Alterations in Integrin Receptor Expression on Chemically Transformed Human Cells: Specific Enhancement of Laminin and Collagen Receptor Complexes

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Abstract. The abilities of malignant tumor cells to bind and migrate through basement membranes are important steps in invasion and metastasis. Malignant tumor cells would therefore be expected to express receptors on their surfaces for basement membrane and stromal components, such as collagens, laminin, and fibronectin, although the pattern of expression of these receptors on the malignant cells may be different from that on their normal progenitors. We report here that chemically transformed tumorigenic human cells express an altered pattern of integrin receptors on their cell surfaces as compared with their untransformed nontumorigenic counterparts. Specificially, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine transformation of HOS cells into highly tumorigenic cells results in a significant specific increase in the expression of (in descending order of level of cell surface expression) the integrins $\alpha6/\beta1$, $\alpha2/\beta1$, and $\alpha1/\beta1$, which are receptors for laminin, collagens, and collagen type IV and laminin, respectively. The level of expression of two fibronectin receptor integrins, $\alpha5/\beta1$ and $\alpha3/\beta1$, are, however, unaltered, whereas the level of expression of vitronectin receptor integrin, $\alpha v/\beta3$, is drastically reduced on the transformed cells. Consistent with the increased expression of laminin and collagen receptors and the decreased expression of vitronectin receptors on the transformed cells, these cells attached three- to fivefold more strongly to laminin and collagen but attached very poorly to vitronectin. The MNNG-HOS cells were also found to have a greater potential for invasion through reconstituted basement membrane, matrigel, the major components of which are laminin and type IV collagen. The invasion of both the HOS and MNNG-HOS cells was inhibited 45–50% by a polyclonal anti-fibronectin receptor antibody. However, although the invasion of HOS cells could be inhibited up to 75% by an anti-$\alpha6$ monoclonal antibody, a similar concentration of this antibody had no effect on the $\alpha6$-overproducing MNNG-HOS cells. A fivefold higher concentration of this antibody did result in partial inhibition of MNNG-HOS invasion. These data indicate a critical role for the $\alpha6/\beta1$ laminin receptor in the invasion of these cells through basement membranes and demonstrate that chemical transformation of nontumorigenic human cells to highly tumorigenic cells is associated with an altered pattern of integrin expression which may play a direct role in the increased capacity of these cells to bind and invade through basement membranes.

MALIGNANT transformation of cells frequently results in decreased expression of extracellular matrix components such as fibronectin, laminin, and collagen (12, 13, 15, 35). In addition, invasive carcinomas consistently exhibit a defective extracellular basement membrane adjacent to the invading tumor cells in the stroma (22). Malignant tumor cells also acquire the capability to actively invade basement membranes and migrate through the surrounding stroma. To form metastases at distant sites, malignant tumor cells must penetrate the subendothelial basement membrane during the processes of intra- and extravasation. Tumor cell interaction with the extracellular matrix therefore occurs at multiple stages in the metastatic cascade. It therefore follows that to interact with basement membranes and tissue stroma, invading tumor cells must express adequate numbers of receptors for basement membrane and stromal components. Thus, although oncogenic transformation may result in decreased expression of extracellular matrix proteins, malignant tumor cells are likely to express receptors for extracellular matrix components, especially for those found in basement membranes such as laminin and type IV collagen. In addition, the pattern of receptor expression is likely to be different for the invasive and metastatic cells as compared with that on the cells of the tissue of origin.

A family of cell surface receptors called integrins, which interact with extracellular matrix proteins, has recently been characterized (3, 14, 27). Integrins are $\alpha\beta$ heterodimeric transmembrane proteins. There are three known β subunits (β1, β2, and β3), each of which is shared by different α subunits which appear to confer ligand specificity. The β1 integrin family includes receptors for fibronectin, laminin, and collagen (10, 14, 29) and the β3 family includes receptors for vitronectin (14).

To determine whether malignant transformation of human cells results in an altered expression of the integrin fibronectin, collagen, laminin, and vitronectin receptors, we compared specific integrin expression on a pair of human cell lines, HOS and MNNG-HOS. HOS is a human osteogenic sarcoma cell line that is nontumorigenic in nude mice (26). MNNG-HOS cell line was derived from HOS cells by treatment of these cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)[1] (26), a known potent carcinogen. The MNNG-HOS cells were found to be highly tumorigenic (26). In addition, DNA from MNNG-HOS cells could transform NIH-3T3 cells, whereas DNA from the HOS cells could not (4), suggesting that MNNG caused genetic alterations in HOS cells that gave rise to a transferable transforming gene. This gene has subsequently been shown to be the *met* oncogene (24) encoding a protein with tyrosine kinase activity (6).

We report here that chemical transformation of HOS cells results in a significant specific increase in α6/β1 (laminin receptor) (29), α2/β1 (collagen receptor) (10), and α1/β1 integrins and a decrease in αv/β3 (vitronectin receptor) expression. This altered pattern of integrin expression on the tumorigenic transformed cells, as compared with the untransformed cells, endows these cells with an increased ability to bind and invade through basement membranes.

