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Adhesive interaction of hemopoietic progenitor cell membrane with the RGD domain of fibronectin

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The binding of two cloned hemopoietic progenitor cell lines, B6Sut (multipotential) and FDCP-1 (bipotential) to dishes coated with fibronectin or its chymotryptic fragments was studied by labeling the cells with ^{51}Cr or [^{35}S]methionine. Intact fibronectin molecule and its 120 kDa fragment, containing the Arg-Gly-Asp (RGD) sequence motif, as well as a synthetic RGD-containing peptide Peptite 2000 all bound progenitor cells. However, the 40 or 45 kDa fragments, containing the heparin-binding and CS-1 domains, failed to bind the cells in a comparable magnitude. The binding of intact fibronectin and its 120 kDa fragment was inhibited in a dose-dependent fashion with increasing concentration of RGD-containing Gly-Arg-Gly-Asp-Ser peptide, but not with Gly-Arg-Gly-Glu-Ser control peptide that does not contain the RGD sequence motif. To explore the nature of the receptor for this fragment of fibronectin, membrane proteins were labeled with ^{125}I and subjected to affinity chromatography using a matrix to which the 120 kDa fragment of fibronectin was covalently bound. Specific competitive elution with RGD yielded two bands with molecular masses of 160 and 110 kDa, corresponding, respectively, to those of $\alpha 5$ and $\beta 1$ chains of integrin molecule. Western blotting of whole-cell-lysate proteins with a monospecific, polyclonal serum specific for vertebrate $\beta 1$ integrins identified a $\beta 1$ integrin in these cells. Thus, it appears that an interaction involving $\alpha 5\beta 1$ integrin with 120 kDa fragment of fibronectin may be involved between hemopoietic progenitor cells and the fibronectin component of extracellular matrix.

Introduction

Hemopoiesis is the result of a cascade of interactions between hemopoietic progenitor cells (HPC) and their supportive microenvironment [1,2]. The latter has a cellular component and an extracellular matrix component (ECM). Several adhesive interactions between progenitors and stromal cells (cell to cell) have been described. These include a recognition system that occurs between homing receptors on the surface of HPC and its ligand on the surface of stromal cells [2,3].

However, interactions occurring between progenitor cells and ECM are not well defined [1,4]. A major component of ECM to which progenitor cells may bind is fibronectin (FN) [5,6]. This concept is supported by several observations demonstrating that hemopoietic cells are capable of binding to FN, either in the extracellular form or membrane-bound [7,8]. Further observations have shown that this binding is lineage-specific and developmentally regulated [8–10].

Hemopoietic progenitor cells may use this binding to FN in search of a specific supportive 'niche' within the hemopoietic microenvironment. This may serve also as a mechanism to strengthen the selective binding of homing protein to its receptor [2,4,9].

Many previous studies have focused on this adhesive interaction in erythroid cells. In the present study we used two murine cloned, factor-dependent, multipotential (B6Sut) and bipotential (FDCP-1) progenitor cell lines to study the nature of the adhesive interactions of HPC with FN.

Fibronectin molecule possesses several domains, each with distinct binding capacity [11]. By chymotrypsin digestion, three major fragments are obtained. A 120 kDa fragment possesses only cell binding capacity that is mediated by the tripeptide sequence motif RGD [11,13]. Other known domains are the 45 kDa

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Abbreviations: FN, fibronectin; HPC, hemopoietic progenitor cells; ECM, extracellular matrix component; D-PBS, Dulbecco's phosphate-buffered saline; FCS, fetal calf serum; RGD, Arg-Gly-Asp, GRGDS, Gly-Arg-Gly-Asp-Ser; GRGES, Gly-Arg-Gly-Glu-Ser.

fragment that binds collagen and the 40 kDa fragment that is known as the heparin binding domain [12,13].

