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Advances in Brief

Histo-Blood Group A/B *versus* H Status of Human Carcinoma Cells as Correlated with Haptotactic Cell Motility: Approach with *A* and *B* Gene Transfection[1]

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Abstract

In a search for the molecular basis of ABH status of tumors as correlated with malignancy, we studied various malignancy-related phenotypes of high H/Le^y-expressing tumor cell lines in comparison with phenotypes of the same lines transfected with histo-blood group *A* or *B* genes. *A* and *B* gene transfectants, prepared independently from different H-active parental cells, showed A or B activity and abolition of H activity. All *A* and *B* gene transfectants, regardless of source, were characterized by significantly reduced Matrigel-dependent haptotactic motility. The level of haptotaxis of all transfectants was similar to that of parental cells in the presence of antibodies against human integrin subunits α3, α6, or β1. These subunits showed high expression of A or B epitope in the *A* and *B* gene transfectants. Enhancement *versus* reduction of malignancy, associated with deletion *versus* induction of A/B epitopes, may be due in part to enhanced haptotaxis sustained by α3, α6, and β1 integrin receptors, the activities of which are regulated by H or A/B glycosylation. These phenotypic changes provide a rationale for the deletion of A and B epitopes as one criterion defining human tumor malignancy.

Introduction

Deletion or reduction of histo-blood group A and B epitopes (1) has been studied in relation to degree of malignancy and metastatic potential in human gastrointestinal, lung, and cervical cancers (2), oral carcinoma (3), and bladder carcinoma (4). Detailed studies of patients with blood group A or AB showed that those with A-negative primary lung carcinoma showed significantly shorter survival than those with A-positive primary lung carcinoma (5). A similar trend was observed for B deletion (6). Survival of patients with primary lung carcinoma showing reactivity with anti-H/Le^y mAb[4] was significantly lower than that of patients whose tumors showed no reactivity (7). Expression *versus* deletion of A and B antigens in tumors is correlated with A and B transferase activity (8). The cDNAs encoding A and B transferases were cloned (9), and the A and B transcript levels were found to be down-regulated in bladder carcinoma cells, in association with growth stimulation of urothelial cell lines (10).

This background information prompted us to investigate the effect of *A* and *B* cDNA transfection on H-expressing human carcinoma cells. Results clearly showed that the conversion of H to A or B inhibits Matrigel-dependent haptotactic motility, in close association with α3, α6, and β1 integrin function, which is presumably affected by H *versus* A/B glycosylation.

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[4] The abbreviations used are: mAb, monoclonal antibody; lamp, lysosome-associated membrane protein; HGF, hepatocyte growth factor.

Materials and Methods

Cell Lines, Antibodies, and Reagents. Human colonic carcinoma cell line HRT18 was purchased from American Type Culture Collection (Rockville, MD). Human gastric carcinoma cell line MKN74 was donated originally by Masakazu Adachi (Japan Immunoresearch Laboratories, Takasaki, Japan). These cells highly express H, Le^y, Le^x, and SLe^x but do not express type 1 chain structures Le^a, Le^b, and SLe^a (11). Anti-A mAb 81FR2.2, anti-B mAb 3E7, anti-H mAb 92FR-A2, and antihuman integrin subunit α3 mouse mAb P1B5 were from Dako (Carpinteria, CA). Antihuman integrin subunit α6 rat mAb GoH3 was from PharMingen (San Diego, CA). Anti-α3 and anti-α6 antibodies were dialyzed to eliminate sodium azide when tested for the inhibitory effect on tumor cell haptotaxis. Antihuman α5 mAb ZH5 and antihuman β1 mAb ZH1 were established in this laboratory as described previously (12). A portion of the antihuman integrin subunit β1 mAb used in immune precipitation was donated by Dr. William Carter (Fred Hutchinson Cancer Research Center, Seattle, WA). Anti-lamp-1 mAb was donated by Dr. Minoru Fukuda (Burnham Institute, La Jolla, CA). Matrigel and human recombinant HGF (scatter factor) were from Collaborative Biomedical Products (Bedford, MA). Fibronectin and mAbs directed to Le^y (AH6), Le^x (SH1), and SLe^x (SNH4, FH6) were prepared in this laboratory (for information regarding sources of mAbs, see Refs. 11 and 13). Peroxidase-labeled lectins from *Ulex europaeus* and *Bandeiraea simplicifolia* were from Sigma Chemical Co.