Materials and Methods

Sodium [^{125}I]iodide and [^{32}P]dCTP were purchased from Amersham Corp. (Arlington Heights, IL). Chemicals used for SDS-PAGE were from Bio-Rad Laboratories (Richmond, CA). Plasmid containing the cDNA encoding the β (λP32) subunit of the human fibronectin receptor (2) and rabbit anti-human fibronectin and vitronectin receptor antisera were from Drs. Erkki Ruoslahti and Michael Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA). The anti-fibronectin receptor antibody reacts with both the α5 and β1 subunits of the fibronectin receptor (2). The anti-vitronectin receptor antibody reacts with both αv and β3 subunits of the vitronectin receptor (30), but the preparation used here does have some antibody cross reacting with β1 subunit. Mouse anti-human VLA-1 monoclonal antibody (TS2/7) was a kind gift from Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA), and anti-VLA-2 α subunit and anti-VLA-3 α subunit (J143) were kind gifts from Dr. Ken Pischel (University of California at San Diego, San Diego, CA) and Dr. A. Albino (Memorial Sloan-Kettering Cancer Center, New York), respectively. Monoclonal antibodies against VLA-5 α subunit and the common β1 subunit were kind gifts from Dr. Carolyn Damsky (University of California at San Fransico, CA). Monoclonal antibody to VLA-6 α subunit (GOH3) was a kind gift from Dr. A. Sonnenberg (Red Cross Blood Transfusions Services, Amsterdam, The Netherlands). HOS (TE-85, clone F5) and MNNG-HOS (TE-85, clone F5) cell lines were obtained from American Type Culture Collection (Rockville, MD). Collagens type I and IV were purchased from Sigma Chemical Co. (St. Louis, MO). Fibronectin and vitronectin were purchased from Telios Pharmaceuticals (La Jolla, CA). Laminin was a gift from Dr. Max Wicha (University of Michigan, Ann Arbor, MI). Hybridoma culture supernatants against major histocompatibility complex antigen (HLADR) and the fluorochrome R-phycoerythrin, used as controls in the antibody inhibition of invasion assays, were obtained from Drs. F. Takei and P. Lansdorp (Terry Fox Laboratory, Vancouver, British Columbia, Canada).

Cell Cultures

HOS, MNNG-HOS, and MG-63 cells were cultured in DME supplemented with 10% heat-denatured FCS, 1% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco Laboratories Grand Island, NY). For routine subculturing, cell monolayers were washed with PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.3) and detached with 1 mM EDTA. Total cell numbers were determined by counting on a counter (model ZBI; Coulter Electronics Inc., Hialeah, FL).

Cell Labeling and Immunoprecipitations

Cells were either labeled with [^{32}P]orthophosphate or were surface labeled with ^{125}I. For ^{32}P labeling, cell monolayers were exposed to [^{32}P]orthophosphate (200 μCi/ml) in phosphate-free DME for 18 h. The medium was then removed, and cells were harvested and extensively washed in PBS containing $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) to remove any free ^{32}P. For surface labeling with ^{125}I, cells were harvested and resuspended in PBS containing $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM). Cells were then surface labeled with ^{125}I as described previously (8). Labeled cells were lysed, and the integrins immunoprecipitated with the respective antibodies and protein A-Sepharose (Sigma Chemical Co.) as described previously (8, 9). The antigen-antibody complexes were dissociated by boiling in sample buffer (200 mM Tris-HCl, pH 6.8, containing 3% SDS, 10% glycerol, and 0.001% bromophenol blue). Samples were analyzed by electrophoresis in 7.5% SDS-polyacrylamide gels (21) followed by autoradiography.

Isolation and Analysis of mRNAs

Total cellular RNA was prepared from HOS, MNNG-HOS, and MG-63 cell lines by the guanidinium/cesium chloride method (23, 32). The RNAs (10 μg) were resolved by agarose gel electrophoresis, transferred to nitrocellulose filters, and probed with ^{32}P-β1 integrin cDNA as described previously (2, 7).

Cell Attachment Assays

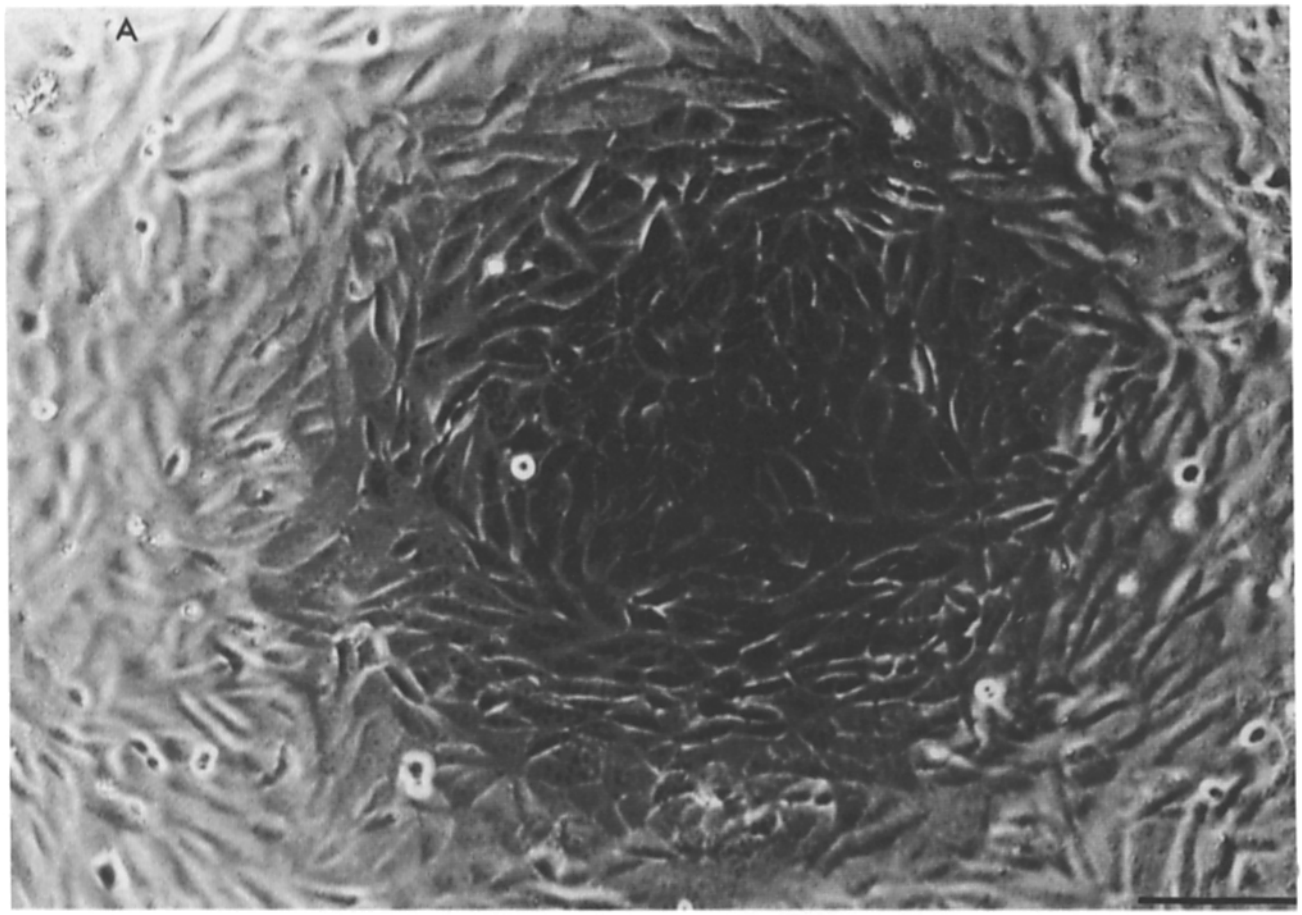
Attachment of HOS and MNNG-HOS cells to protein-coated microtiter wells was carried out as described by Ruoslahti et al. (28).

