligand-binding properties of $\alpha 2\beta 1$, as demonstrated in static cell adhesion washing assays, is still unclear. However, binding of stimulatory $\beta 1$-specific mAb Ts2/16 induced a functional transition of KX2C2 so that these cells now bind both collagen and laminin (Chan and Hemler, 1993). In comparison, results from the present study detected JBS2 stimulation of $\alpha 2\beta 1$ binding to collagen but not laminin. In addition, we have consistently observed that Ts2/16 stimulated more KX2C2 adhesion to collagen than JBS2 (our unpublished observation). Stimulation of $\alpha 2\beta 1$ function by JBS2 and Ts2/16 likely involved an induction of conformational changes that favor ligand binding (Arroyo *et al.*, 1993; Chan and Hemler, 1993; Stupack *et al.*, 1994). In addition, $\alpha 2\beta 1$ appears to interact with collagen more readily than with laminin as supported by our observation that KX2C2 exhibited constitutive migratory function on collagen but not laminin and binding of JBS2 stimulated adhesion of collagen but not

laminin. Although JBS2 did not stimulate a detectable increase in the binding of laminin, $\alpha 2\beta 1$-mediated KX2C2 cell movement on laminin was enhanced. A preferential ligand interaction has also been demonstrated in studies of the other $\beta 1$ integrins; for example, $\alpha 4\beta 1$ binds vascular cell adhesion molecule-1 (VCAM-1) more readily than fibronectin, and $\alpha 3\beta 1$ binds epiligrin/kalinin more readily than the other ECM proteins (Masumoto and Hemler, 1993; Weitzman *et al.*, 1993; Delwel *et al.*, 1994). In addition, based on static cell adhesion washing assays, $\alpha 2\beta 1$ expressed on K562 cells had been described as "nonfunctional" because no stable adhesion of KX2C2 to collagen or laminin was detectable (Elices and Hemler, 1989; Chan and Hemler, 1993). Results from the present study demonstrated that KX2C2 cells were constitutively more migratory on collagen when compared with KX2C2(I^-). Thus, it is apparent that $\alpha 2\beta 1$ mediated a low level of interaction of K562 cells with collagen that enhanced cell movement.

It is well established that the inserted (I)-domains are critical for integrin function (Diamond *et al.*, 1993; Kamata *et al.*, 1994; Lee *et al.*, 1995). The I-domain in $\alpha 2$ integrin subunit has been shown to be important for interaction with collagen (Kamata *et al.*, 1994). We have previously expressed the $\alpha 2\beta 1$ variant lacking $\alpha 2$ I-domain on RD cells and showed that $\alpha 2$ I-domain was also important for interaction with laminin (Hangan *et al.*, 1996). We show herein that $\alpha 2$ I-domain was critical for $\alpha 2\beta 1$ response to stimulation by JBS2. However, results from these studies cannot exclude the possibility of a global structural disruption of $\alpha 2\beta 1$ as a result of the removal of $\alpha 2$ I-domain. This is, however, unlikely since binding of HAS4 to the previously described RDX2C2(I^-) (Hangan *et al.*, 1996) and in the present study KX2C2(I^-) cells was still detectable, indicating that at least certain epitopes of $\alpha 2\beta 1$ are conserved after the deletion of $\alpha 2$ I-domain. The integrin $\alpha 2\beta 1$ function was not restored by a substitution with the I-domain of αL integrin subunit (our unpublished observation). Mutation of only two amino acids (Asp-151 and Asp-254) within $\alpha 2$ I-domain was sufficient to abolish $\alpha 2\beta 1$ binding of collagen (Kamata *et al.*, 1994) and recombinant $\alpha 2$ I-domain has been shown to bind collagen (Tuckwell *et al.*, 1995).

