Brief Definitive Report

The Heat-stable Antigen Can Alter Very Late Antigen 4–mediated Adhesion

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Summary

The integrin very late antigen, (VLA-4) $\alpha_4\beta_1$ and its counter receptor vascular cell adhesion molecule 1 (VCAM-1) are involved in B cell maturation and pre-B cell attachment to bone marrow stroma cells. We have analyzed whether heat-stable antigen (HSA), a marker for immature leukocytes, is involved in such cell adhesion phenomena. HSA is a glycolipid-anchored, highly glycosylated surface protein differentially expressed on cells during the maturation of both the hematopoietic and nervous systems. We found that pre-B cells lacking HSA (due to targeted disruption of both alleles) can still bind via VLA-4 to tumor necrosis factor α–stimulated endothelioma cells. This binding, however, cannot be blocked by an anti-VCAM-1 antibody. Restoration of HSA expression restores the inhibitable VCAM-1 binding. We also found that pre-B cells lacking HSA did not bind to the FN40 fragment of fibronectin but reexpression of HSA restored VLA-4–mediated binding to fibronectin. Thus, expression of HSA on pre-B cells modifies the binding specificity of VLA-4 for two known ligands.

Regulated adhesion of lymphocytes is crucial both for maturation in primary lymphoid organs as well as for effector functions in the periphery. Several cell surface receptors have been reported to be involved in this adhesion, depending on the cell type and the maturation stage. During the differentiation of B cells, attachment of pre-B cells to bone marrow stroma cells can be blocked by antibodies against the integrin very late antigen, (VLA-4) $\alpha_4\beta_1$ or its counter receptor vascular cell adhesion molecule 1 (VCAM-1) suggesting that they play a major role in the binding (1–4). The heat-stable antigen (HSA; mouse CD24) is a heavily glycosylated surface protein expressed on immature neural (5) and hematopoietic cells and is widely used in the mouse as a maturation marker for lymphocytes (6, 7). Results have been published suggesting the involvement of HSA in lymphocyte adhesion and in delivery of costimulatory signals for T cells (8, 9). Since HSA is most strongly expressed on immature B cells in the bone marrow, we asked whether it could also influence pre-B cell adhesiveness.

Materials and Methods

Reagents and Antibodies. The mAbs used in this study were M1/69 directed against HSA (mCD24; reference 10), MK-2 against mouse VCAM-1 (CRL 1909; American Type Culture Collection [ATCC], Rockville, MD; reference 1) and PS-2 against the integrin chain α_4 (2), M17/4.4.11.9 against CD11a (TIB217; ATCC; reference 11); 9EG7 directed against the integrin chain β_1 (12), 13CA7 directed against an as yet undefined, TNF-α–inducible antigen on the surface of brain-derived endotheliama (bEND.3) cells (Hahne, M., and D. Vestweber, unpublished observations). The mouse E- and P-selectin antibodies have been recently described (13). Human recombinant TNF-α (8.72×10^6 U/mg) was a generous gift of the Knoll AG (Ludwigshafen, Germany).

Cell Culture and Transfection. The brain endothelioma bEND.3 (a gift from W. Risau, Max-Planck-Institute for Physiological and Clinical Research, Bad Nauheim, Germany, and L. Williams, Ludwig Institute for Cancer Research, Melbourne, Australia) was cultured as described (13). The Abelson murine leukemia virus (A-MuLV)–transformed pre-B lines were cultured in vitro in IMDM (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FCS, 50 μM 2-ME at 37°, and 7.5% CO_2. The HSA expression plasmid pHSEX62.8 was described previously (14) and DNA preparation was done according to standard procedures (15). Pre-B cell lines were transfected by electroporation of 2×10^7 cells in 350 μl medium without FCS using 50 μg Xmn I-linearized pHSEX62.8 (950 μF, 225 V) in a 0.4-cm electroporation cuvette (Bio-Rad Laboratories, Richmond, CA). Stable expression was achieved by coelectroporation with pHyg72 (contains the hygromycin-resistance gene under the control of the HSV-tk promoter) and selection with 1 mg/ml hygromycin B (Calbiochem Corp., La Jolla, CA). Monoclonal hygromycin-resistant HSA^+ cell lines were obtained by limiting dilution.