In Vitro Invasion Assay

The relative abilities of HOS and MNNG-HOS cells to invade through basement membranes was assayed quantitatively using an in vitro invasion assay. The assay was modified from that described by Albini et al. (1). Basement membrane matrigel (Collaborative Research, Bedford, MA) which consists of laminin, type IV collagen, heparan sulfate proteoglycan, and entactin (nidogen) (20) was used as a barrier for the invading cells. The details of the assay will be published elsewhere (Saulnier, R., and S. Dedhar, manuscript in preparation). Briefly, Transwells (Costar, Cambridge, MA) which consist of upper and lower compartments separated by polycarbonate filters, were used in the assay. The 13-mm-diameter filters were coated with type I collagen to facilitate the attachment of the invaded cells. These filters were overlayed with polyvinylpyrolidone-free polycarbonate filters (12 μm pore size; Nucleopore, CA), which were coated with a 1:3 dilution of matrigel. The optimum concentration of matrigel used was determined empirically. Cells were labeled in standard tissue culture medium for 24 h. with [^{3}H]thymidine (1 μCi; 20 Ci/mmol), tested for viability by trypan blue exclusion, washed extensively to remove unincorporated label, resuspended

1. *Abbreviation used in this paper*: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Figure 1. Morphology of HOS and MNNG-HOS cells. The photomicrographs represent the morphology of cells growing under standard tissue culture conditions: i.e., DME in 10% FBS. (*A*) HOS cells; (*B*) MNNG-HOS cells. Bar, 25 μm.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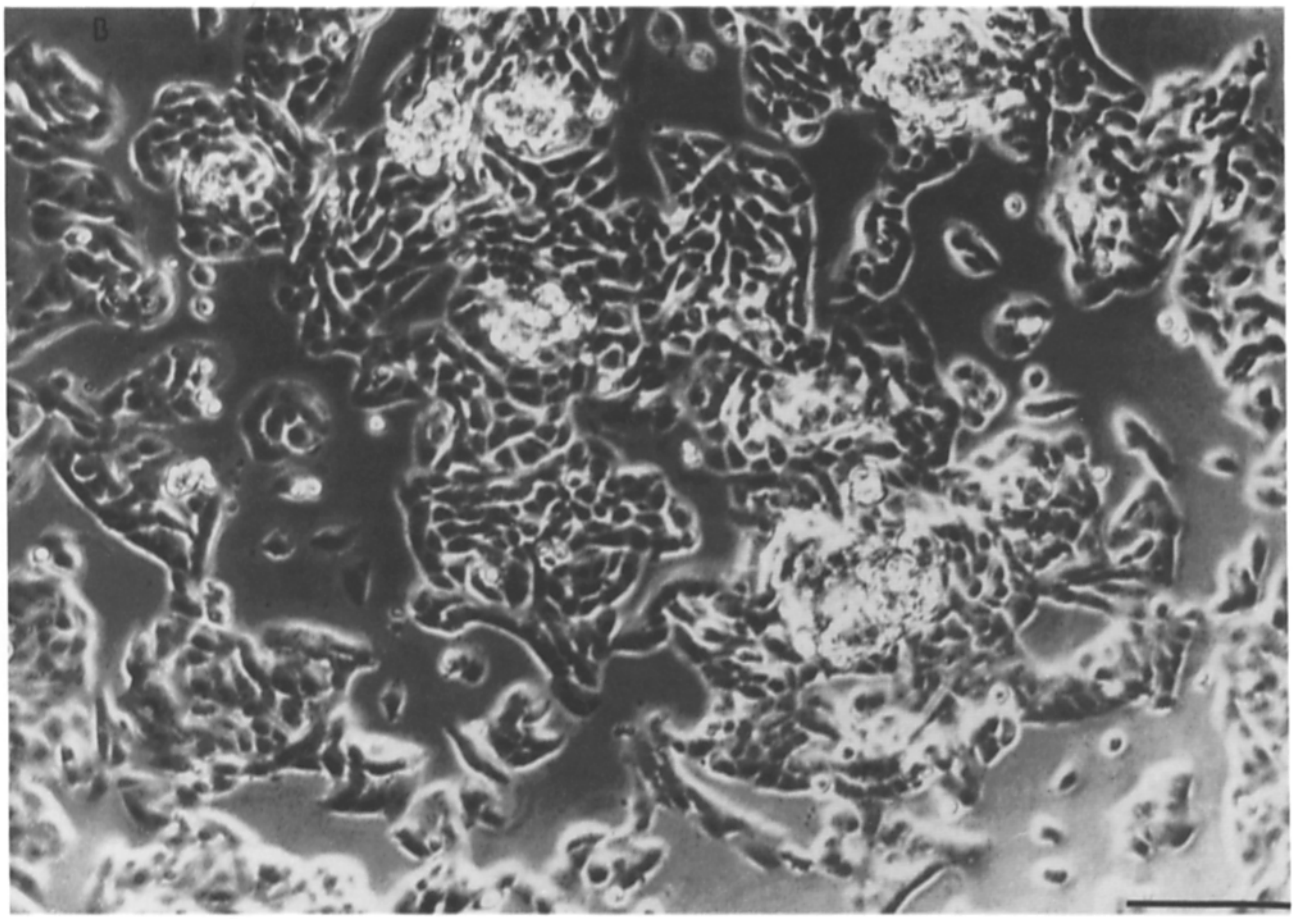
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in DME containing 0.1% BSA and applied onto the matrigel-coated filter (10^5 cells/well). An aliquot of the radiolabeled cells was used to determine the initial radioactivity applied.