We studied the adherence of these progenitor cell lines to surfaces coated with intact FN or its chymotryptic fragments. We now report that the adherence of the cells to the 120 kDa fragment or to its equivalent synthetic peptide, Peptite 2000, is similar in magnitude to intact FN molecule. In both the 120 kDa fragment and the synthetic peptide the binding is exclusively mediated by the RGD sequence. We further investigated the characteristics of the binding of the progenitor cells to the 120 kDa fragment. Our results indicate that in both progenitor cell lines, an RGD-type integrin receptor is expressed which can bind the RGD sequence in FN, and this accounts for HPC binding to FN.

Materials and Methods

Cell cultures

The IL-3/GM-CSF-dependent murine cell line B6Sut proliferates in liquid phase, but under appropriate conditions in semisolid medium differentiates into mixed colonies containing erythroid, granulocyte-macrophage and basophil/mast cells, and, thus, is considered pluripotential. B6Sut cells were maintained in Kincaid's medium supplemented with 15% fetal calf serum (FCS) and 10% 5 × concentrated WEHI-3 conditioned medium as the source of growth factor [14,15]. The IL-3/GM-CSF-dependent murine cell line FDCP-1 can differentiate into granulocyte and macrophage lineages and, therefore, can be considered bipotential [16]. FDCP-1 cells were maintained in RPMI medium supplemented with 10% FCS and 10% 5 × concentrated WEHI-3 conditioned medium as the source of growth factor [16,17]. Both cell lines were subcultured twice weekly at 10^4 cells/ml, and were grown at 31°C (B6Sut) and 37°C (FDCP-1), under 5% CO_2. Both cell lines were provided through the courtesy of Dr. J. Greenberger (Worcester, MA, USA).

As positive control cells which are known to possess an $\alpha 5\beta 1$ integrin on their membrane, the mouse embryo fibroblast Swiss 3T3 cells (ATCC) were used. These cells were maintained as an adherent layer in DMEM (high glucose) with 10% FCS, and grown at 37°C, 5% CO_2. They were passaged weekly following trypsinization.

Cell adhesion assay

B6Sut and FDCP-1 cells grow in suspension and do not attach to plastic culture dishes, even in the presence of exogenous soluble FN in the culture medium. However, they do attach to the pericellular fibronectin matrix on murine stromal cells [17,18]. Thus, a cell adhesion assay was developed to measure the ability of HPC to attach to culture plates coated with intact FN or its chymotryptic fragments.

48-well plates were coated with either intact human FN (15 μg/ml, Collaborative Research, Bedford, MA, USA) or with the human FN proteolytic fragments of 120 kDa, 45 kDa and 40 kDa (15 μg/ml each, Telios, San Diego, CA, USA), or Peptite 2000 synthetic peptide (Telios, 10 μg/ml), or gelatin (2 mg/ml, Sigma, St. Louis, MO, USA) by incubation of the plates with a solution of each protein in Dulbecco's phosphate-buffered saline ((pH 7.4) D-PBS) for 1 h at room temperature. The remaining protein adsorption sites were saturated by incubation for 30 min with 10 mg/ml heat-denatured BSA in D-PBS.

Cells were labeled with ^{51}Cr using standard methods [19]. The final level of radioactivity achieved was approx. 30000 cpm/10^5 cells. In parallel experiments, metabolic labeling with [^{35}S]methionine produced similar results to ^{51}Cr labeling. Therefore, only results using the latter technique will be described.

Coated plates were overlaid with a suspension of ^{51}Cr-labeled cells ($3 \cdot 10^5$/well) in D-PBS containing 0.1% BSA. After incubating for 45 min at room temperature, the non-adherent cells were removed by washing the plates three times with D-PBS followed by mechanical agitation. Cells remaining attached to the plate were released by either trypsinization (0.1%, 10 min, 37°C) or by solubilization (0.1% SDS, 0.1 M NaOH), and the radioactivity was measured. The results of adhesion assays are expressed as a percentage of the cells plated, after correction for nonspecific binding to BSA-coated dishes, which usually accounted for 2–5% of the total cells added.