Abelson Virus Transformation. Bone marrow cells from isolated femurs were flushed with medium, counted, centrifuged, and resuspended at $2–3 \times 10^6$ cells in 0.25 ml medium. They were mixed with 1 ml fresh supernatant of a transformed NIH3T3 cell line (54C12) that produces a replication defective A-MuLV (16, 17).

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Polybrene was added to a final concentration of 10 mg/ml and the resulting mixture was incubated at 37°C and 7.5% CO_2 for 3 h. After dilution with medium (1:10) and addition of 3% molten low melt SeaPlaque agarose (FMC BioProducts, Rockland, ME) to a final concentration of 0.6% the cells were placed in 1-ml aliquots on a 1-ml 0.6% soft agarose bottom layer in 3.5-cm petri dishes (Falcon Labware, Oxnard, CA). The dishes were incubated for 12 d and transformed clones were picked and propagated in liquid suspension culture. The clones were classified by flow cytometry as pre-B because they were surface positive for the B-lineage marker B220 (CD45) and negative for surface IgM.

Flow Cytometry. Analysis was performed on a FACScan® cytometer (Becton Dickinson & Co., Mountain View, CA) using LYSISII software. Cells were stained with the following mAB conjugates: M1/69-FITC, PS-2-biotin, 9EG7-FITC. Biotinylated mAbs were revealed with streptavidin coupled to PE (Southern Biotechnology Associates, Birmingham, AL). Dead cells were excluded by electronic gating on propidium iodide–negative cells.

Cell Adhesion Assays. Endothelioma cells were plated at a density of 2×10^4 cells/well on 96-well microtiter plates two d before the test. TNF-α activation of the endothelioma cells was for 16 h with human recombinant TNF-α followed by two washes in HBSS (Biochrom, Berlin, Germany). Pre-B cells were harvested, washed once in 37° HBSS, and added to the microtiter plate (2×10^5 cells in 200 μl/well) that was rotating at 75 rpm (with 1 cm orbit diameter). After 20 min at 37°C, unbound pre-B cells were removed by gentle flicking. Bound cells were fixed with 1% glutaraldehyde in PBS for 2–3 h at 7°C, followed by three more washes with HBSS. None of the mAbs against HSA tested (J11d, M1/69, 79, B2A2) had any inhibitory effect in our binding system (data not shown).

Fibronectin Adhesion Assay. 96-well plates were coated with FN-40, 1 μg/well (Telius Pharmaceuticals, San Diego, CA), or ovalbumin, 1 μg/well in PBS overnight at 37°C. Plates were subsequently blocked by incubation with 10% heat-inactivated FCS in PBS (attachment buffer) at 37°C. Pre-B cells (2×10^5 cells) were added in 200 μl attachment buffer to each well and allowed to bind for 30 min at 37°C in 5% CO_2. Nonadherent cells were then washed off with attachment buffer and bound cells counted as described above.

Results and Discussion

Pre-B cell clones deficient for HSA were generated by transforming bone marrow–derived cells from a chimeric mouse generated by the injection of ES cells carrying deletions in both alleles of the HSA gene (Wenger, R. H., M. Kopf, L. Nitschke, M. C. Lamers, G. Köhler, and P. J. Nielsen, manuscript submitted for publication). The HSA deficiency of one of these clones (termed N232.18) is shown in Fig. 1 using the anti-HSA mAb M1/69 in FACS® analysis. This clone was transfected with an HSA expression plasmid (14) and numerous clones were obtained that stably expressed HSA on the surface (three cell lines, 18H8, 18H15, and 18H18, are shown in Fig. 1).

We tested the ability of HSA^- N232.18 pre-B cells, and the stable HSA^+ transfectants 18H8, 18H15, and 18H18 to bind to bEND.3. As shown in Fig. 2, both the HSA-deficient N232.18 cells and the HSA^+ transfectants strongly bound to bEND.3 cells activated for 16 h with TNF-α, but not to unstimulated cells.