***A* and *B* Transferase Genes and Transfection.** The construct of cDNAs encoding blood group A or B transferases, as described previously (9, 14), was used. The full-length cDNAs pAAAA and pBBBB were obtained through excision by digestion with *Eco*RI and cloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA), which was previously digested with *Eco*RI and treated with calf intestine alkaline phosphatase.

The pcDNA3 vector containing pAAAA and pBBBB, as well as the vector alone, were transfected into both cell lines using lipofectin (Life Technologies, Inc.) according to the manufacturer's instructions. Transfected cells were selected in medium containing G418 (1.0 mg/ml; Life Technologies). Individual colonies were isolated by repeated limiting dilution and expanded. Expression of blood group antigens was tested using the antibodies described above.

Haptotactic and Chemotactic Activity Assays. Haptotactic assay was performed using a 6.5-mm Transwell chamber (8-μm pore size; Costar, Cambridge, MA) as described previously (15, 16). Briefly, the lower surface of the filter was coated with 25 μl of Tris-HCl buffer containing various quantities of Matrigel (0.5, 1.5, or 5.0 μg per membrane filter), and the filter was dried at room temperature. Cells were harvested and placed in the upper compartment of the Transwell chamber. For haptotactic migration of HRT18 cells and transfectants, cells were incubated for 24, 48, and 72 h. For migration of MKN74 cells and transfectants, cells were incubated for 12, 24, and 36 h. Cells that had not migrated from the upper surface of the filter were removed by cotton swab, and cells that had migrated to the lower surface were fixed in methanol and stained in 1% toluidine blue. The stain was solubilized in 10% acetic acid, and the color was measured by an ELISA reader at 630 nm.

Chemotactic assay was performed using the same Transwell chamber with 50 μg of Matrigel per filter. HGF (scatter factor; 10 ng/ml) was added to the lower chamber, and cells, harvested and suspended in DMEM with 0.25% BSA, were placed in the upper chamber. After 24 h, cells migrated to the lower surface of the filter were measured as described above (15).

Determination of Antigen Expression. The expressions of ABH, Le^x, Le^y, and SLe^x were determined using flow cytometry with specific primary and secondary antibodies as described previously (11, 13).

Western Blotting Analysis. Cells were harvested and solubilized in lysis buffer (140 mM NaCl, 10 mM Tris-HCl, and 5 mM EDTA, pH 7.8) containing 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 0.05–0.1 trypsin inhibition units per ml of aprotinin. Protein content of the lysates was determined with a BCA kit (Pierce, Rockford, IL). Lysates were subjected to SDS-PAGE, electrophoretically transferred to Immobilon-P (Millipore, Bedford, MA), blocked overnight in PBS containing 5% defatted milk at 4°C, and washed in PBS containing 0.05% Tween 20 (PBS/Tween). Membranes were incubated with antibodies to proteins or carbohydrates, washed in PBS/Tween, and then incubated with horseradish peroxidase-labeled secondary antibodies, and blotted bands were detected by a chemiluminescence system (Pierce, Rockford, IL).

For immunoadsorption, cell lysates (20 μg of protein) were precleared with protein A-Sepharose beads (Pharmacia) for 2 h at 4°C. After centrifugation, the supernatant was incubated with anti-integrin-α3, -α6, or -β1 subunit or anti-lamp-1 antibody for 2 h at 4°C and then incubated with protein A-Sepharose beads to collect the immunocomplex. In the experiment with GoH3, beads were preincubated with rabbit antirat immunoglobulin (Dako) and washed. The fractions immunoadsorbed on beads were washed five times with lysis buffer, and bound protein was eluted by boiling in electrophoresis sample buffer containing 5% 2-mercaptoethanol and subjected to 8% SDS-PAGE. Separated proteins were subjected to Western and lectin blotting as described above.