To study the effect of inhibitors of cell attachment, the synthetic peptide GRGDS (Telios) [12], control peptide GRGES (Telios) and the FN peptide CS-1 (Peninsula, Belmont, CA, USA) were added to the adherence assay at the concentrations indicated in the figure legends. GRGES does not compete for the RGD sequence as a result of the change in one residue in its sequence [12]. FN fragment CS-1 binds to the cell via an $\alpha 4\beta 1$ integrin, and is contained within the 40 kDa chymotryptic fragment of FN [20].

Cell surface iodination and affinity chromatography

For identification and isolation of a putative RGD-type FN receptor in progenitor cells, affinity chromatography studies were performed. Cells (10^8) were radioiodinated using 1 mCi of ^{125}I in the presence of *N*-chloro-benzenesulfonamide derivatized beads (Iodobeads, Pierce, Rockford, IL, USA), according to manufacturer's recommendations. After the labeling period, cells were washed three times with D-PBS containing 1 mM NaI. Cells were lysed in 1 ml column buffer (10 mM sodium phosphate, 150 mM NaCl, 1 mM $CaCl_2$, 1 $MgCl_2$ (pH 7.5)) containing 200 mM octylglucoside [8]

and a cocktail of proteinase inhibitors [3]. After a 30 min incubation at 4°C, cells were pelleted by centrifugation at 15000 × *g* for 15 min, and the cell extract thus obtained was stored at −80°C.

The 120 kDa FN fragment was immobilized on CNBr-activated Sepharose beads (Sigma) according to standard procedures, forming a matrix containing 2 mg/ml of the 120 kDa fragment. The cell extract was then applied to the affinity column with 1 ml bed volume, which had been pre-equilibrated with the column buffer containing 50 mM octylglucoside and the cocktail of proteinase inhibitors (starting buffer). Unbound proteins were removed by washing the column with 50 bed volumes of the starting buffer. Bound proteins were eluted by washing the column sequentially with starting buffer containing the control peptide GRGES (0.5 g/ml) and the active peptide GRGDS (1 mg/ml).

Fractions were collected, concentrated and the proteins were precipitated overnight at −80°C with 10 volumes of acetone to exclude the detergent. The pellet was resuspended in a small volume of D-PBS, aliquots were mixed with either reducing or non-reducing gel sample buffer, and subjected to SDS-PAGE analysis on a 7.5% gel [21]. Gels were stained with either silver or Coomassie blue, and autoradiograms of dried gels were prepared.

Western blotting

Whole cell octylglucoside extracts were prepared from B6Sut, FDCP-1 or 3T3 cells similarly to those which were prepared for affinity chromatography (above). In this case, D-PBS was the buffer system. Freshly prepared extracts were subjected to reducing conditions, loaded onto 12% polyacrylamide mini gels (SDS-PAGE gels), 25 μg of protein per lane, and the gels were run according to the Laemmli system. Proteins were transferred electrophoretically to nitrocellulose at 100 V for 1 h, using a mini-transblot apparatus (Bio-Rad, Richmond, CA, USA). Nitrocellulose blots were blocked for non-specific binding in 3% milk-0.5% Tween-PBS ((pH 7.4), Blotto) for 30 min at room temperature. They were probed for specific binding by an antibody directed against the β1 chain of vertebrate integrins, kindly provided by R.O. Hynes (MIT, Cambridge, MA, USA). This rabbit monospecific, polyclonal serum was prepared against a 39-amino-acid synthetic peptide from the C-terminus of the chicken integrin β1 subunit, coupled to KLH [22,23]. The serum was diluted 1:400 in Blotto, and incubated with the blots overnight at 4°C. Blots were washed three times for five min each in Tween-PBS, and re-blocked for 5 min in Blotto. An [^{125}I]anti-rabbit IgG (ICN) was diluted in Blotto such that approx. 2 μCi of radioactivity was incubated with each blot. Blots were incubated for 1 h at room temperature, washed as above, allowed to air dry, and subjected to autoradiography for three days.

