

Heparan Sulfate Proteoglycan on Leukemic Cells is Primarily Involved in Integrin Triggering and Its Mediated Adhesion to Endothelial Cells

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Summary

Leukocyte migration from circulation into tissue depends on leukocyte integrin-mediated adhesion to endothelium, but integrins cannot function until activated. However, it remains to be understood how tumor cells adhere to endothelium and infiltrate into underlying tissue. We studied mechanisms of extravasation of leukemic cells using adult T cell leukemia (ATL) cells and report the following novel features of cell surface heparan sulfate proteoglycan on ATL cells in ATL cell adhesion to endothelium: ATL cells adhere to endothelial cells through already activated integrins without exogenous stimulation; different from any other hematopoietic cells, ATL cells express a characteristic heparan sulfate capable of immobilizing heparin-binding chemokine macrophage inflammatory protein (MIP)-1 β , a potent T cell integrin trigger, produced by the cells themselves; competitive interruption of endogenous heparan sulfate proteoglycan synthesis reduces cell surface MIP-1 β and prevents ATL cells from integrin-mediated adhesion to endothelial cells or intercellular adhesion molecule-1 triggered through G-protein. We propose that leukemic cells adhere to endothelial cells through the adhesion cascade, similar to normal leukocyte, and that the cell surface heparan sulfate, particularly on ATL cells, is pivotally involved in chemokine-dependent autocrine stimulation of integrin triggering by immobilizing the chemokine on them.

Leukocyte migration from circulation into tissue is understood to involve, in a coordinated sequence of events, stabilizing adhesion molecules both on the leukocyte and on the endothelium (1–4). The adhesion is mediated by leukocyte integrin binding to endothelial ligands (5). However, leukocyte integrins cannot function until they are activated and integrin triggers are essential to the integrin-mediated adhesion in which a signal transduced to the leukocyte converts the functionally inactive integrin to an active adhesive configuration (1, 3). We have reported that the chemokine macrophage inflammatory protein-1 β (MIP-1 β)¹ triggers integrin and induces adhesion of T cell

subsets to endothelial integrin ligands (6). Recent papers and reviews have supported the potential importance of chemokines in inflammatory responses that various other chemokines, as well as MIP-1 β , produced in large amounts from inflamed tissues trigger integrins on leukocytes and monocytes which leads to their accumulation in the tissues (4, 7). Furthermore, we and others have proposed that chemokines MIP-1 β and IL-8 recruit leukocytes most efficiently when immobilized on the luminal surface of endothelium and that heparan sulfate proteoglycan on the endothelium immobilize the chemokines in this process without being washed away by the blood flow (6, 8–10).

Heparan sulfate proteoglycan is posttranslationally modified by the addition of heparan sulfate glycosaminoglycan side chains made up of long and linear disaccharide subunits at serine residues of core protein, some of which are in the extracellular matrix, while others are integral membrane proteins (11, 12). Various cytokines and growth factors such as all the chemokines, IL-7, GM-CSF, fibroblast growth

¹Abbreviations used in this paper: ATL, adult T cell leukemia, FGF, fibroblast growth factor; HEX-D-xyloside, hexyl- β -D-xyloside; HUVEC, human umbilical vein endothelial cells; ICAM, intercellular adhesion molecule; MIP-1 β , macrophage inflammatory protein-1 β ; NAP-D-xyloside, (6-hydroxyl)-2-naphthyl- β -D-xyloside, NAP-L-xyloside, (6-hydroxyl)-2-naphthyl- β -L-xyloside.

factor (FGF), TGF, and hepatocyte growth factor possess heparin-binding sites, which allow these proteins to bind to heparan sulfate proteoglycan (13–17). These factors interact in functionally important ways with heparan sulfate in several contexts: (a) extracellular matrix heparan sulfate proteoglycan binds these factors such as FGF as a reservoir (18, 19); (b) cell surface heparan sulfate interacts with these factors, e.g., FGF, to facilitate their binding with the primary high affinity receptor on the same cells; and (c) we and others have proposed that cell surface heparan sulfate immobilize the factors such as GM-CSF and MIP-1 β and present them to their primary receptor on another cell (6, 17). Furthermore, heparan sulfate enhances formation of cytokine multimers, which facilitates cross-linking of the cytokine receptors.

The extravasation of tumor cells which is one important step of tumor metastasis is mediated by tumor cell adhesion to endothelial cells (20, 21). However, it remains to be understood how tumor cells adhere to endothelium and subsequently infiltrate into underlying tissue. We have addressed the mechanism of extravasation of leukemic cells using adult T cell leukemia (ATL) cells. ATL is a unique and useful model to assess the extravasation, since ATL is a peripheral CD4⁺ T cell malignancy caused by infection with HTLV-I and since ATL shows a marked increase of peripheral ATL cells with monoclonal growth and severe infiltration into multiple organs in an acute phase (22–24). The present report demonstrates that heparan sulfate proteoglycans are abundantly expressed on the surface of ATL cells and ATL cell lines, whereas any other hematopoietic cells and cell lines which we have screened do not express them, and proposes that the heparan sulfate proteoglycans on ATL cells which immobilize integrin-triggering chemokine in an autocrine mechanism play a pivotal role in the continuous triggering of integrin and facilitation of ATL cell adhesion to endothelial cells.

Materials and Methods

ATL Cells and ATL Cell Lines. 18 patients with ATL and one case of chronic T cell leukemia which is not infected with HTLV-I as a control, four established HTLV-I-infected T cell lines, MT-1, MT-2, HUT-102, and SALT-3 (from K. Sagawa, University of Kurume, Kurume, Japan, and I. Miyoshi, Kochi Medical University, Kochi, Japan) were used. ATL was diagnosed according to the clinical features, hematological findings, serum antibodies against HTLV-I, and monoclonal integration of HTLV-I proviral genome (22, 23). Highly purified CD4⁺ T cells and ATL cells were prepared by exhaustive negative selection (3) from PBMC of normal donors and ATL patients using magnetic beads (Dynal, Oslo, Norway) and multiple-antibody cocktail consisting of CD19 mAb FMC63, CD16 mAb 3G8, CD11b mAb NIH11b-1, and CD14 mAb 63D3.