Invasion of the cells through the matrigel was allowed to proceed for 18 h at 37°C. The top, matrigel-containing filter was then removed, and the amount of radioactivity associated with the bottom, collagen-coated filter was determined by liquid scintillation counting. Invasion of the cells was confirmed by fixing and staining the cells on the bottom filter with 3.7% paraformaldehyde, 0.5% toluidene blue and visualizing the cells under an inverted microscope. The percent invasion was quantitated by dividing the number of ^3H counts per minute on the bottom filter by the initial ^3H counts per minute applied. Each experiment was carried out in quadruplicate.

For antibody inhibition assays, the cells were preincubated with the reagents in 100 μl of DME containing 0.1% BSA for 30 min at 4°C. Antibodies were also present in the upper chamber during the invasion assay and were also incorporated into the matrigel in similar concentrations.

Results

To determine whether chemical transformation of human cells into tumorigenic and invasive cells resulted in alterations in the integrins, we analyzed integrin phosphorylation and the pattern of specific integrin expression at the cell surface of HOS and MNNG-transformed HOS cells, the differences in cell morphology of which are shown in Fig. 1. The reason for determining the status of integrin phosphorylation upon chemical transformation was twofold. First, the protooncogene, *met*, which is activated upon MNNG transformation of HOS cells encodes tyrosine kinase activity (6), and, second, Rous sarcoma virus transformation of chicken cells has been shown to result in the phosphorylation of both the α and β1 chicken integrin homologues (11). The consequence, if any, of this phosphorylation on cell adhesion, however, has yet to be determined.

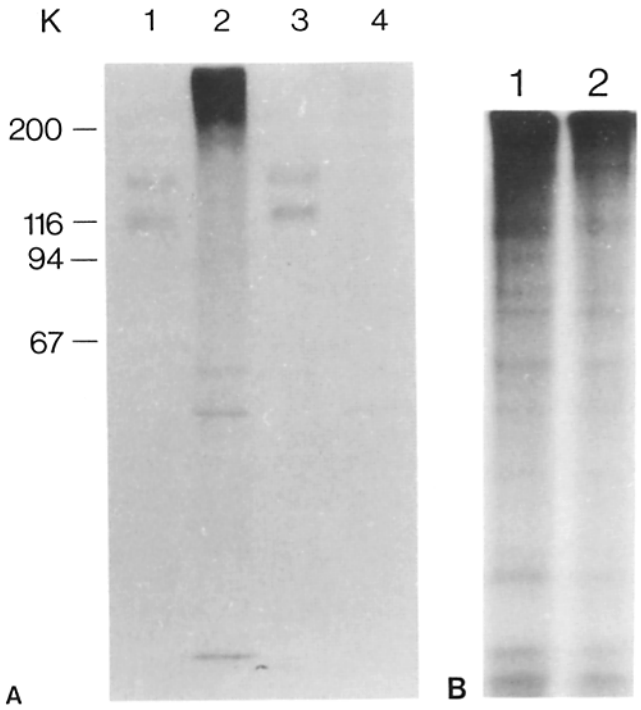

Figure 2. Immunoprecipitation of integrins from ^{32}P-labeled and ^{125}I-surface labeled HOS and MNNG-HOS cells. Cells were radioactively labeled, extracted, and immunoprecipitated with a rabbit anti-human fibronectin receptor antiserum as described in Materials and Methods. Immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions, and the bands were visualized by autoradiography. (*A*, lanes *1* and *3*) ^{125}I-surface labeled cells; (lanes *2* and *4*) [^{32}P]orthophosphate-labeled cells. Lanes *1* and *2* are HOS cells, and lanes *3* and *4* are MNNG-HOS cells. (*B*) SDS-PAGE of nonimmunoprecipitated ^{32}P-labeled HOS (lane *1*) and MNNG-HOS (lane *2*) lysates. Molecular weight markers are myosin heavy chain (200K), β-galactosidase (116K), phosphorylase β (94K), and albumin (67K).

As shown in Fig. 2, we were unable to demonstrate phosphorylation of integrins in either of the cell lines, HOS or MNNG-HOS. In this experiment, cells from parent cultures were subcultured; one half were labeled with [^{32}P]orthophosphate, and the other half were surface labeled with ^{125}I. Integrins could be clearly immunoprecipitated from ^{125}I-labeled cells but not from ^{32}P-labeled cells (Fig. 2 *A*), demonstrating that, although β1 integrins are present on both cell lines, they are not phosphorylated. As shown in Fig. 2 *B*, many other membrane-associated proteins were ^{32}P labeled by this procedure, and therefore the lack of demonstration of ^{32}P labeling of integrins was not due to incomplete labeling. It would therefore appear that MNNG-induced activation of a tyrosine kinase–encoding protooncogene does not result in the phosphorylation of β1 integrins.

The immunoprecipitation of ^{125}I-labeled cells with anti-fibronectin receptor antibody, which contains antibodies against the β1 and α5 integrin subunits (2), suggested that the MNNG-HOS cells may express an altered pattern of integrin expression as compared with HOS cells (Fig. 2 *A*, lanes *1* and *3*). To analyze the patterns of integrin expression on these two cell lines in more detail, we immunoprecipitated each of the integrin heterodimers from both cell lines using α subunit–specific monoclonal antibodies.