Results

Isolation of A- and B-expressing Clones following *A* and *B* Gene Transfection and Alteration of Carbohydrate Epitopes in These Clones. Transfection of cDNA encoding A and B transferases into colonic carcinoma cell line HRT18 led to the isolation of three clones expressing A antigen (A2, A3, and A9) and three clones expressing B antigen (B5, B7, and B9). Similarly, transfection of *A* and *B* cDNA

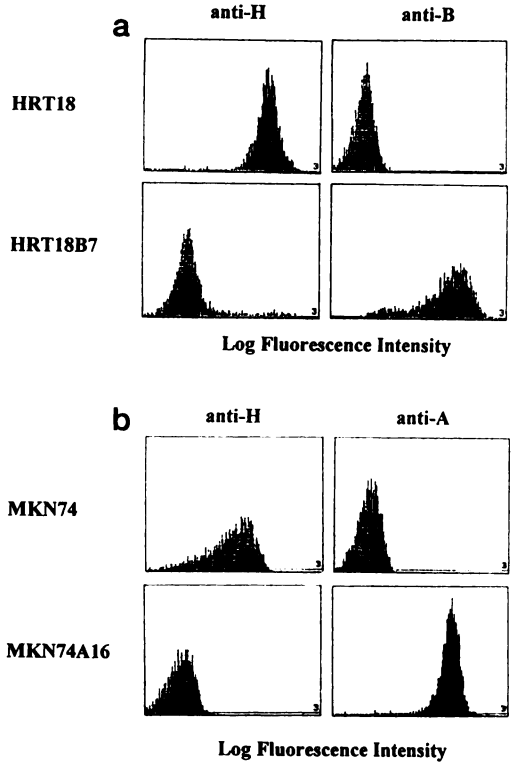

Fig. 1. Cytofluorograms of transfectants from human cancer cell lines. *a*, colonic carcinoma cell line HRT18 and its transfectants. *b*, gastric carcinoma cell line MKN74 and its transfectants. Procedures for staining and cytofluorometric analysis were as described previously (11, 13).