Antibodies and Other Reagents. The following mAbs were used as purified Ig in preparation of T cells and ATL cells, staining and analysis of cell surface molecules, blocking of cellular adhesion; anti-heparan sulfate mAb HK249 which was locally established

(25) and 10E4 (Seikagaku Kogyo, Tokyo, Japan), activated LFA-1 mAb NKI-L16 (C. Figdor, University Hospital, Nijmegen, Netherlands) (26), CD19 mAb FMC63 (H. Zola, Flinders Medical Center, Bedford Park, Australia), CD11b mAb NIH11b-1, CD49d (very late antigen [VLA]-4) mAb NIH49d-1, CD54 (intracellular adhesion molecule [ICAM]-1) mAb 84H10 (S. Shaw, National Institutes of Health [NIH], Bethesda, MD), CD49d mAb HP2/1 (F. Sanchez-Madrid, The Princess Hospital, Madrid, Spain) (27, 28), CD16 mAb 3G8 (D. Siegel, Bethesda, MD), CD2 mAb MAR206 (A. Moretta, University of Genova, Genova, Italy), CD62L (L-selectin) mAb Leu-8 (Becton Dickinson Japan, Tokyo, Japan), CD14 mAb 63D3, CD11a (LFA-1 α) mAb TS1/22, MHC class I mAb W6/32, control mAb Thy1.2 (ATCC, Rockville, MD). ICAM-1 was purified by affinity column chromatography from the Reed-Sternberg cell line L428 as previously described (3, 29). (6-hydroxyl)-2-naphthyl- β -D-xyloside (NAP-D-xyloside) which is a competitive inhibitor capable of interrupting endogenous heparan sulfate- and chondroitin sulfate-proteoglycan biosynthesis, its nonfunctional isomer (6-hydroxyl)-2-naphthyl- β -L-xyloside (NAP-L-xyloside), and hexyl- β -D-xyloside (HEX-D-xyloside) which competitively interrupts endogenous chondroitin sulfate- but not heparan sulfate-proteoglycan synthesis were developed as previously described (30, 31, and manuscript in preparation).

Adhesion Assay. Adhesion assay of ATL cells and cell lines to human umbilical vein-derived endothelial cells (HUVEC) or purified ICAM-1 glycoproteins was performed as previously described (3, 29). HUVEC were applied to 48-well culture plates (Costar Corp., Cambridge, MA) coated with 2% gelatin and were cultured to confluence in DMEM (Nissui, Tokyo, Japan) containing 100 U/ml penicillin G, 100 U/ml streptomycin, 20% heat-inactivated FCS, endothelial mitogen 20 μ g/ml (Biomedical Technologies, Stoughton, MA) and heparin (10 U/ml). After washing with PBS, HUVEC were stimulated with 1 ng/ml IL-1 β (Otsuka, Tokyo, Japan) for 4 h at 37°C. Purified ICAM-1 (50 ng/well) was applied to the 48-well plates in Ca/Mg-free PBS at 4°C overnight. Binding sites on the plastic were subsequently blocked with Ca/Mg-free PBS/3% HSA (Green-Cross, Osaka, Japan) for 2 h at 37°C to reduce nonspecific attachment. The plates were washed three times with PBS before the addition of T cells. 2×10^5 T cells, ATL cells, and ATL cell lines, which were labeled with ⁵¹Cr (DuPont NEN, Wilmington, DE) in RPMI 1640 (Nissui) with 1% HSA, were added to the culture with or without relevant adhesion-blocking mAb in the presence or absence of PMA (10 ng/ml, Sigma Chemical Co., St. Louis, MO) which is a pharmacologically relevant trigger for integrin adhesiveness. All mAbs were used at a saturating concentration of 10 μ g/ml, which was shown in previous studies to maximally inhibit the relevant adhesive interaction (3). After a settling phase of 30 min at 4°C, which also allowed mAb binding, plates were rapidly warmed to 37°C for 30 min. Then, the plates were gently washed twice with RPMI 1640 at room temperature to remove nonadherent T cells and ATL cells completely. Contents of each well containing adherent T cells or ATL cells were lysed with 250 μ l of 1% Triton X-100 (Sigma), and γ emissions of well contents were determined. Data are expressed as mean percentage of binding of indicated cells from a representative experiment.

Flow Microfluorometry. Staining and flow cytometric analyses of freshly obtained ATL cells or normal T cells were carried out by standard procedures as already described using a FACScan[®] (Becton Dickinson and Co., Mountain View, CA) (3). Briefly, cells (2×10^5) were incubated with negative control mAb thy1.2,

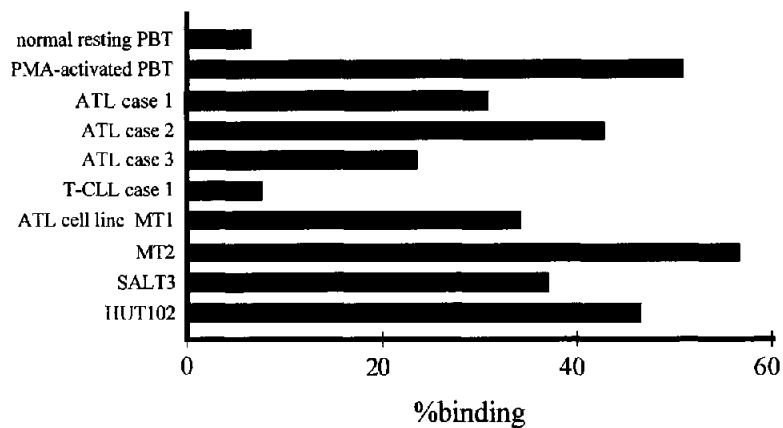


Figure 1. Spontaneous adhesion of ATL cells to IL-1 β -activated HUVEC. Adhesion of resting or PMA-activated peripheral normal CD4⁺ T cells, ATL cells freshly obtained from periphery of three representative ATL patients, CD4-positive leukemic cells from a patient with HTLV-I-negative T cell leukemia and four ATL cell lines, MT1, MT2, SALT-3, and HUT-102 which were labeled with ⁵¹Cr to IL-1 β -activated HUVEC, was assessed after the 30-min incubation at 37°C. γ Emissions of the lysates of only adherent cells were determined. Data are expressed as mean percentage of binding of indicated cells from a representative experiment

L-selectin (CD62L) mAb Leu-8, LFA-1 (CD11a) mAb TS1/22, VLA-4 (CD49d) mAb NIH49d-1, CD2 mAb MAR206, activated LFA-1 mAb NKI-L16, anti-heparan sulfate mAb HK249 (25), or 10E4 in FACS[®] media consisting of HBSS (Nissui, Tokyo, Japan), 0.5% HSA, and 0.2% NaN₃ (Sigma) for 30 min at 4°C. After washing the cells with FACS[®] media three times, the cells were further incubated with FITC-conjugated goat anti-mouse IgG Ab for 30 min at 4°C. After washing and incubating with irrelevant mAbs, the cells were further incubated with CD4-PE (Fujisawa, Osaka, Japan) for 30 min at 4°C. The staining of cells with the mAbs was detected by the gating on CD4-positive cells using a FACSscan[®]. Amplification of the mAb binding was provided by a three-decade logarithmic amplifier.