Results

The adhesive properties of progenitor cells were examined in short-term adhesion assays to plastic surfaces coated with intact FN or with the various chy-

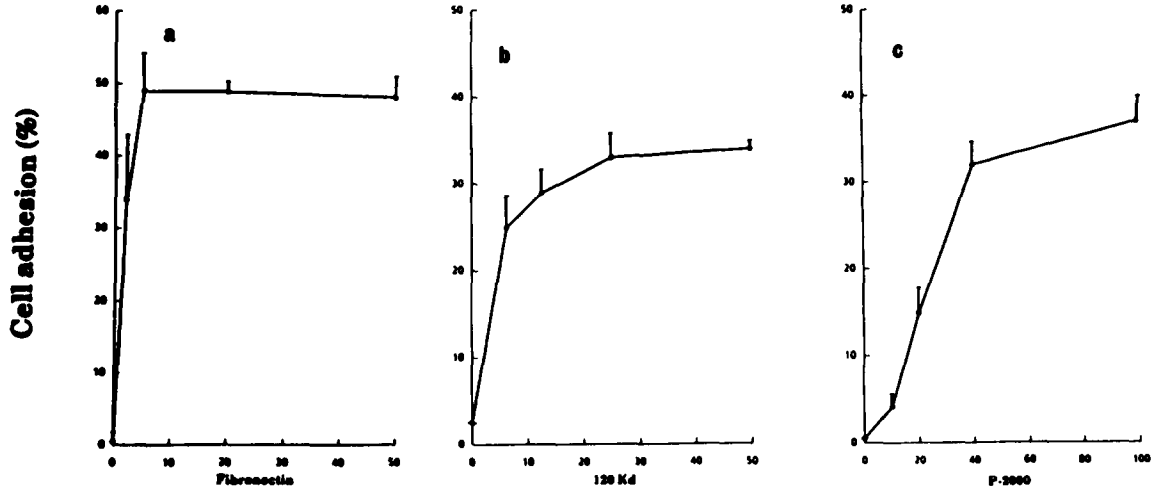

Fig. 1. Adhesion of B6 cells to increasing coating concentrations of FN, 120 kDa and Peptite 2000. Cells ($3 \cdot 10^5$) were added to multiwell dishes coated with various concentrations of FN (a), 120 kDa (b) and Peptite 2000 (P 2000) (c). Each value is the mean ± S.E. of triplicate experiments.

motryptic fragments of fibronectin (120, 45 and 40 kDa) containing the cellular, collagen and heparin-binding domains, respectively [12,13].

Both FDCP-1 and B6Sut cells showed a maximum attachment to FN-coated dishes on a cell basis of 60 ± 8.5% (FDCP-1) and 55 ± 5.3% (B6Sut) of the cell input. Both cell lines also attached, in a comparably high proportion, to the 120 kDa chymotrypsin fragment containing the central cell binding domain. In this case, adhesion was approx. 80 to 90% of that observed for intact FN, when one normalizes the binding to FN control as 100%. The extent of binding to the 40 kDa fragment, containing the heparin-binding site, varied according to the progenitor cell. Adherence of B6Sut cells to 40 kDa fragment was almost twice that of FDCP-1 cells (50% vs. 20%, respectively, as compared to binding to intact FN). Neither cell line bound the 45 kDa fragment containing the collagen-binding domain, nor to gelatin; binding to BSA-coated dishes was 2–5%.

Dose–response experiments showed that progenitor cells bound in a saturable manner to substrates containing the RGD sequence motif. As seen in Fig. 1 for B6Sut cells, coating concentrations of FN and 120 kDa higher than 20 μg/ml permitted maximal cell binding (Fig. 1a,b). Progenitor cells also bound in a saturable manner to a synthetic peptide (Peptite 2000) containing the RGD cell binding sequence. Results in Fig. 1c showed that adhesion to dishes coated with this peptide became saturable at concentrations close to 50 μg/ml. Similar dose–response results were obtained with FDCP-1 cells (not shown).