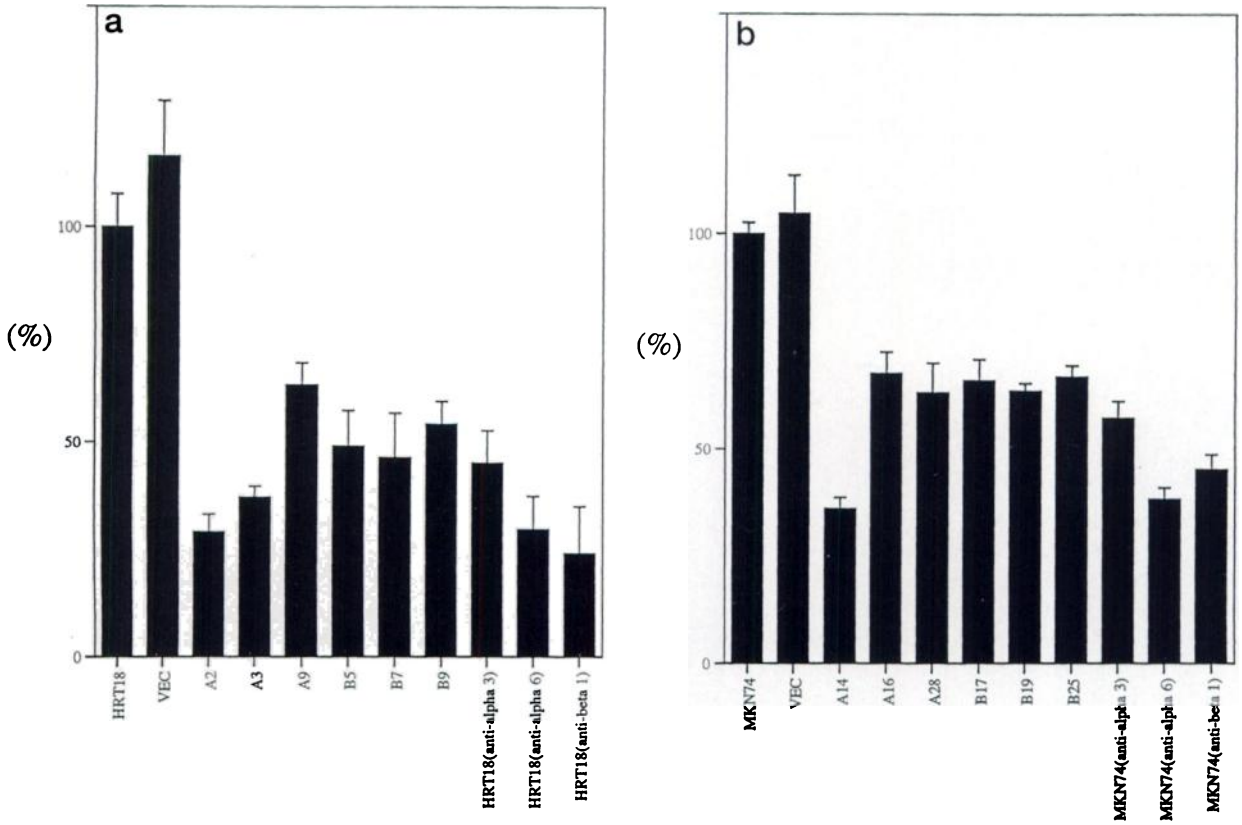

Fig. 2. Matrigel-dependent haptotactic motility of *A* and *B* gene transfectants and control vectors as compared to parental cells. *a*, HRT18 (*column 1*; columns numbered from left to right) and its transfectants. *Column 2*, transfectant with pcDNA3 vector only; *columns 3–5*, *A* gene transfectants; *columns 6–8*, *B* gene transfectants. Parental cells incubated with antibodies (concentration, 10 μg/ml) directed toward integrin subunits α3 (*column 9*), α6 (*column 10*), and β1 (*column 11*). *b*, MKN74 (*column 1*) and its transfectants. *Column 2*, transfectant with pcDNA3 vector only; *columns 3–5*, *A* gene transfectants; *columns 6–8*, *B* gene transfectants. Parental cells incubated with antibodies (concentration, 10 μg/ml) directed toward integrin subunits α3 (*column 9*), α6 (*column 10*), and β1 (*column 11*). *Columns*, means; *bars*, SD.