The migratory function of $\alpha 2\beta 1$ appeared to be, at least in part, regulated by its adhesive properties for matrix protein ligands. Results from the present study of $\alpha 2\beta 1$ migratory function is consistent with the concept that the adhesive strength of cell–matrix interactions affects cell movement in a biphasic manner; cell movement occurs when interaction with matrix proteins is optimal (DiMilla *et al.*, 1993; Huttenlocher *et al.*, 1996). The biphasic cell movement upon modulation of $\beta 1$ integrin adhesive function has been demonstrated in vitro using echistatin, an integrin-binding competitor, and divalent cations (Grzesiak *et al.*, 1992; Wu *et al.*, 1994). More recently, the speed of $\alpha 5\beta 1$- and αIIb$\beta 3$-mediated cell movement has been defined in terms of the concentration of the substrate, the level of integrin expression, and the extent of ligand binding (Palecek *et al.*, 1997). As shown in the present study with KX2C2, stimulation of $\alpha 2\beta 1$ by mAb JBS2 resulted in increases in both cell adhesion and movement on collagen; in the presence of Mn^{2+}, adhesion was further increased, whereas $\alpha 2\beta 1$-mediated migration was reduced. The removal of adherent KX2C2 cells from collagen at the different g forces revealed that JBS2 stimulation increased the adhesive strength to be closer to the optimal level required for migration of KX2C2 on collagen; addition of Mn^{2+} further increased adhesive strength beyond this optimal level and thus reduced cell movement. This observation may explain the variable adhesive and migratory function of $\alpha 2\beta 1$ reported for the different cells. With adhesive strength beyond optimal interaction, $\alpha 2\beta 1$ expression on mammary carcinoma Mm5 mT resulted in increases in adhesion but a reduction in cell movement (Zutter *et al.*, 1995). In the present study, RDX2C2 interaction with collagen was beyond the optimal level for cell movement. Decreases in adherence allowed RDX2C2 cells to approach optimal interaction with collagen and thus an increase in cell movement. In addition to our in vitro studies, we have also demonstrated for the first time that a $\alpha 2\beta 1$-modulated increase in adhesion can also affect cell movement in vivo. Thus, JBS2 stimulation of $\alpha 2\beta 1$ adhesion was associated with an increase in KX2C2 cell migration to the subcapsular region of the liver.

Cell migration in vitro involves protrusion of the leading edge of the cell that forms an adhesive complex with the matrix. Subsequently, the cell releases the adhesive bonds at the rear of the cell, which allows the cell to move over the substrate (Stossel, 1993; Lee *et al.*, 1994). The pseudopodial-like projections, termed invadopodia, may contain membrane-bound proteinases to facilitate cell movement by localized degradation of the matrix components (Kelly *et al.*, 1994; Monsky *et al.*, 1994). The $\beta 1$ integrins may also play a role in this process. $\alpha 6\beta 1$ integrin, as a receptor for laminin, has been shown to be involved in the formation of invadopodia with localized ECM degredation activities (Nakahara *et al.*, 1996). In vivo we also find that extravasated RD and mammary carcinoma cells frequently send out pseudopodial projections before they migrate (Morris *et al.*, 1994; Hangan *et al.*, 1996). Thus, in the liver, these cells first send these projections to the subcapsular region and then the body of the cell follows. RD transfectant cells expressing $\alpha 2\beta 1$ fail to migrate after extravasation; they generally wrap around blood vessels and remain firmly attached there (Hangan *et al.*, 1996). Our in vivo results and our in vitro findings suggest that limited adhesion with the

extracellular matrix via $\beta 1$ integrins may allow the pseudopodial projections to form and cell migration to occur. However, if the adhesive forces become too strong, then cells will tend to wrap around structures such as the basement membrane of blood vessels and prevent further cell movement. Thus, the ability of $\alpha 2\beta 1$ to confer stationary or motile phenotypes between different cell types may have determining effects on the distribution of cells in tissues after extravasation. The presence of cytokines and growth factors at sites where cancer cells eventually reside may have direct impact on the outcome of tumor metastasis. It has been shown that the metastatic potential of melanoma, osteosarcoma, rhabdomyosarcoma, and lung tumors correlated with increases in $\alpha 2\beta 1$ expression; whereas, decreases in $\alpha 2\beta 1$ expression has been associated with epithelial malignant transformation (Dedhar and Saulnier, 1990; Chan *et al.*, 1991; Klein *et al.*, 1991; Mortarini *et al.*, 1991; Pignatelli *et al.*, 1991; Danen *et al.*, 1993; Chen *et al.*, 1994; Santala *et al.*, 1994; Zutter *et al.*, 1995). Our current work and our recent studies of RD cells may explain why both an enhancing and inhibitory effect of $\alpha 2\beta 1$ on tumor metastasis have been reported. Thus, the extent of the interaction between $\alpha 2\beta 1$ and matrix proteins may confer differing migratory and adhesive properties among different tumor cell types; these differences, in turn, can cause them to reside in different tissue sites with various degrees of permissiveness to tumor foci formation. However, a complete understanding of cell movement in vivo will require addition determination of the role of the other adhesion molecules and their regulation by localized production of chemotactic and growth factors.

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