Northern Blot Analysis. For Northern blot analysis, total cellular RNA was isolated from freshly obtained normal T cells, ATL cells, and ATL cell lines by using single-step isolation procedure (32). The RNA (10 μ g) was electrophoresed on 1% agarose gel and was blotted onto nylon membrane filters (Amersham Corp., Arlington Heights, IL). EcoRI fragment of pAT 744 MIP-1 β cDNA (U. Siebenlist, NIH, Bethesda, MD) (33) and β -actin cDNA (P.E. Auron, Harvard Medical School, Boston, MA) were labeled with [³²P]dCTP and subsequent Northern blot analysis was performed.

ELISA Assay of MIP-1 β in Supernatant or Cytosol of ATL Cells. Freshly obtained normal T cells and ATL cells (10⁶) were washed in PBS and lysed with 250 μ l of PBS containing 2% *N*-octyl- β -D-glucopyraide (Sigma). The culture supernatants were collected from normal T cells (10⁶) and ATL cells (10⁶) after the 24-h incubation in RPMI 1640 with 5% FCS at 37°C without any stimulation. The MIP-1 β protein levels in each sample were measured by MIP-1 β ELISA system (R&D Systems, Minneapolis, MN). The sensitivity of the assay is 4.0 pg/ml of MIP-1 β and it was not affected by the presence of *N*-octyl- β -D-glucopyraide at the concentration used to lyse cells. Results were expressed in nanograms per milliliter per 10⁵ cells.

Immunofluorescence Staining of Cell Surface Heparan Sulfate and MIP-1 β . Freshly obtained ATL cells and ATL cell line MT1 were cultured with or without 1 mM NAP-D-xyloside for 5 h at 37°C or were treated with the mixture of 100 mU/ml heparinase I and II for 2 h at 37°C. After putting the cells on glass slides by cytopsin, indirect immunofluorescence was performed with anti-heparan sulfate mAb HK249 and PE-conjugated anti-rat IgG (Fujisawa) or anti-MIP-1 β Ab (U. Siebenlist) (33) and FITC-conjugated anti-rabbit IgG (Fujisawa). The staining was analyzed

with a fluorescence microscope (Epi-fluorescence Condense IV FI; Zeiss, Oberkochen, Germany).

Results

ATL Cells and Cell Lines Spontaneously Adhered to HUVEC. ATL is a characteristic leukemia which shows a marked increase of peripheral ATL cells and their severe infiltration into multiple organs in an acute phase (22–24). Leukocyte migration from circulation into tissue depends on leukocyte integrin-mediated adhesion to endothelial cells. Because leukocyte integrins cannot function until activated, the regulation of integrin-dependent adhesion is critical to the migration of virtually all the hematopoietic cells (1, 3). For instance, as shown in Fig. 1, resting peripheral T cells did not bind to IL-1-activated HUVEC, whereas T cells activated with PMA, which is one potent integrin trigger, adhered to them well. CD4-positive leukemic cells from a patient with HTLV-I-negative T cell leukemia also did not bind to HUVEC. However, both ATL cells freshly obtained from three representative ATL patients and four ATL cell lines, MT1, MT2, SALT-3, and HUT-102, spontaneously bound to HUVEC without any exogenous stimuli after the 30-min incubation.

ATL Cells and Cell Lines Expressed Activated LFA-1 and Heparan Sulfate on the Cell Surface. Leukocyte selectins and integrins mediate its adhesion to endothelial cells (1). Next, we assessed the expression of these adhesion molecules on freshly obtained ATL cells using a flow cytometer (Fig. 2). Although it was assumed that ATL cells (Fig. 2, group B) might express these adhesion molecules more than normal T cells (Fig. 2, group A), integrin LFA-1 and CD2 were equally expressed on ATL cells with normal CD4⁺ T cells (Fig. 2, b and d) and L-selectin and VLA-4 were rather decreased on ATL cells compared with CD4⁺ T cells (Fig. 2, a and c). However, it is of interest that NKI-L16 mAb which binds to functionally activated LFA-1 (26, 34) reacted significantly highly with most of the freshly obtained ATL cells, compared with resting CD4⁺ normal T cells (Fig. 2 e). The mean fluorescence intensity for NKI-L16

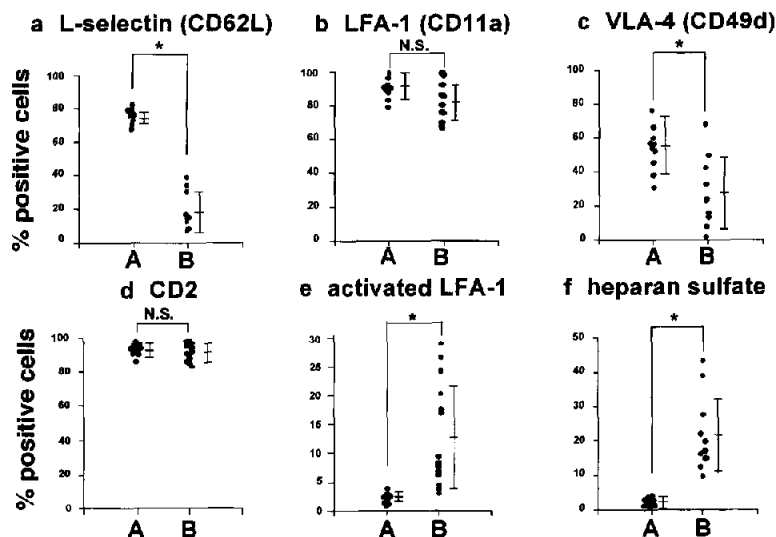


Figure 2. Phenotypic analysis of fresh ATL cells by flow cytometer. Staining and flow cytometric analyses of 10 resting peripheral normal CD4⁺ T cells (group A) and ATL cells freshly obtained from periphery of 18 ATL patients (group B) were carried out with (a) L-selectin (CD62L) mAb Leu-8, (b) LFA-1 (CD11a) mAb TS1/22, (c) VLA-4 (CD49d) mAb NIH49d-1, (d) CD2 mAb MAR206, (e) antiactivated form of LFA-1 mAb NKI-L16, (f) anti-heparan sulfate mAb HK249 using FACScan[®]. Each point shows the percent positivity of the binding of indicated mAbs compared with control mAb Thy1.2 from individual persons. The bars indicate mean \pm SD of each group and the asterisks show an overall statistical significance of $P < 0.01$ (Student's *t* test).

staining was also higher in ATL cells than that in normal CD4⁺ T cells (285 ± 79 vs. 146 ± 31 , $P < 0.01$ by Student's *t* test). All the ATL cells also expressed MHC class II antigens, CD25 and CD69, regarded as activation markers (data not shown). The results suggest that spontaneous adhesion of ATL cells to endothelium depends on already triggered/activated integrins rather than the quantity of the integrins expressed on ATL cells.