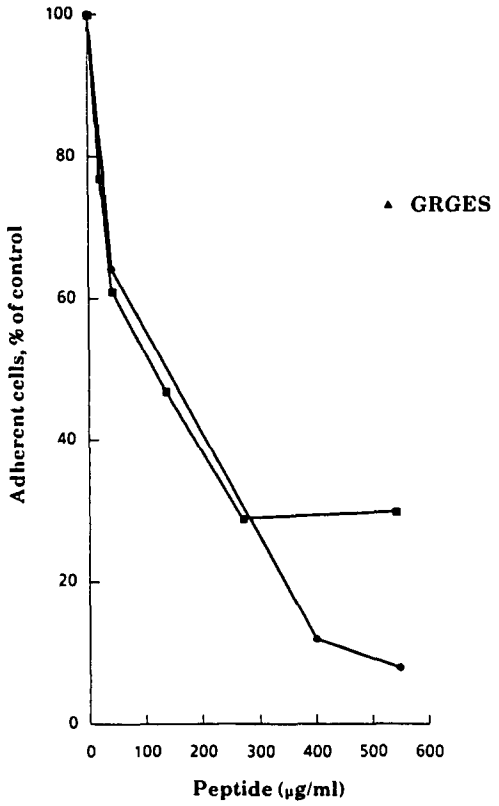

Fig. 2. Effect of the peptide GRGDS on the adhesion of B6 and F1 cells to FN-coated dishes. The adhesion of B6 (■) and F1 (●) cells to FN-coated dishes was assessed in the presence of increasing concentrations of the peptide GRGDS. For each cell line, adhesion in the absence of the inhibitory peptide was taken as 100%. Each value is the mean of three experiments. In all cases, S.E. (not indicated) was less than 10%. GRGES at a concentration of 550 μg/ml did not affect cell adhesion and was used as a negative control (▲).

The above results as well as those obtained after co-culturing progenitor cells with stromal cells [18] strongly suggested that hemopoietic progenitor cells could adhere equally well to both FN or the 120 kDa fragment immobilized on plastic surfaces and to the pericellular FN matrix on stromal cells. These data, moreover, indicated the Arg-Gly-Asp-Ser (RGDS) sequence as a potential site for cell binding [18].

This contention was further supported by experiments in which the synthetic peptide GRGDS was used as a competitor of adhesion of cells to surfaces coated with FN or with the 120 kDa fragment. As seen in Fig. 2, GRGDS at concentrations higher than 300 μg/ml inhibited almost completely the attachment of both FDCP-1 and B6Sut cells to intact FN. GRGDS inhibited cell adhesion to FN with an ID_{50} (inhibition dose that inhibits 50% of binding) of approx. 150 μg/ml (0.25 mM). The inactive control peptide GRGES at a high concentration (550 μg/ml) produced a small decrease in cell adherence.

As seen in Fig. 3 for FDCP-1 cells, adhesion to dishes coated with the 120 kDa fragment was also inhibited in a dose-dependent manner by the GRGDS peptide. The calculated ID_{50} was close to 150 μg/ml (0.25 mM). The inactive control peptide GRGES was ineffective and at a concentration of 500 μg/ml produced only a small inhibition (20%) of adhesion.

Solutions (up to 170 μg/ml) of the synthetic peptide CS-1 (Glu-Ile-Leu-Asp-Val-Pro-Ser-The) which spans part of the III CS region of FN [18], did not affect HPC adhesion either to intact FN or to the 120 kDa fragment (not shown). These results suggested that these HPC lines lack the non-RGD adhesion receptor for an alternative cell binding domain (CS-1) in FN [20,24].

Together, these data suggest that HPC adhesion to FN or to the RGD-containing 120 kDa fragment is mediated by a receptor with characteristics of an RGD-type integrin.