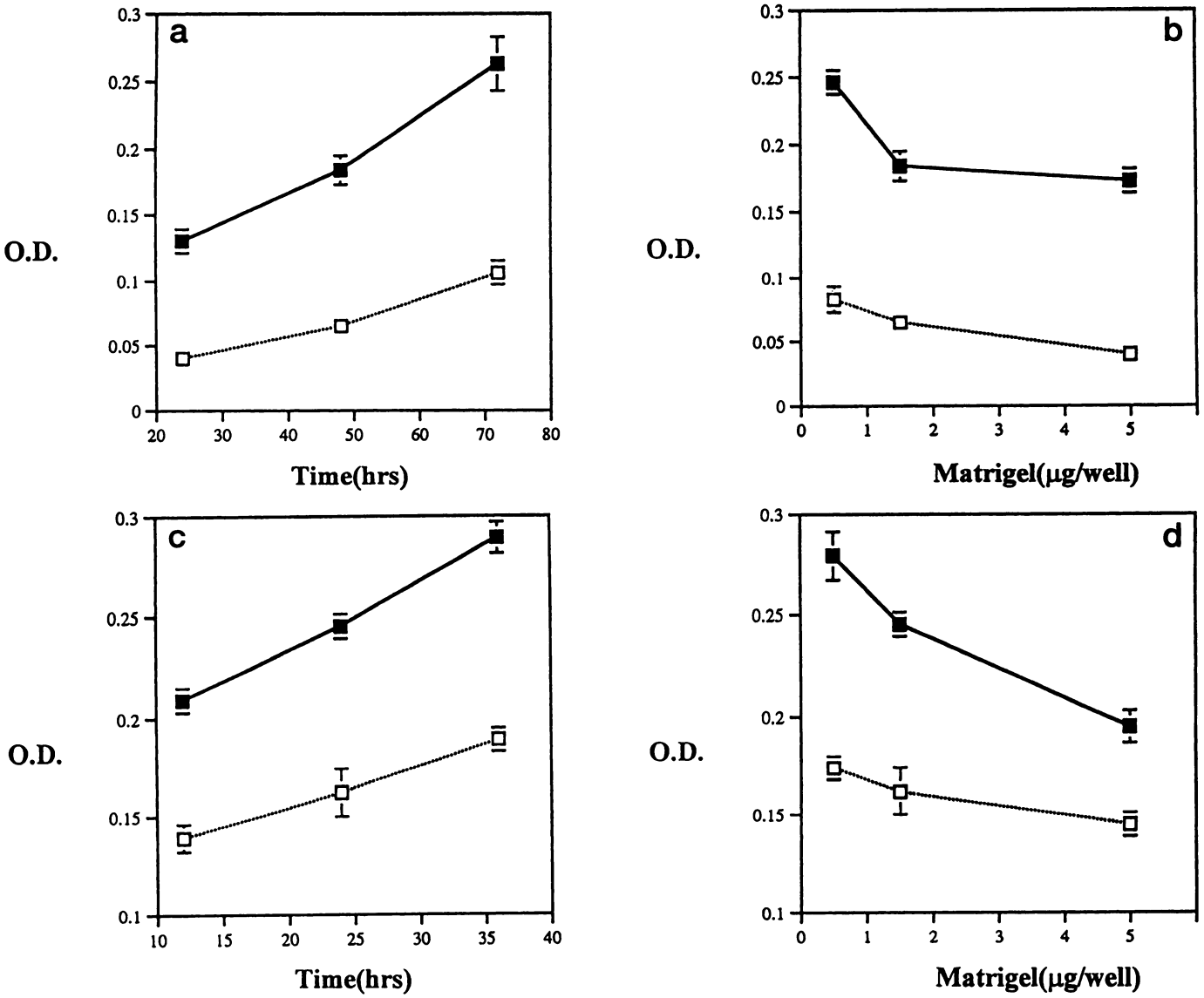

Fig. 3. Matrigel-dependent haptotactic motility of *A* and *B* gene transfectants as a function of time and of Matrigel quantity. *a* and *b*, HRT18 (■) and transfectant HRT18A2 (□). *c* and *d*, MKN74 (■) and transfectant MKN74B19 (□). *a* and *c*, time-dependent changes. *b* and *d*, Matrigel quantity-dependent changes. *Data points*, means; *bars*, SD.

into human gastric carcinoma cell line MKN74 led to the isolation of three A-expressing (A14, A16, and A28) and three B-expressing (B17, B19, and B25) clones. All these clones were characterized by the permanent expression of A or B antigens, associated with the abolition of H antigen (see for example Fig. 1), and decrease of Le^y antigen expression (data not shown). Le^x expression was not changed by *A/B* gene transfection (data not shown).

Reduction of Matrigel-dependent Haptotactic Motility in *A* and *B* Gene Transfectants. All of the *A* or *B* gene transfectants prepared from two different H-expressing colonic carcinoma cell lines showed consistent, strong reduction of Matrigel-dependent haptotactic motility relative to parental cells. Haptotactic motility of *A* or *B* transfectants was reduced to 29–63% of parental level in HRT18 transfectants and to 36–67% in MKN74 transfectants (Fig. 2). All differences in the haptotactic motility of *A* or *B* gene transfectants *versus* parental cells were statistically significant ($P < 0.01$, one-tailed *t* test), whereas the haptotactic motility of the vector alone ("mock transfectant") was not statistically distinguishable from that of parental cells. The difference in haptotaxis of parental cells *versus A* or *B* gene transfectants was observed regardless of incubation time or quantity of Matrigel applied. Examples are shown in Fig. 3, *a* and *b*. The reduction in activity in the transfectants was in the same range as that observed in parental cells that were incubated with antihuman integrin subunit α3, α6, or β1 antibody (Fig. 2, *a* and *b*, *columns 9–11*, columns numbered from left to right). Reduction of haptotaxis by anti-integrin subunit α6 was the most remarkable and showed clear dose dependence (Fig. 4). This was consistent with the observation that the motility-inhibitory effect of anti-α3 (P1B5) was lower than that of anti-α6.

In contrast, levels of Matrigel-dependent adhesion were essentially the same for parental cells *versus A* or *B* gene transfectants (data not shown). Adhesion to fibronectin-coated plates was 20–40% higher for transfectants than for parental cells (data not shown). Chemotactic activity induced by HGF (scatter factor) or by "conditioned medium" was essentially the same for parental cells as for *A* or *B* gene transfectants (data not shown).