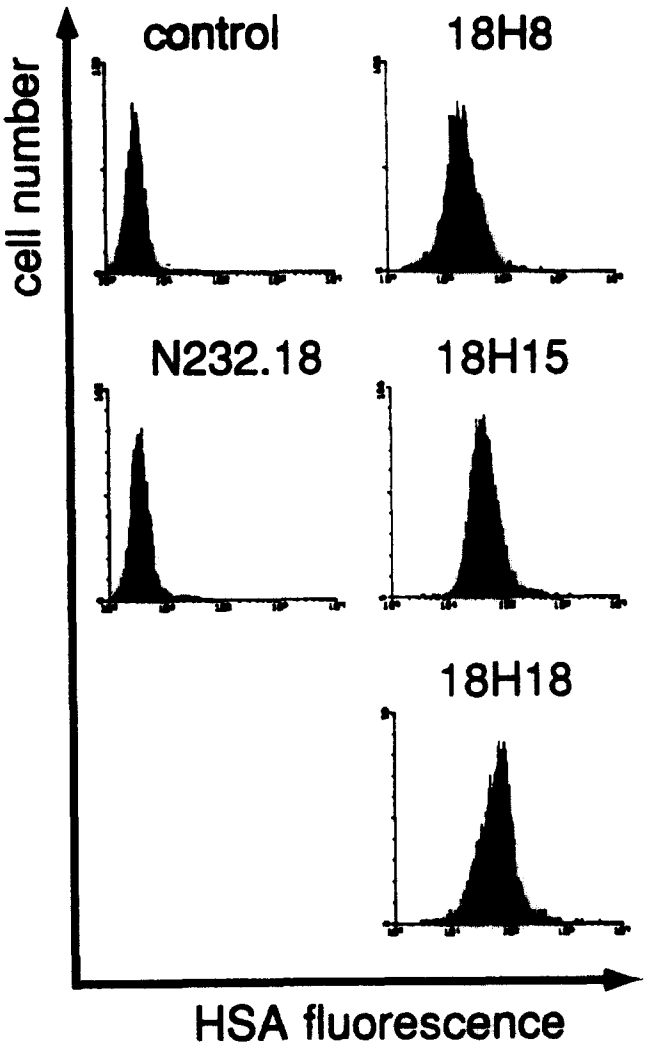

Figure 1. Flow cytometric (FACS®) analysis of the HSA^- pre-B cell line N232.18 and the HSA^+ transfectants 18H8, 18H15, and 18H18. Cells were stained with FITC-conjugated anti-HSA mAb M1/69 (19) and analyzed on a FACscan® cytometer. Control staining were with goat anti-rat Ig (H+L) coupled to FITC.

It has been recently reported that bEND.3 cells express VCAM-1 at maximal levels after 16 h of TNF-α stimulation whereas intercellular adhesion molecule-1 (ICAM-1) is hardly induced on these cells and E- and P-selectin are transiently induced and return again to basal levels, 16 h after stimulation (13). Therefore, we tested whether VCAM-1 mediated the binding of the four pre-B cell clones. As shown in Fig. 3 *A*, mAb MK-2 against mouse VCAM-1 indeed blocked the binding of the three HSA transfectants. However, binding of the HSA deficient pre-B cell clone N232.18 could not be blocked with this antibody (Fig. 3 *A*). Binding of all four pre-B cell clones was also not blocked by adhesion blocking antibodies against mouse E- and P-selectin (not shown) or by the control mAb 13CA7.

Despite the fact that the anti-VCAM-1 mAb could not block adhesion of the HSA-deficient pre-B cells to activated bEND.3 cells, this adhesion was completely blocked by the anti-VLA-4 mAb PS/2 (Fig. 3 *B*). The same inhibitory effect