The results are shown in Fig. 3. Equivalent numbers of HOS and MNNG-HOS cells (~70% confluent) were labeled with ^{125}I and lysed, and equivalent ^{125}I radioactive counts were immunoprecipitated with the antibodies described in Fig. 3. Immunoprecipitation with an anti-β1 monoclonal antibody (Fig. 3, lanes *1* and *2*) demonstrates that increased numbers of β1 integrins are expressed on MNNG-HOS cells as compared with HOS cells. More specifically, the heterogeneous α band, which includes α subunits 2–6 because they all have very similar molecular weights (10), is more diffuse in the MNNG-HOS cells than in the HOS cells. Immunoprecipitation with α subunit–specific monoclonal antibodies demonstrated that there is no difference in the amounts of α3/β1 (Fig. 3, lanes *3* and *4*) and α5/β1 (lanes *5* and *6*) integrin expressed on these two cell lines. α3/β1 integrin has been shown to be a low affinity receptor for fibronectin, laminin, and collagen (10, 25, 33), whereas α5/β1 is a higher affinity receptor specific for fibronectin (10, 31). The α5/β1 integrin is expressed at much lower amounts on both cell lines than is the α3/β1 integrin.

Immunoprecipitation with anti-α6 and anti-α2 monoclonal antibodies, however, provided what appears to be the most significant difference in integrin expression on these two cell lines. As shown in Fig. 3, there is very little expression of α6/β1 on HOS cells, but a >10-fold increased expression of this integrin on MNNG-HOS cells (Fig. 3, lanes *7* and *8*). In fact, α6 is the most abundant α subunit expressed on MNNG-HOS cells. α6/β1 has been shown to be a specific receptor for laminin (29). Similarly, the integrin α2/β1,

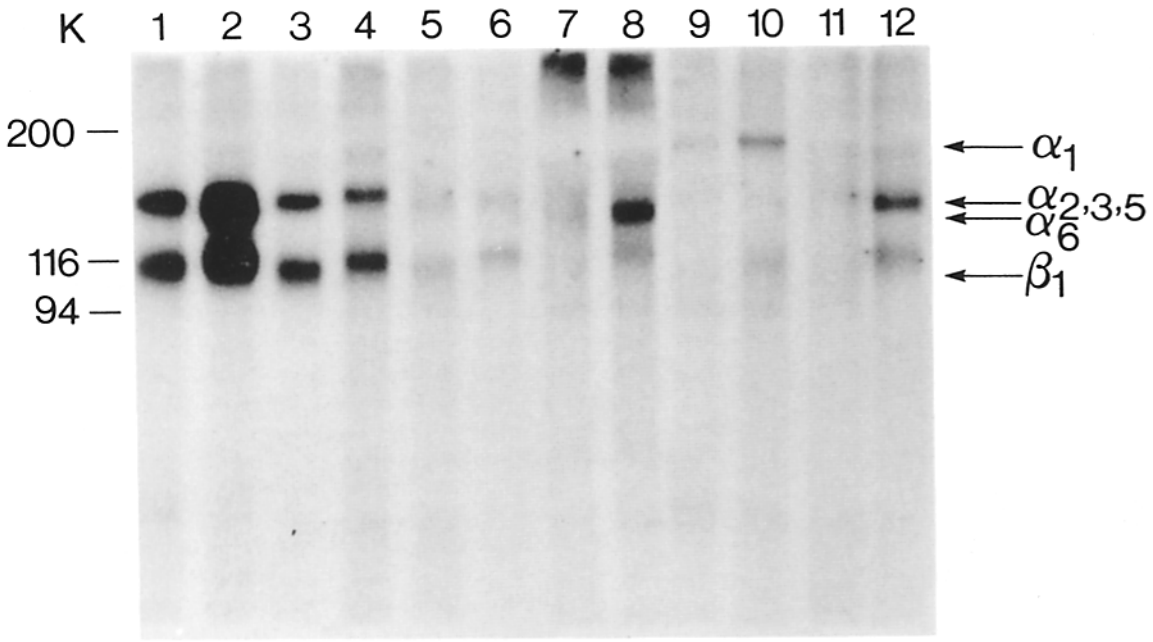

Figure 3. Immunoprecipitation of specific integrins from ^{125}I-surface labeled HOS and MNNG-HOS cells. Cells were labeled, extracted, and immunoprecipitated with the appropriate antibodies as described in Materials and Methods. Immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions, and the bands were visualized by autoradiography. Lanes *1*, *3*, *5*, *7*, *9*, and *11* are HOS cells, and lanes *2*, *4*, *6*, *8*, *10*, and *12* are MNNG-HOS cells. (Lanes *1* and *2*) Anti-human β1 monoclonal antibody; (lanes *3* and *4*) anti-human α3 monoclonal antibody; (lanes *5* and *6*) anti-human α5 monoclonal antibody; (lanes *7* and *8*) anti-human α6 monoclonal antibody; (lanes *9* and *10*) anti-human α1 monoclonal antibody; (lanes *11* and *12*) anti-human α2 monoclonal antibody. Molecular weight standards are similar to those described in Fig. 2.

which is a collagen receptor (10), is expressed at an approximately fivefold higher level on MNNG-HOS cells than on HOS cells (Fig. 3, lanes *11* and *12*). A twofold increase in the expression of α1/β1 in MNNG-HOS cells is also apparent when immunoprecipitation is carried out with an α1 subunit-specific monoclonal antibody (Fig. 3, lanes *9* and *10*). The identity of the ligand for integrin α1/β1 remains unclear, although it may be a human homologue of a laminin-binding rat heterodimer with similar relative molecular masses (16). The human α1/β1 integrin may also be a receptor for collagen (18).

Another interesting feature of the β1 integrins expressed by MNNG-HOS cells is that the common β1 subunit expressed by these cells has a slightly higher apparent relative molecular mass than the β1 subunit expressed on HOS cells (Fig. 3, lanes *1–12*). The difference in the relative molecular mass may be due to the expression of a different mRNA or to differences in posttranslational modifications such as phosphorylation or glycosylation. The difference is unlikely to be due to phosphorylation since the β1 integrins are not phosphorylated in either cell line (Fig. 2). We therefore carried out Northern blot analysis of total RNA from the two cell lines to determine whether the MNNG-HOS cells express an altered β1 mRNA. As shown in Fig. 4 the size and the intensity of the mRNA from both cell lines is identical and is similar to that expressed in another osteosarcoma cell line, MG-63. The altered size of the β1 subunit is therefore unlikely to be due to the expression of a larger mRNA and is likely to be due to increased glycosylation. In addition, whereas immunoprecipitation with anti-β1 antibody demonstrated more β1 expressed on the MNNG-HOS cell surfaces, β1 mRNA levels appear to be the same in both cell types. This suggests that MNNG transformation may have resulted in posttranslational modification of β1 processing and transport, leading to increased β1 expression on the cell surface.