Furthermore, it is worthy of note that all the ATL cells and cell lines expressed heparan sulfate proteoglycan on the cell surface detected by HK249 mAb, which possesses sharp

specificity for tumor type (O-sulfate deficient) heparan sulfate chains (25), but resting T cells did not at all (Fig. 2*f*). As far as we have done, none of both resting and cytokine or lectin-activated cells from hematopoietic origin expressed the heparan sulfate (data not shown). The heparan sulfate on one representative ATL patient and ATL cell line MT1 was also detected by another anti-heparan sulfate mAb 10E4 (Fig. 3, B and E). The expression was reduced by the pretreatment of ATL cells or MT1 with the mixture of 100 mU/ml heparitinase I and II for 2 h at 37°C (Fig. 3, C and F), whereas the expression of LFA-1 on ATL cells

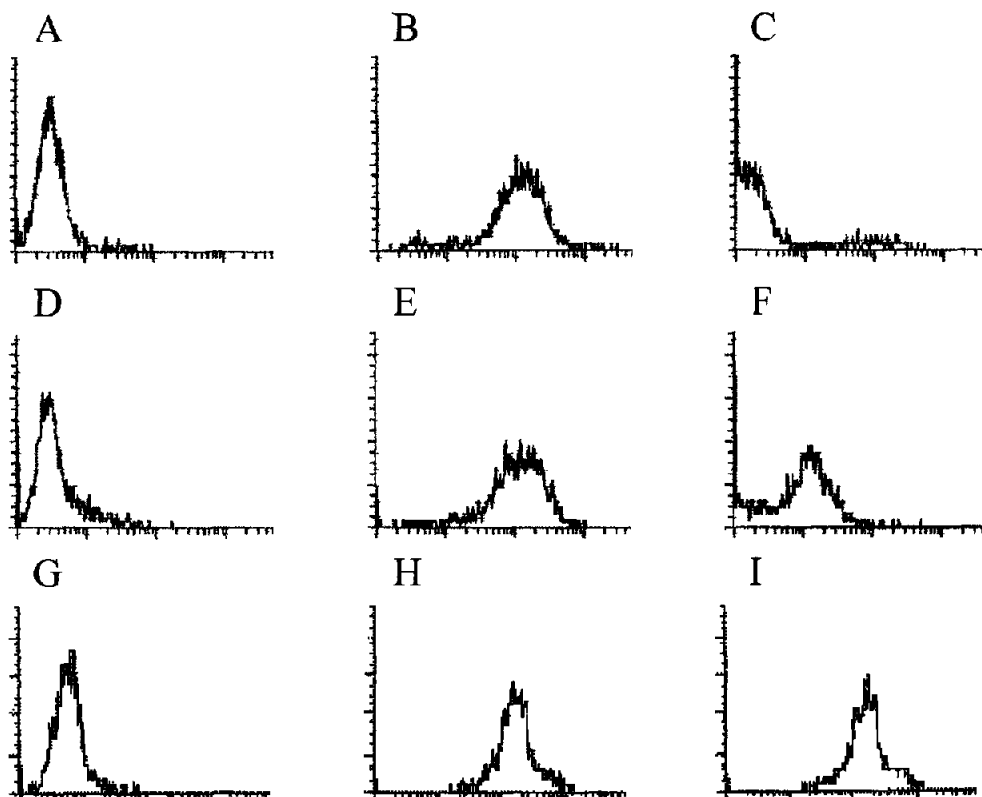


Figure 3. Expression of heparan sulfate on ATL cells and ATL cell line MT1. The expression of heparan sulfate on ATL cell line MT1 (A, B, C, G, H, and I) and one representative ATL patient (D-F) which were pretreated with (C, F, and I) or without (B, E, and H) the mixture of 100 mU/ml heparitinase I and II for 2 h at 37°C was detected by anti-heparan sulfate mAb 10E4 (B, C, E, and F) or anti-LFA-1 (CD11a) mAb TS1/22 (H, I) using FACScan[®]. γ -axis represents the histogram of cell number stained with mAbs in each logarithmic scale of fluorescence amplifier. Thy1.2 mAb was used as a negative control of the staining (A, D, and G).

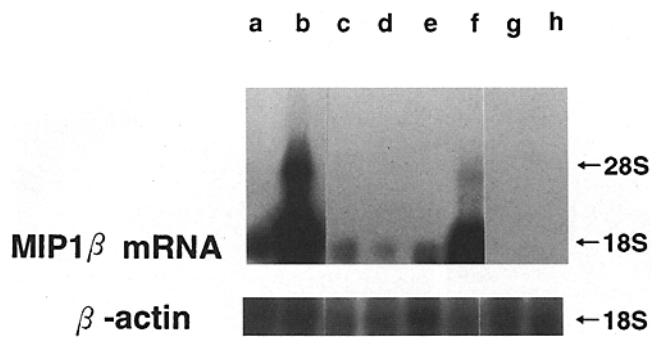


Figure 4. Spontaneous MIP-1 β mRNA transcription on ATL cells and cell lines. For the Northern blot analysis, total cellular RNA was isolated from HUT-102 (lane *a*) and MT2 (lane *b*), three representative ATL patients (lanes *c-f*), and normal CD4⁺ T cells (lanes *g* and *h*) and Northern blot analysis was performed using pAT 744 MIP-1 β cDNA and β -actin cDNA.

was not affected by the treatment (Fig. 3, *H* and *I*). Thus, different from any hematopoietic cells which we have examined, ATL cells characteristically and specifically expressed heparan sulfate proteoglycan on their surface.