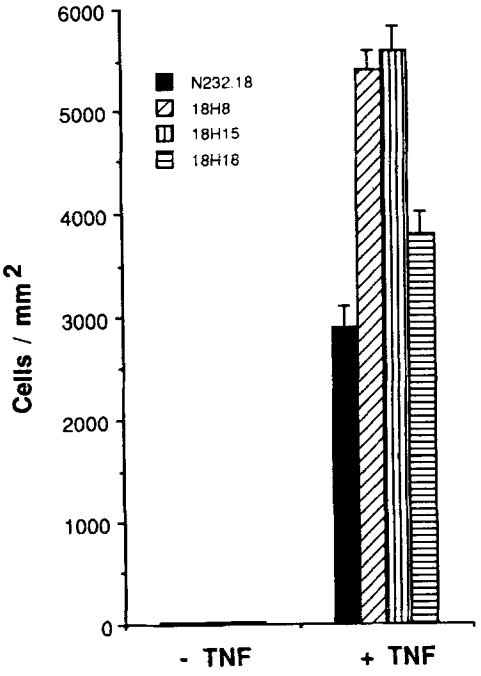

Figure 2. Adhesion of the HSA⁻ pre-B cell line N232.18 and the HSA⁺ transfectants 18H8, 18H15, and 18H18 to brain endothelioma cells. Pre-B cells were incubated with untreated and 16 h TNF-activated bEND.3 endothelioma cells in 96-well microtiter plates. Each measurement was done in three wells and one of three similar series of experiments is depicted in the graph.

was seen on the binding of all three HSA transfectants. (Fig. 3 *B*). Thus, independent of the expression of HSA, all tested pre-B cell clones bound via VLA-4 to activated endothelioma cells. However, these cells apparently bind to VCAM-1 only when they express HSA. We conclude that the lack of HSA expression changes the binding characteristics of VLA-4 such that MK-2–inhibitable binding to VCAM-1 is abolished. It is unclear, at present, which endothelial ligand is bound by VLA-4 in the absence of HSA. It could be either another domain of VCAM-1 not blocked by MK-2 or an independent counter receptor, the existence of which has been recently suggested (18, 19).

Fibronectin is a second ligand for VLA-4 and binds to a different site on VLA-4 than VCAM-1 (20). We tested whether the HSA- deficient pre-B cell clone N233.18 differs from the HSA transfectants in its ability to bind to the 40-kD trypsin fragment of plasma fibronectin (FN-40) that contains the CS-1 binding site for VLA-4 (21). As shown in Fig. 4, only the HSA⁺ transfectants but not the HSA deficient pre-B cells bound to FN-40. The binding of the HSA⁺ transfectants could be inhibited by the anti-α_4 mAb PS/2 but not by a control antibody against lymphocyte function–associated antigen 1 (LFA-1).

The binding of VLA-4 to both of its two known ligands, VCAM-1 and fibronectin, was altered by the loss of HSA. The mechanism by which HSA expression influences the binding specificity of VLA-4 is at present unknown. Interest-

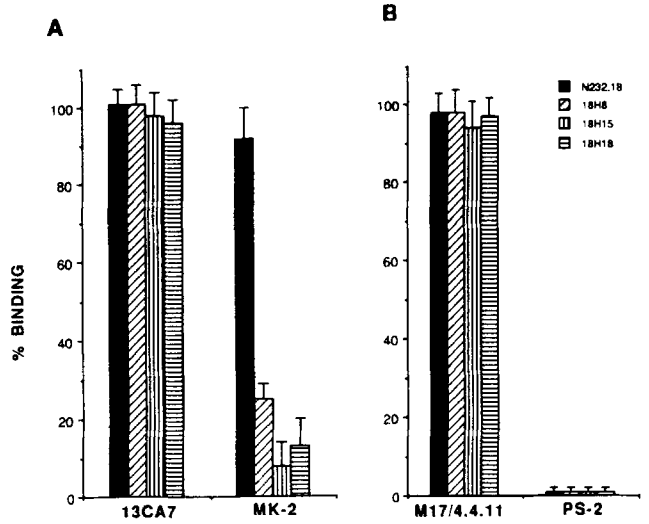

Figure 3. The effect of antibodies on the adhesion of the HSA⁻ N232.18 pre-B cells and the HSA⁺ transfectants 18H8, 18H15, and 18H18 to TNF-α–activated bEND.3 cells. Cell binding in the absence of antibodies was defined as 100%. Inhibition of cell adhesion with antibodies was tested with fivefold concentrated hybridoma supernatants of mAb MK-2 (anti-VCAM-1 [1]) (*A*), and plain supernatant of the mAb PS-2 (anti-α_4 [2]) (*B*). As a control, the endothelial cells were incubated with fivefold concentrated hybridoma supernatants of mAb 13CA7 (recognizes an as yet unidentified surface marker on TNF-α–activated bEnd.3; Hahne, M., and D. Vestweber, unpublished observations) (*A*) or plain supernatant of the mAb M17/4.4.11 (anti-LFA-1 [18]) (*B*). Antibody incubations were for 15 min on ice before the adhesion assays. None of the anti-HSA mAbs tested (M1/69, J11d, B2A2, 79) inhibited binding of pre-B cells to bEND.3 cells.