We also determined the expression of the vitronectin receptor, an integrin belonging to the β3 integrin family (14, 27), on the two cell lines. Immunoprecipitation of ^{125}I-surface labeled cells with a rabbit anti-human vitronectin receptor antiserum (30) demonstrated that the MNNG-HOS cells express greatly reduced levels of this receptor as compared with the HOS cells (Fig. 5). In particular, the β3 subunit is present in much lower amounts on the surface of MNNG-HOS cells.

To determine the biological consequences of these changes in integrin expression, attachment of HOS and MNNG-HOS cells was carried out on various ligands. As can be seen from Fig. 6, both cell lines attach well to fibronectin, but the MNNG-HOS cells demonstrated stronger attachment to collagen type I and much stronger attachment to the basement membrane components collagen type IV and laminin, correlating well with the increased expression of the receptors for these two ligands on these cells. In addition, consistent with the demonstrated expression of reduced vitronectin receptors on MNNG-HOS cells, these cells attached very poorly to vitronectin as compared with the untransformed HOS cells (Fig. 6).

To determine whether there were any differences in the ability of HOS and MNNG-HOS cells to invade a reconstituted basement membrane, invasiveness of the two cell lines was quantitatively determined with the use of an in vitro invasion assay through basement membrane matrigel. Matri-

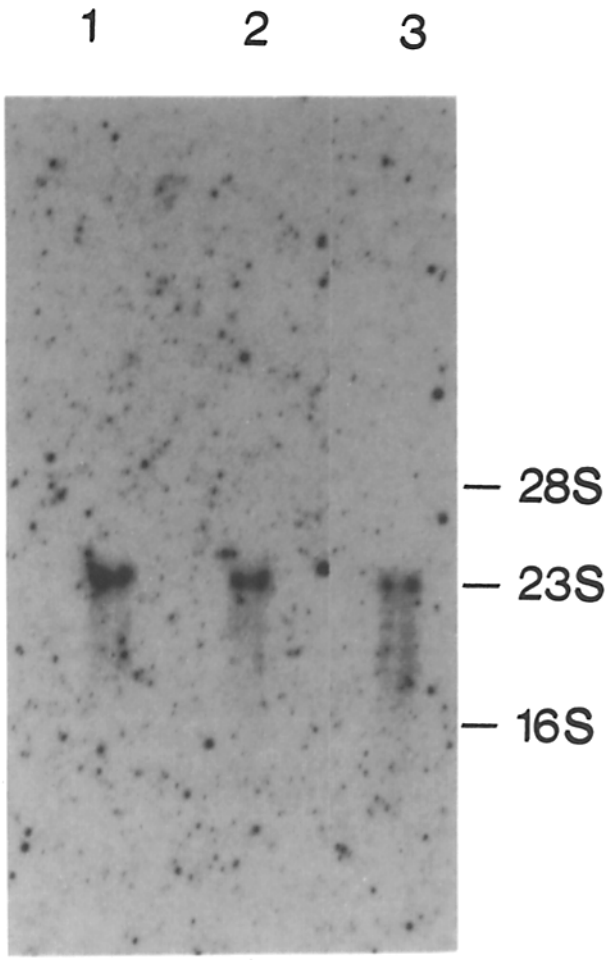

Figure 4. Identification of β1 integrin mRNA in HOS and MNNG-HOS cells. Cytoplasmic RNA was extracted as described in Materials and Methods. The RNA (10 μg/lane) was resolved by electrophoresis on 0.8% agarose gel containing formaldehyde (6%), transferred onto a nitrocellulose filter, and probed with ^{32}P-labeled fibronectin receptor β subunit (β1) cDNA (2). (Lanes *1–3*) MNNG-HOS, HOS, and MG-63, respectively.

gel has been shown to consist of laminin, type IV collagen, heparan sulfate proteoglycan, and entactin (nidogen) (20). As shown in Table I, the MNNG-HOS cells were significantly ($p < 0.05$) and uniformly more invasive than HOS cells in five different experiments. Although the percent invasion fluctuated from one experiment to another, the MNNG-HOS cells were more invasive relative to the HOS cells in all five experiments. The invasive potential of the two cell lines were compared relative to the invasiveness of a human lung fibroblast cell line (IMR-90), the invasive potential of which was found to be negligible (Table I).

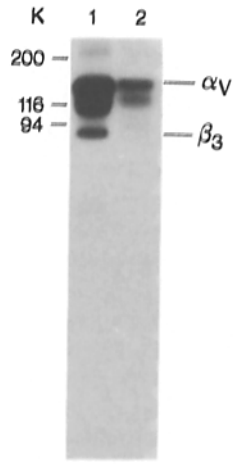

Figure 5. Immunoprecipitation of the vitronectin receptor (αv/β3) from ^{125}I-surface labeled HOS and MNNG-HOS cells. Cells were labeled, extracted, and immunoprecipitated with a rabbit anti-human vitronectin receptor antiserum as described in Materials and Methods. The immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions, and the bands were visualized by autoradiography. (Lane *1*) HOS cells; (lane *2*) MNNG-HOS cells. Molecular weight markers are similar to those described in Fig. 2.

The invasion of both HOS as well as MNNG-HOS cells was inhibited by an anti-fibronectin receptor antiserum, which contains antibodies against both α5 and β1 subunits (2), when these cells were preincubated with this antiserum and when this antibody was incorporated in the matrigel (Table II). Invasion of the HOS cells was also profoundly inhibited by the anti-α6 monoclonal antibody (GOH3) (29) under similar conditions (Table II). However, the invasion of the α6-overproducing MNNG-HOS cells was not inhibited by similar concentrations of this antibody. The inability of the invasion of MNNG-HOS cells to be inhibited by the anti-α6 antibody is most likely due to the much higher level of expression of α6 on these cells. The use of fivefold higher concentrations of antibody did result in partial inhibition of MNNG-HOS cell invasion (Table II). These data thus indicate that the integrin α6/β1 plays an important role in the invasion by these cells through basement membranes. The increased invasion of the MNNG-HOS cells may thus at least partially be due to the very high level of expression of the α6/β1 integrin on these cells. The partial inhibitory effect of the anti-fibronectin receptor antiserum on invasion may be due to the inhibition of the common β1 function rather than a specific effect of this antiserum on α5/β1 (fibronectin receptor). Similar experiments with adhesion-perturbing antibodies to α2 (unavailable at the present time) should reveal whether this integrin is also involved in the basement membrane invasion by these cell lines.