ATL Cells and Cell Lines Spontaneously Produced Chemokine MIP-1 β . We and others have reported that ATL cells produce multiple cytokines including IL-1, IL-6, and TNF- α (35–37). ATL cell lines MT2 and HUT-102 and four representative freshly obtained ATL cells spontaneously synthesized large amounts of MIP-1 β mRNA by Northern blot analysis, whereas normal T cells did not (Fig. 4). Furthermore, freshly obtained ATL cells from periphery of the patients produced significantly high amounts of MIP-1 β protein in the culture supernatant as well as in the cytosol without any stimulation, compared with normal T cells (Fig. 5). MIP-1 β is a member of chemokine family which is now understood to trigger leukocyte integrin and to induce its adhesion, e.g., MIP-1 β and MIP-1 α for T cell subsets and IL-8 for neutrophils (6, 9, 38), all of which

were also produced by ATL cells (data not shown). Thus, ATL cells spontaneously produced multiple proadhesive chemokines.

MIP-1 β Was Expressed on the Surface of ATL Cells and Its Expression Was Reduced by the Interruption of Heparan Sulfate-Proteoglycan Synthesis. As shown in Fig. 6 *A*, heparan sulfate was strongly detected on ATL cell line MT1, by immunofluorescence staining using anti-heparan sulfate mAb HK249. Of note is that MIP-1 β was also comparably stained with anti-MIP-1 β Ab on the surface of not only MT1 (Fig. 6 *D*) but also freshly obtained ATL cells (Fig. 6 *G*). Chemokines including MIP-1 β possess heparin-binding sites (39) and we and others have proposed that cell surface heparan sulfate proteoglycan immobilizes chemokine (6, 8–10). Interestingly, when MT1 or ATL cells were pretreated with the mixture of 100 mU/ml heparitinase I and II for 2 h, the expression of MIP-1 β as well as heparan sulfate on the surface was markedly reduced (Fig. 6, *B*, *E*, and *H*). Furthermore, pretreatment of MT1 and ATL cells with 1 mM NAP-D-xyloside for 5 h which interrupts endogenous heparan sulfate proteoglycan biosynthesis (30, 31, and manuscript in preparation), resulted in suppression of cell surface MIP-1 β (Fig. 6, *F* and *I*). These results indicate that ATL cell-derived MIP-1 β appeared to be retained on the ATL cell surface through binding to heparan sulfate chains of proteoglycan expressed on ATL cells.

Heparan Sulfate Was Involved in the Spontaneous Adhesion of ATL Cells. Finally, we assessed if the heparan sulfate is involved in the spontaneous adhesion of ATL cells and MT1 to HUVEC. MT1 (Fig. 7 *A*) and freshly obtained ATL cells (Fig. 7 *B*) without stimulation and PMA-activated peripheral normal T cells (Fig. 7 *C*) adhered to IL-1-activated HUVEC well. However, when MT1 and ATL cells were pretreated with NAP-D-xyloside, the adhesion was inhibited in a concentration-dependent manner, whereas its nonfunctional isomer NAP-L-xyloside did not affect the adhesion (Fig. 7, *A* and *B*). Neither NAP-D-xyloside nor

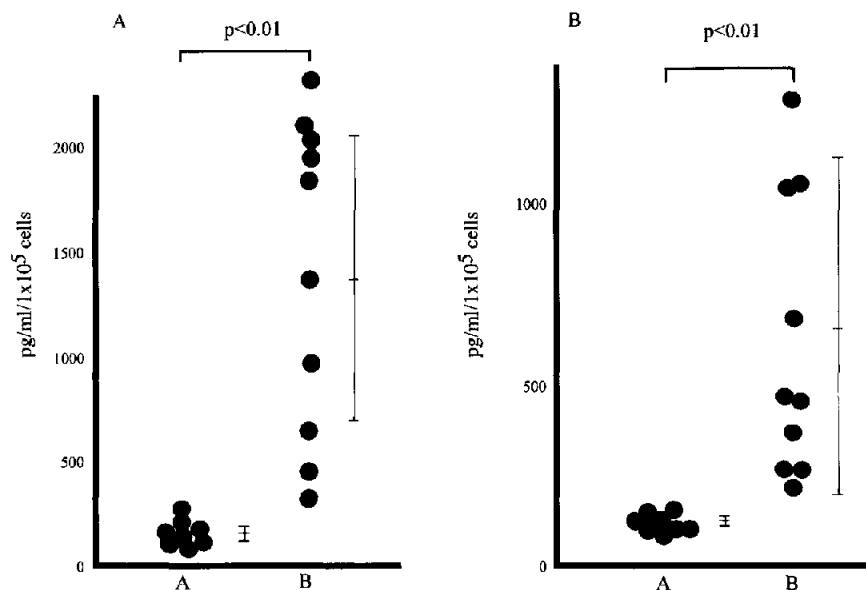


Figure 5. Spontaneous MIP-1 β production from ATL cells. The cytokine levels in cytosol (*A*) of normal CD4⁺ T cells (group *A*) and ATL cells freshly obtained from periphery of ATL patients (group *B*) or culture supernatants (*B*) collected from the normal CD4⁺ T cells (group *A*) or the ATL cells (group *B*) after the 24-h incubation at 37°C without any stimulation were determined by MIP-1 β ELISA system. Each point shows the quantity of MIP-1 β in the lysate or the supernatant derived from 10⁵ cells of individual persons. The bars indicate mean \pm SD of each group and the asterisks show an overall statistical significance of $P < 0.01$ (Student's *t* test).