To identify and isolate the putative RGD-type integrin receptor in HPC, affinity chromatography studies were performed. These studies were based on the fractionation of a non-ionic detergent extract of ^{125}I-surface-labeled progenitor cells by affinity chromatography. Chromatography was done on a matrix containing the 120 kDa fragment that was immobilized by coupling to agarose beads. Under these conditions the

fragment retained its cell-attachment promoting activity [7,13] and, therefore, could bind the RGD-type integrin present in the cell extract.

Treatment of the affinity column with column buffer containing the peptide GRGDS eluted a major radioactive protein, around fraction 90. This was collected and concentrated by Amicon. The protein was then precipitated with 10 vols. cold acetone and redissolved in a small volume of D-PBS. It was subjected to SDS-PAGE under non-reducing conditions (Fig. 4). A major radioactive species was noted with calculated molecular mass of 160 kDa under non-reducing conditions, which approximately corresponds to the molecular mass of the $\alpha 5$ chain of RGD-dependent integrin that is an $\alpha 5\beta 1$. A second band (not seen here) with much less radioactivity was also noted with a molecular mass of about 110 kDa. This molecular mass could correspond to that of $\beta 1$ chains of integrin. The affinity of this protein for the matrix was unaffected by treatment with a solution containing the control peptide GRGES. These bands were also faintly observed on Coomassie blue or silver-stained gels as well, and

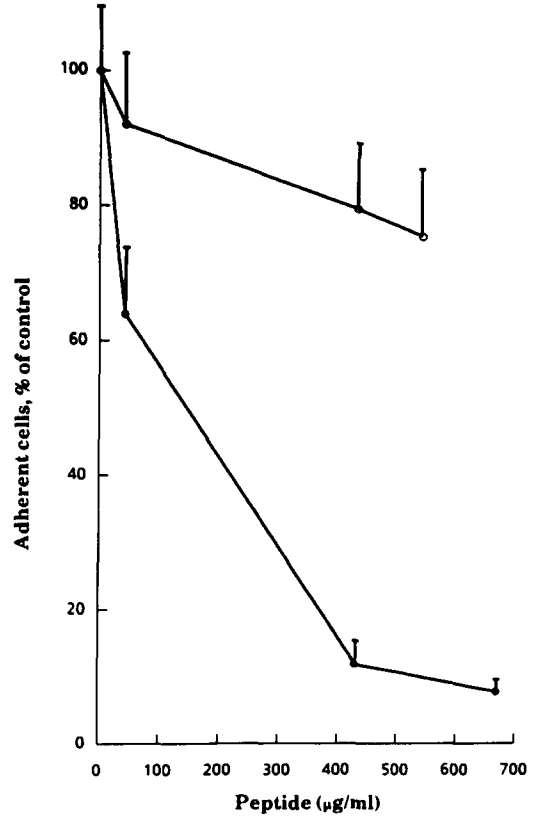

Fig. 3. Effect of peptides GRGDS and GRGES on the adhesion of F1 cells to 120-kDa-coated dishes. The adhesion of F1 cells to 120-kDa-coated dishes was assessed in the presence of increasing concentrations of the active peptide GRGDS (●) or in the presence of the control peptide GRGES (○). Adhesion in the absence of the peptides was taken as 100%. Each value represents the mean ± S.E. of triplicate experiments.