Discussion

The behavior of malignant tumor cells is quite different from that of their parental normal counterparts. Apart from their ability to proliferate in an uncontrolled manner at the primary site, some of the malignant tumor cells also acquire phenotypes which make them less adhesive to the surrounding stroma at the primary site but at the same time are able to attach and migrate through basement membranes. It is therefore highly likely that such cells would express on their cell surfaces receptors (integrins) for various components of the basement membrane such as laminin and type IV collagen. In addition, it is likely that the pattern of integrin expression on the malignant cells would be different from that on the parental untransformed cells, reflecting the altered phenotype of these cells.

To determine whether transformation of human cells into malignant cells results in an altered pattern of integrin expression, we analyzed specific integrin expression on the cell surfaces of HOS and MNNG-HOS cells (26). Unlike rodent cells, malignant transformation of human cells is very difficult. The availability of chemically transformed malignant cells (MNNG-HOS) and their parental nontumorigenic counterparts (HOS cells) provides a suitable model system for comparative studies of tumorigenic vs. nontumorigenic human cells.

Analysis of integrin expression on these two cell lines has

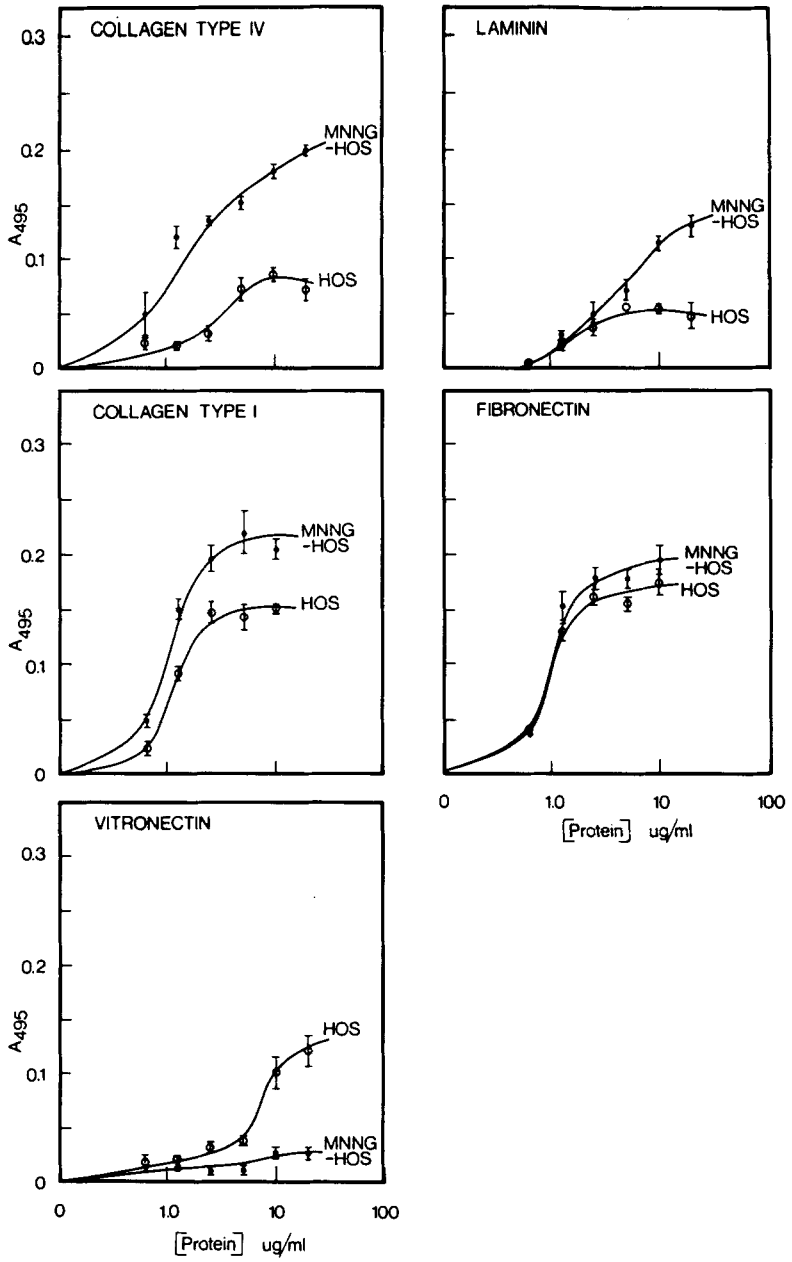

Figure 6. Attachment of HOS and MNNG-HOS cells to collagen type IV, laminin, collagen type I, fibronectin, and vitronectin. Attachment assays were carried out on ligand-coated microtiter wells as described by Ruoslahti et al. (28). Cells (3×10^4) were plated in wells coated with increasing concentrations of the indicated ligand in the presence of 2.5 mg/ml BSA. The plates were incubated at room temperature for 3 h in an atmosphere of 7% CO_2. Nonattached cells were washed away with PBS, and the attached cells were fixed with 3% paraformaldehyde and stained with 0.5% toluidene blue in 3.7% paraformaldehyde. Cell attachment was measured as a function of absorbance of destained cells at 495 nM.