Glycosylation Patterns of Integrins and Other Motility Receptors. All parental and transfectant cell lines were characterized by three or four glycoprotein bands, with a M_r 140,000 and a M_r 200,000 band in common, and by the presence of α3, α6, and β1 integrin

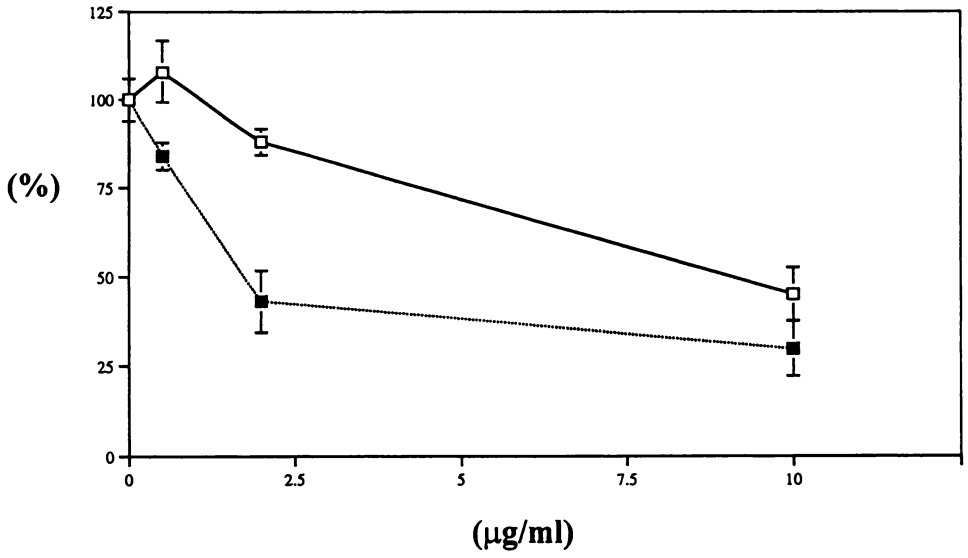

Fig. 4. Dose-dependent effect of anti-integrin subunit antibodies on haptotactic motility of HRT18 cells. *Abscissa*, antibody (μg/ml); *ordinate*, percent inhibition. ■, anti-α6; □, anti-α3. *Data points*, means; *bars*, SD.

receptors, which control Matrigel-dependent haptotactic motility. α5 was not detectable. Immunoprecipitation of cell extracts followed by Western blotting with anti-H and anti-A antibodies revealed that α3, α6, and β1 from A transfectants were heavily A-glycosylated, whereas integrins from parental cells were H- but not A-glycosylated (Fig. 5). Expression of Le^x in integrin receptors of parental cells *versus* transfectants was essentially the same (data not shown). A- and B-glycosylation were observed not only for integrin receptors but also for lamp-1 (data not shown; see "Discussion").

Discussion

Of the many types of aberrant glycosylation expressed in human cancers, deletion or reduction of A and B epitopes has been the most clearly correlated with invasive and metastatic properties of tumors and with survival rates of patients (17, 18). These phenotypic changes are under the control of yet-unidentified mechanisms that inhibit or stop *A* or *B* gene transcription (10), presumably through aberrant expression of transcription factors. Does A/B *versus* H expression of primary tumor cells correlate consistently with certain malignant phenotypes of the cells? Comparison of A/B glycosylation pattern with multiple malignant phenotypes (see below) of primary human tumors may reveal significant relationships between these parameters because A/B deletion reduces average patient survival.