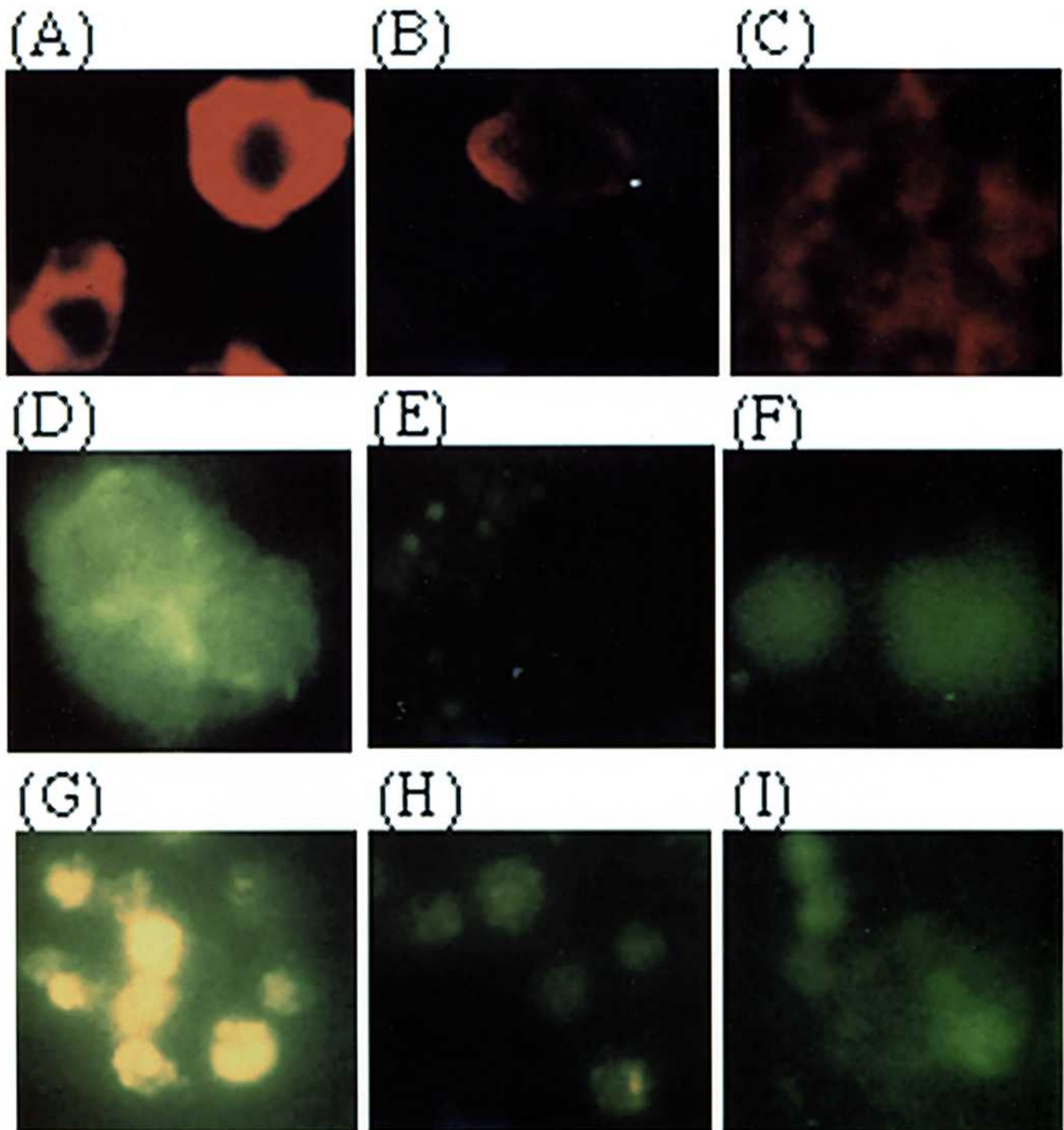


Figure 6. Expression of heparan sulfate and MIP-1 β on ATL cells and MT1 subjected to xyloside interruption of heparan sulfate proteoglycan biosynthesis and enzymatic digestion of heparan sulfate chains. MT1 (A-F) and fresh ATL cells from one representative patient (G-I) were cultured without (A, D, and G) or with 1 mM NAP-D-xyloside for 5 h at 37°C (B, E, and H) or were treated with the mixture of 100 mU/ml heparitinase I and II for 2 h at 37°C (C, F, and I). Indirect immunofluorescence was performed with anti-heparan sulfate mAb HK249 and PE-conjugated anti-rat IgG (A-C) or anti-MIP-1 β Ab and FITC-conjugated anti-rabbit IgG (D-I) and the expression was observed by fluorescence microscopy ($\times 1,000$).

NAP-D-xyloside affected the adhesion of PMA-activated normal T cells, which did not express heparan sulfate to HUVEC (Fig. 7 C).

MT-1 also spontaneously adhered to purified ICAM-1, an endothelial ligand for the integrin LFA-1 and it was in-

hibited by the pretreatment with NAP-D-xyloside, whereas neither NAP-D-xyloside nor HEX-D-xyloside did affect the adhesion (Fig. 8). Furthermore, both the adhesion of MT1 to purified ICAM-1 (Fig. 8 A) and to IL-1-activated HUVEC (Fig. 8 B) was reduced by the pretreatment of MT1 with

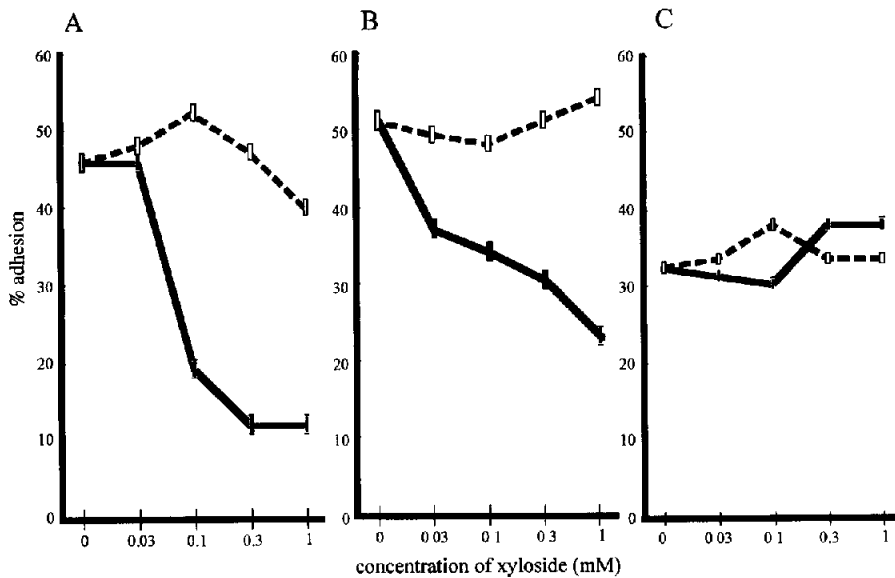


Figure 7. The effect of particular xyloside on the adhesion of ATL cells and MT1 to IL-1-activated HUVEC. (A) MT1, (B) fresh ATL cells, and (C) PMA-activated normal T cells were preincubated with or without indicated concentration of NAP-D-xyloside (*dense line*) or NAP-L-xyloside (*dotted line*) for 5 h at 37°C and adhesion assay of obtained cells to IL-1-activated HUVEC was performed as in Fig. 1. Data are expressed as mean percentage of binding of indicated cells from a representative experiment.