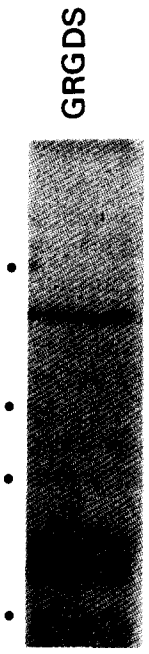

Fig. 4. Autoradiogram of SDS-PAGE analysis of components eluted from a 120 kDa fragment affinity matrix. Cells (10^8) were ^{125}I-surface labeled and extracted in a non-ionic detergent-containing buffer. Cell extract was applied to an affinity chromatography column containing Sepharose-4B coupled to the 120 kDa fibronectin fragment. Affinity-adsorbed material was eluted sequentially with the synthetic peptides GRGES and GRGDS. Fractions were collected and aliquots were analyzed by SDS-PAGE under non-reducing conditions, followed by silver staining and autoradiography. GRGDS selectively eluted a major radioactive species of approx. 160 kDa. Molecular mass markers, indicated on the left are: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa and BSA, 66 kDa.

both staining techniques showed that the 160 kDa and 110 kDa proteins were detected only when the column was eluted with the GRGDS peptide (not shown).

Fig. 5 presents the results of Western blotting experiments in which detergent cell extracts of HPC, which had been separated by SDS-PAGE under reducing conditions, were blotted with an antibody against the vertebrate $\beta 1$ integrin chain [22,23]. The fibroblast cell 3T3 is known to contain an $\alpha 5\beta 1$ integrin, and displayed specific binding of the antibody (panel A). The lower band, approx. 95 kDa, is thought to contain the intracellular precursor form of this molecule [23]. The upper band of approx. 115–120 kDa represents the intact, membrane bound form of the $\beta 1$ integrin chain. FDCP-1 (panel B) and B6Sut cells were both positive for the integrin, displaying the same two molecular species of the integrin.

Discussion

The initiation of hemopoiesis requires several molecular interactions, through which progenitor cells 'home' to the bone marrow stroma [1–3]. It is through these interactions that intravenously transplanted stem

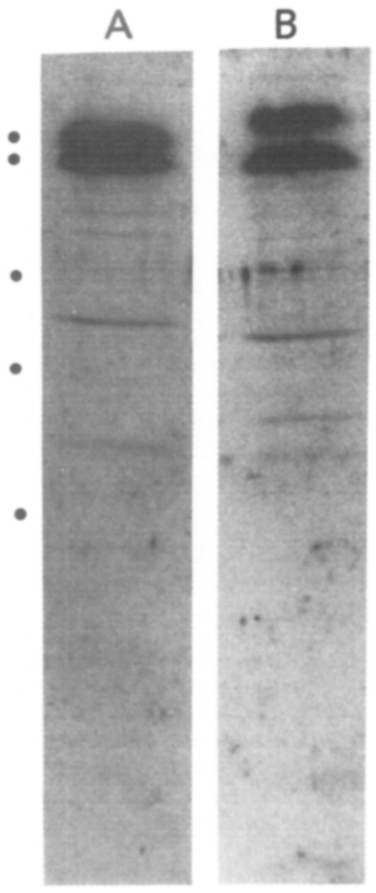

Fig. 5. Autoradiogram of Western blotting experiments using anti-β1 integrin serum. Detergent extracts of 3T3 cells (positive control) and FDCP-1 cells, were separated by SDS-PAGE under reducing conditions on 12% gels, electrophoretically transferred to nitrocellulose and immunoblotted with rabbit anti-β1 integrin serum. The blots were visualized using an [^{125}I]anti-rabbit IgG secondary antibody and autoradiography. The lower band (95 kDa) is thought to represent the intracellular pool of the integrin, while the upper (115–120 kDa) is the mature membrane form. Panel A, 3T3 cells; panel B, FDCP-1 cells. Molecular mass markers, indicated on the left are: β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; BSA, 66 kDa and ovalbumin, 45 kDa.

cells, although moving through many organs via the blood stream, are recognized and selectively 'home' to the bone marrow. Homing appears to be a multi-step event initiated by cell-cell interactions via a membrane recognition protein on the surface of progenitor cells interacting with a membrane glycoconjugate on stromal cells [2,3]. Probably this early recognition step is followed by other adhesive processes in which the binding of cells to ECM is involved [4,8,10].