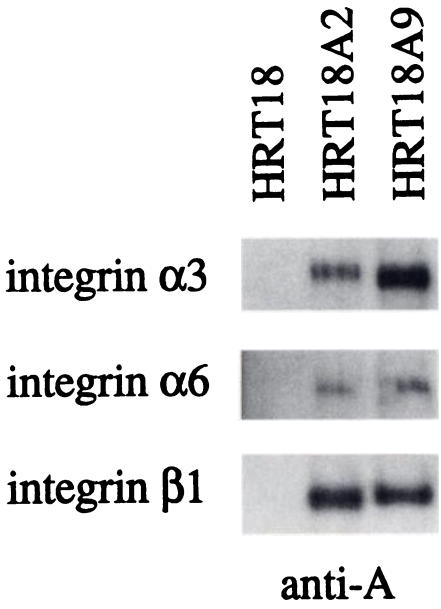

Fig. 5. Histo-blood group A glycosylation present in integrin α3, α6, and β1 subunits in human colonic carcinoma HRT18 and its two *A* gene transfectants, A2 and A9. The immune-adsorbed fractions from cell extracts were prepared with protein A-Sepharose beads after addition of primary mAb. Fractions were subjected to SDS-PAGE followed by Western blot analysis using anti-A mAb 81FR2.2 as described in "Materials and Methods." The stained bands corresponding to α3, α6, and β1 are shown.

Invasive properties of tumor cells are highly complex yet well-coordinated mechanisms. They include proteolytic activity, haptotactic and chemotactic motility, ability to cause angiogenesis and activate endothelial cells and platelets, aberrant glycosylation, ability to bind selectins and other lectins, and others (18). Some of these mechanisms may be under the control of a common transcription factor, although this factor may affect expression of each gene to a different degree. Therefore, comparison of multiple phenotypes among various tumor cell lines showing different expression of A/B *versus* H epitopes may not reveal a simple, straightforward relationship between malignant phenotypes and A/B glycosylation despite the fact that haptotactic motility of A-deleted cells was significantly higher than that of A-persistent cells from the same tumor cell lines derived from histo-blood group A patients.[4] The present approach, using transfection of *A* or *B* genes, affects only terminal glycosylation, *i.e.*, H *versus* A/B, and no other genes or factors were involved. Our results clearly indicate that addition of A or B epitope by transfection of *A* or *B* gene results in consistent reduction of Matrigel-dependent haptotactic motility in all transfectants derived from two independent H-expressing parental tumor cell lines. The reduction of haptotactic motility was observed regardless of incubation time or quantity of Matrigel, although the level of Matrigel-dependent adhesion of the transfectants was similar to that of parental cells.

Matrigel-dependent haptotactic motility is considered a standard measure of tumor cell motility and invasiveness, which are controlled primarily by integrin chains α3, α6, and β1. Reduced or enhanced haptotaxis of tumor cells may be closely associated with A or B *versus* H glycosylation of these integrin receptors. This concept is supported by the observations that haptotactic motility of parental cells was inhibited to the same degree as that of *A* and *B* gene transfectants in the presence of antibodies against integrins, particularly α6 and β1, and that A and B epitopes are highly expressed in α3, α6, and β1 of

[4] D. Ichikawa, K. Handa, and S. Hakomori, unpublished data.

transfectants, whereas parental cells express H epitope. However, all of the transfectants and parental cells showed similar chemotactic activity toward HGF, another frequently-used criterion for tumor cell invasiveness (15). Thus, chemotactic receptor function of tumor cells may not be affected by H *versus* A/B glycosylation.

N-glycosylation of integrin is essential for maintenance of integrin function (19, 20) or, more precisely, for correct assembly of α and β subunits as typically demonstrated for $\alpha5\beta1$ (12). *N*-glycosylation is crucial in mutual interaction of $\alpha6\beta1$ with laminin (20). The interaction between α and β may be reduced by addition of or enhanced by deletion of A and B epitopes, which effect haptotaxis of tumor cells. Glycosylation, *i.e.*, H *versus* A/B, of integrin subunits may affect degree of $\alpha6$ interaction with $\beta1$ subunit or $\alpha3$ interaction with $\beta1$ subunit, which control haptotactic motility of colonic tumor cells.

Effect of H *versus* A/B glycosylation may occur with not only integrins, but also some other receptors. For example, lamp-1, defining trafficking of components between lysosomal and plasma membranes, is a carrier of fucosyl-polylactosamine (21), and its expression is positively correlated with metastatic potential of colonic carcinoma cell lines (22). H/Le^y glycosylation was reported to be high in lamp-1 and was correlated with motility and invasiveness of rat carcinoma cells (23). In fact, we found H *versus* A/B glycosylation differences in lamp-1 in parental cells *versus* their transfectants (data not shown). The overall effect of H *versus* A/B glycosylation in various types of tumors is complex, and further studies along this line are in progress.

Acknowledgments

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