1 µg/ml pertussis toxin, ADP ribosylates which uncouple certain G-protein from its complex. mAb-blocking studies, in which MT1-adhesion to ICAM-1 or activated HUVEC was inhibited by anti-LFA-1/VLA-4 mAbs or anti-LFA-1 mAb (10 µg/ml), respectively, indicated that the adhesion was mediated by ATL cell integrins and their endothelial ligands. Thus, the results suggested that heparan sulfate was involved in integrin-mediated adhesion of ATL cells to endothelial integrin ligands, which might be activated through G-protein.

Discussion

The cell surface heparan sulfate in the form of proteoglycan is known to immobilize heparin-binding cytokines or growth factors, affecting cell growth/adhesion and modu-

lating clinically relevant events such as inflammation and tumor metastasis (11, 14, 18). Here we demonstrate three pivotal points for the mechanisms of how heparan sulfate amplifies leukemic cell adhesion to endothelium, using ATL cells, which show a spontaneous adhesion to HUVEC in vitro and a tendency of severe infiltration into tissue in vivo: (a) suitable heparan sulfate is expressed on ATL cells, whereas none of resting and activated cells from hematopoietic origin do express heparan sulfate; (b) cell surface heparan sulfate immobilizes integrin-triggering chemokine on ATL cells, since the expression of cell surface chemokine is abolished by xyloside-induced interruption of heparan sulfate-proteoglycan biosynthesis or by heparitinase digestion of cell surface-associated heparan sulfate chains; and (c) the artificially decreased cell surface heparan sulfate results in the integrin-mediated adhesion of ATL cells to en-

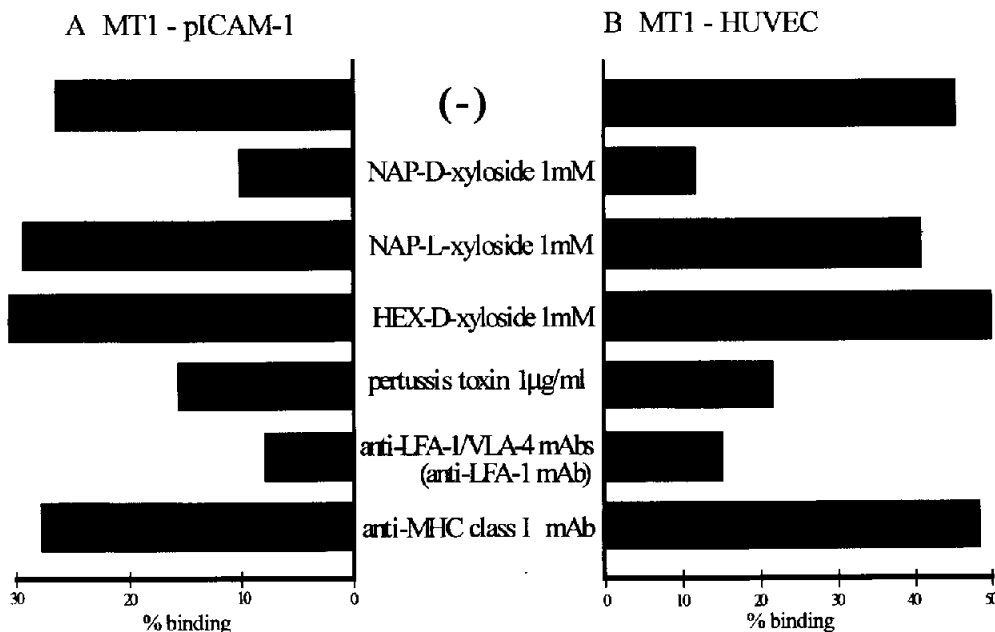


Figure 8. The adhesion of MT1 to IL-1-activated HUVEC and purified ICAM-1. The adhesion assay of MT1 which were pretreated with or without 1 mM NAP-D-xyloside, NAP-L-xyloside, HEX-D-xyloside or 1 µg/ml pertussis toxin to (A) purified ICAM-1 or (B) IL-1-activated HUVEC was carried out in the presence or absence of indicated adhesion-blocking mAbs (10 µg/ml). Data are expressed as mean percentage of binding of indicated cells from a representative experiment.

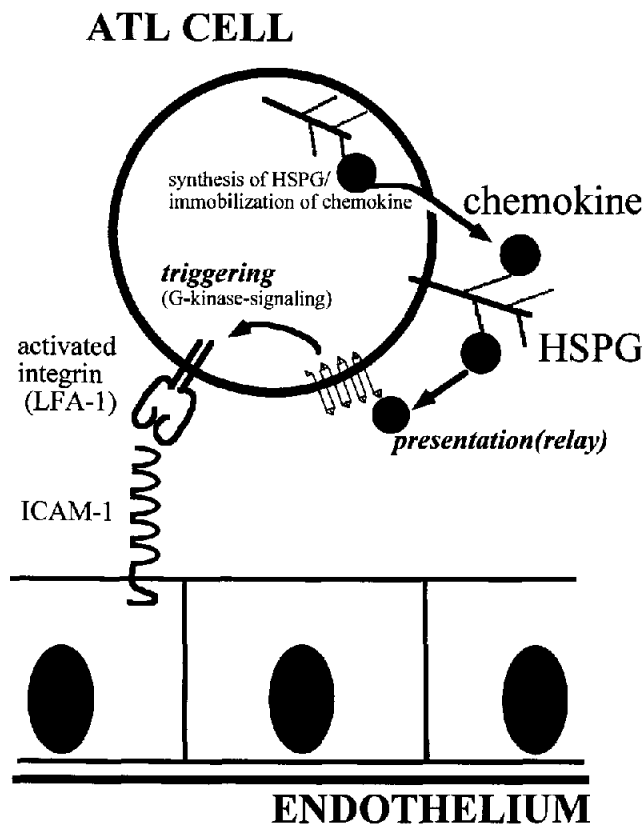


Figure 9. Diagram showing the significance of heparan sulfate in ATL cell infiltration. We propose that heparan sulfate on ATL cells immobilizes heparin-binding chemokine MIP-1 β derived from ATL cells, by which ATL cell integrins are efficiently triggered to adhere to ligands on endothelium

endothelial cells and purified ICAM-1. Thus, we propose that heparan sulfate proteoglycan on the surface of ATL cells can be involved in integrin triggering and the cellular adhesion to endothelial ligands through immobilizing chemokines (Fig. 9).