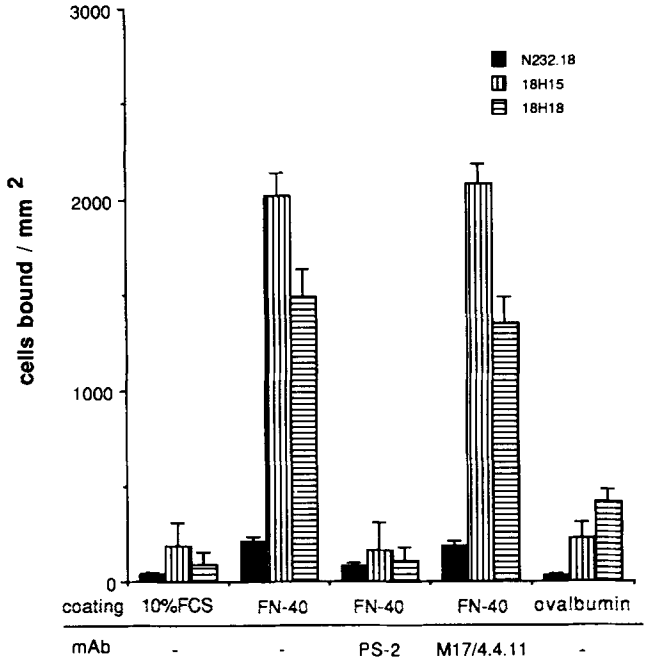

Figure 4. Adherence of the HSA⁻ N232.18 pre-B cells and the HSA⁺ transfectants 18H15 and 18H18 to the 40-kD chymotryptic fragment of fibronectin (FN-40). Cell binding in the absence of antibodies was defined as 100%. Inhibition of adhesion was tested with plain supernatants of PS-2 (anti-α_4 [2]) or M17/4.4.11 (anti-LFA-1 [11]) as a control. Bound cells were counted as described above.

ingly, a similar observation has been made for CD43, another heavily glycosylated surface protein, where it seems that expression of CD43 can interfere with adhesion between the integrin LFA-1 and ICAM-1 (24). It is well documented that the binding activity of the integrins is regulated through conformational changes which are influenced either by extracellular ligands or by modifications transmitted from within the cell (see 23–25 for reviews). Conceivably, HSA could induce such conformational changes either directly by association with VLA-4 on the cell surface, or indirectly by transmitting a signal into the cell. Adhesion mediated by the integrin VLA-4 can be activated by incubation of cells with Mn^{2+} and this activation correlates with increased binding of the mAb 9EG7 (12). HSA does not seem to change the ability to activate VLA-4 since, for HSA^- as well as HSA^+ cells, binding of mAb 9EG7 was stimulated to a comparable extent by Mn^{2+} (not shown). The surface expression of VLA-4 was also not changed by loss of HSA expression since VLA-4 levels on all four pre-B cell lines described were similar when examined by FACS® using the anti-α_4 mAb PS-2 and the anti-β_1 mAb 9EG7 (not shown).

The influence of HSA on VLA-4–mediated adhesion should be examined in other pre-B lines as well as in cells representing different stages of the B and T cell maturation since this would have important implications both for the development of hematopoietic cells as well as their function in the periphery. It has been shown that both murine and human bone marrow stromal cells and human bone marrow–derived fibroblasts express VCAM-1 and that the adhesion of B cell precursors to accessory cells in the bone marrow is mediated by the VCAM-1/VLA-4 ligand–receptor pair (1–4). Blocking these interactions prevents B cell development. The regulation of HSA expression during cell maturation could be a mechanism for associating cells of various maturation stages with specific developmental compartments. Similarly, the down-regulation of HSA during the maturation of peripheral B cells and monocytes, could change their ability to home and extravasate. These possibilities are being investigated in homozygote HSA-knockout mice.

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