Adherence of cells to ECM is known to be mediated by several molecular mechanisms [25] including FN receptors, collagen and membrane-associated proteoglycans [18]. In the case of adherence to FN, a heterogeneous binding potential exits that is the consequence of the presence, in this glycoprotein, of several domains with distinct biological properties [12,24,26]. The interaction involving CS-1 domain of FN with $\alpha 4\beta 1$ species of integrin has been described by Williams et al. [27] who, however, failed to detect an interaction involving the $\alpha 5$ chain of integrin.

The data presented here indicate that FN, which is produced and organized into a matrix by stromal cells [5,28], is a major stromal component for adhesive interactions with HPC. These data also provide some insight into the nature of at least one of the various membrane-associated molecules in progenitor cells involved in adhesive interactions with FN [4,18].

Our observations further indicate that in the adhesion of progenitor cells to FN, the RGD binding domain of FN is involved. Evidence for this contention was provided by adherence studies of progenitor cells to surfaces coated with either intact FN or with the 120 kDa chymotryptic fragment of FN. The adhesion of progenitor cells to FN and 120 kDa-coated dishes was competitively blocked by a synthetic peptide, which contains the Arg-Gly-Asp-Ser sequence found in the cell-binding fragment of FN [11]. This inhibition was not observed when adhesion was assayed in the presence of a related peptide (GRGES) in which the Asp residue is replaced by Glu (Arg-Gly-Glu-Ser), as well as in the presence of a synthetic peptide, CS-1. The latter contains the binding sequence of a non-RGD site that is located close to the heparin-binding region in FN [20].

These results predict the existence of an RGD-type integrin ($\alpha 5\beta 1$) molecule in the progenitor cell. Our affinity chromatographic studies showed a band with a molecular mass of 160 kDa (similar to that of $\alpha 5$ integrin) and a less appreciable band with a molecular mass of approx. 110 kDa (similar that of the $\beta 1$ chain of integrin) [26]. These bands could be eluted increasingly with time by competitive concentrations of GRGDS, but not with the control peptide. This indicates that these HPC express an $\alpha 5\beta 1$ integrin capable of interacting with the cell-binding domain of FN. In further experiments (shown in Fig. 5) the $\beta 1$ integrin was identified immunologically by Western blotting using a monospecific polyclonal serum which identifies this integrin in vertebrate species [22,23]. There are fewer of such immunological reagents available to identify murine integrin α chains. The $\beta 1$ integrin was detected immunologically in both FDCP-1 cells (Fig. 5) and B6Sut cells (not shown). The presence of an $\alpha 5\beta 1$-containing integrin has been described previously in murine leukemic cells, $CD34^+$ myeloid precursors and erythroid progenitors [7–9,29].

Thus, there appear to be at least three molecular interactions involved in the adherence of progenitor cells to a pericellular matrix of FN, produced by stromal cells [5,28]. The first one is through an $\alpha 5$-containing integrin receptor in progenitor cells interacting with the RGD sequence of the cell binding domain of

FN. The second one is a membrane-associated chondroitin sulfate proteoglycan on the side of progenitor cells [30,31] interacting with the heparin-binding domain of FN [18]. A third interaction described by Williams et al. [27] involves $\alpha 4$ integrin with CS-1 (40 kDa chymotryptic fragment of FN).

Whereas the extent of binding of both cell lines to 120 kDa fragment was similar, the magnitude of binding to the 40 kDa fragment was higher in B6 cells. This higher binding deserves a comment. The 40 kDa fragment contains the heparin-binding domain of FN and binds to membrane-associated chondroitin sulfate proteoglycan (CS-PG) [18]. In a previous study, we have documented that the membrane density of CS-PG is higher in pluripotent than in bipotent HPC [29]. This may explain the higher binding of B6 cells to the 40 kDa fragment. Selective regulation of the expression of these FN receptors on the surface of HPC could be important in the modulation of cytoadhesion, as well as in related events such as cell differentiation and migration.

Acknowledgements

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