Although the relevance of cell surface heparan sulfate proteoglycan to heparin-binding cytokines is emerging (13–17), it was difficult to investigate the involvement of heparan sulfate in cellular functions. It has been considered that suitable xylosides as well as heparitinases should be useful to perform these studies. We newly developed NAP-D-xyloside, which appears to be appropriate for functional assessments of heparan sulfate proteoglycan, since this compound, but not its L-isomer, is capable of interrupting endogenous heparan sulfate- and chondroitin sulfate-proteoglycan biosynthesis in cytoplasmic organelle such as Golgi bodies and also can inhibit the immobilization of heparin-binding cytokines there in advance to posting and transferring them to the cell surface (16, 40). Actually, NAP-D-xyloside inhibited not only the chemokine expression on the ATL cell surface but also the integrin-mediated adhesion through triggering by the posting chemokines. Furthermore, the control, NAP-L-xyloside, and HEX-D-xyloside, which selectively interrupts chondroitin sulfate-pro-

teoglycan synthesis, did not affect the chemokine expression and integrin-mediated adhesion of ATL cells. Thus, the newly established xyloside would allow us to execute precise functional investigations in the contexts with heparan sulfate on cellular functions.

It is becoming clearer that the heparan sulfate proteoglycan expressed on cellular surface is connected to cytokine network by their own bioactive functions as indicated by the following: (a) cell surface heparan sulfate interacts with basic FGF to facilitate their interaction with high affinity receptors on fibroblasts in an autocrine mechanism (41); (b) heparan sulfate on stromal cells in the bone marrow presents IL-3 and GM-CSF to myeloid progenitors in a paracrine mechanism (17); (c) heparan sulfate on vessels binds and presents not only chemokines such as MIP-1 β and IL-8 but also hepatocyte growth factor to passing (tethering) leukocytes without being washed away by the blood flow in a juxtacrine system as proposed by us and others (6, 8, 9, 38, 43). The concept that heparan sulfate proteoglycan can bind/hold and present/relay cytokines to the specific receptors and can induce cellular function have come into the involvement of heparan sulfate on ATL cells in chemokine-mediated integrin triggering. ATL cells produced a high quantity of chemokine MIP-1 β and the chemokine induced the integrin-mediated adhesion. The chemokine receptor is known to be a "serpentine" receptor with seven transmembrane domains and is a GTP-binding protein which is involved in integrin triggering (43–45). The results that pertussis toxin inhibited the integrin-mediated adhesion of ATL cells, but not PMA-activated T cell adhesion, and decreased the expression of the activated form of LFA-1 on ATL cells recognized by the NKI-L16 mAb (manuscript in preparation) suggest that MIP-1 β transduced signaling through certain G-protein by the binding to its serpentine receptor in an autocrine mechanism. The autocrine mechanism of the chemokine mediated by heparan sulfate proteoglycan would be emphasized in circulating leukocytes or leukemia cells. Heparan sulfate proteoglycan is known to be synthesized and to bind any heparin-binding cytokines in the cytoplasmic organelles such as Golgi bodies and to be transferred to the cell surface holding the cytokines (16, 40). Once "free" chemokines are secreted into the circulation, they would be washed away by the blood flow. However, by the binding to the heparan sulfate, chemokine could be practically accumulated on the cellular surface and be presented to the particular chemokine receptor in an autocrine mechanism efficaciously. Alternatively, it can be interpreted that the competitive interruption of heparan sulfate-proteoglycan synthesis by NAP-D-xyloside might reduce the binding of leukemic heparan sulfate to endothelial heparin-binding molecules such as CD31. However, the adhesion of ATL cells to purified ICAM-1 which does not have heparin-binding sites was also inhibited by the reduction of heparan sulfate synthesis equally to the adhesion of ATL cells to HUVEC. Thus, we propose here that heparan sulfate proteoglycan can be involved in chemokine-mediated autocrine mechanisms in the circulating leukemia cells.

The spontaneous production of MIP-1 β might be a characteristic feature of ATL cells, since the HTLV-I viral product tax induces MIP-1 β (46). However, as described, ATL cells produce multiple cytokines including heparin-binding chemokines in addition to MIP-1 β , and anti-MIP-1 β Ab only slightly inhibited the adhesion of ATL cells (data not shown), suggesting that MIP-1 β is one representative chemokine and the involvement of heparan sulfate proteoglycan in ATL cellular adhesion would be generalized to the adhesion mediated by multiple chemokines.

An adhesion cascade consisting of tethering, triggering, and adhesion, has been proposed to explain the mechanism of normal leukocyte adhesion to endothelium (1–4, 7, 25). The present communication also suggests that this concept can be expanded to leukemic cell migration. Circulating ATL cells first tether to endothelium through the loose tethering of selectin to its ligand such as sialyl Lewis^x, since ATL cells express a high density of sialyl Lewis^x (47), which can effectively lead to the second step of triggering. The trigger appears to be essential for the adhesion of ATL cells similarly to leukocytes, in which signal transduction converts the functionally inactive integrin to an active adhesive configuration through the G-protein by the heparan sulfate-immobilized chemokine. The efficient triggering brings high affinity adhesion of ATL cells to endothelium and subsequent infiltration into underlying tissues (Fig. 9). The densities of heparan sulfate on ATL cells varied among patients who had different clinical features and activation

status. Several reports suggest that expression of heparan sulfate depends on the type of tumor cells and that the accumulation of cell surface heparan sulfate is correlated with metastatic properties of some tumor cells (11, 48). It has also been reported that the heparin-binding cytokines such as GM-CSF and IL-8 determine the metastatic potential of various tumor cells (49, 50). Structural diversity of cell surface proteoglycan in regard to protein core and heparan sulfate chains may provide additional specificity for cytokine immobilization (39), and differential production of heparin-binding cytokines from tumor cells might prescribe ability of integrin triggering, adhesion to endothelium, and metastasis.

Taken together, we propose that cell surface heparan sulfate on circulating leukemic cells can induce integrin-mediated adhesion through chemokine-mediated autocrine mechanisms by posting and relaying chemokine to the receptor on the cells. Heparan sulfate appears to have a junctional role between cytokines and adhesion molecules, the two greatest mediators of cellular communication. This is the first report to explore the significance of interaction among heparan sulfate proteoglycan and heparin-binding cytokine for circulating tumor adhesion. Our findings warrant further studies, to see whether different proteoglycans and cytokines might bring enormous flexibility to the process of leukemic cell infiltration and tumor metastasis, and would introduce new pharmacological approaches to control them.

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