Table I. In Vitro Invasion of HOS, MNNG-HOS, and IMR-90 Cells through Reconstituted Basement Membrane Matrigel

Experiment	Cells invaded		
	HOS	MNNG-HOS	IMR-90
	%	%	%
1	1.71	4.65	0.70
2	1.81	2.41	0.82
3	4.97	8.90	0.59
4	2.40	3.70	–
5	1.96	5.74	–

The increased invasiveness of MNNG-HOS cells was statistically significant ($p < 0.05$) as determined by the Mann-Whitney test. The invasion assay was carried out and quantitated as described in Materials and Methods.

revealed, first, that β1 and β3 integrin heterodimers are not phosphorylated on either cell line despite the activation of a tyrosine kinase-encoding oncogene (*met*) in the MNNG-HOS cells. Second, when individual integrin heterodimer expression was analyzed, it was found that the MNNG-treated cells expressed similar levels of fibronectin receptor structures, α3/β1 and α5/β1, but expressed greatly increased levels of laminin and collagen receptors, namely integrins α6/β1, α2/β1, and α1/β1 (Table III). Third, the common β1 subunit of all of these receptor structures appears to have a slightly higher relative molecular mass in the MNNG-HOS cells than the β1 subunit in HOS cells. This difference in molecular mass is not due to the expression of a different size mRNA but rather appears to be due to altered posttranslational modification. Since this alteration is not due to phos-

Table II. Inhibition of In Vitro Invasion of HOS and MNNG-HOS Cells by Anti-integrin Antibodies

Cells	Cells invaded*	Inhibition
	%	%
HOS		
Control	5.0 ± 1.2	–
Anti-fibronectin receptor antiserum (1:50)	2.7 ± 0.8	46
Anti-α6 antibody (1:2)	1.5 ± 0.4	70
MNNG-HOS		
Control	8.1 ± 1.5	–
Anti-fibronectin receptor antiserum (1:50)	5.2 ± 1.1	36
Anti-α6 antibody (1:2)	8.3 ± 1.7	–
Anti-α6 antibody (fivefold more concentrated)	4.8 ± 1.2	41

The effect of anti-fibronectin receptor antiserum was controlled for by carrying out replicate assays in the presence of equivalent dilutions of normal rabbit serum, which did not effect the invasion of either cell line. The effect of anti-α6 hybridoma culture supernatant was controlled for by carrying out replicate assays in the presence of equivalent dilutions of hybridoma culture supernatants containing antibodies against human major histocompatibility complex antigen (HLADR) or the fluorochrome R-phycoerythrin, neither of which had a significant effect on the invasion of either cell line.

*Values represent the mean ± SD of three separate experiments. Each experiment was carried out in quadruplicate. Percent invasion was determined as described in Materials and Methods.

phorylation, it is likely to be due to altered glycosylation, although we do not have any evidence for this as yet. Last, the expression of αv/β3 integrin, which mediates attachment of cells to vitronectin, is greatly reduced in the MNNG-transformed cells. Since we do not at the present time have cDNA probes available for the α6, α2, and α1 subunits, we are unable to correlate the levels of expression of these subunits at the cell surface with their mRNA levels. It is therefore conceivable that the alterations in the levels of cell surface expression of these subunits occur at a posttranslational level in the processing and transport of these proteins or even be due to alterations resulting in increased surface exposure.

The functional consequence of this difference in the pattern of integrin expression on these cells is manifested in a much stronger binding of MNNG-transformed cells to laminin and type IV collagen, both of which are components of basement membranes. In addition, the MNNG-transformed HOS cells were found to be significantly more invasive than the untransformed HOS cells, as determined by the ability of these two cell lines to invade through a reconstituted basement membrane matrigel, the major components of which are laminin and type IV collagen. Since the invasion assay used here determines all three parameters of invasion through a basement membrane—i.e., attachment to the matrix, degradation of the matrix, and migration through the matrix (22)—it is likely that the increased expression of laminin and collagen receptor integrins on the transformed cells may serve to facilitate the first and third parameters of basement membrane invasion. This conclusion is partially supported by the ability of anti-α6 antibodies in inhibiting the invasion of HOS cells and, at much higher concentrations of antibody, of MNNG-HOS cells through matrigel. The requirement of much higher concentrations of anti-α6 antibody for the invasion of the α6-overexpressing MNNG-HOS cells suggests an important role for this integrin in basement membrane invasion by these cells.

Table III. Summary of Alterations in Integrin Subunits upon Chemical Transformation of Human Cells with MNNG

Integrin subunit	Change	Ligands
β1	Increased/altered relative molecular mass	–
α3	Unchanged	FN/LM/COLL
α5	Unchanged	FN
α6	Greatly increased	LM
α2	Increased	COLL
α1	Increased	LM/COLL IV
αv	Decreased	VN
β3	Greatly decreased	–

FN, fibronectin; *LM*, laminin; *COLL*, collagen; *VN*, vitronectin.

Interestingly, a nonintegrin 68,000-M_r laminin-binding protein, expressed on normal epithelial, skeletal muscle, and neuronal cells, is also increased on highly metastatic tumor cells (22, 34), and tumor-promoting phorbol esters also enhance attachment of NIH-3T3 cells to laminin and type IV collagen (17). Integrin-related complexes mediating attachment to laminin, collagen, and fibronectin have also been shown to be expressed on metastatic B16 melanoma cells (19). Furthermore, poorly differentiated human colon carcinoma cell lines have been recently shown to attach and spread more strongly on laminin and matrigel than moderately differentiated cell lines which attach poorly to these substrates (5).

Our results differ from those described recently by Plantefaber and Hynes (25) in that MNNG transformation of human cells does not result in a decrease in the expression of α5/β1 (fibronectin receptor) integrin, as is the case in virally transformed rodent cells (25). However, the level of expression of α5/β1 is quite low relative to α3/β1 in both the HOS and MNNG-HOS cells, and, because of greatly increased laminin and collagen receptors on the MNNG-HOS cells, the effective ratio of fibronectin receptors to laminin receptors or to collagen receptors is greatly decreased on these cells. Since Plantefaber and Hynes (25) did not determine the level of expression of α6 and α1 subunits on their transformed cell lines, it is not clear whether oncogenic transformation is generally associated with increased laminin receptor expression.

Taken together, the results presented here indicate that MNNG transformation of HOS cells results in an altered pattern of cell surface integrin expression. The greatly increased expression of laminin and collagen IV receptors on these cells likely endow these cells with an increased capability of interacting with basement membranes during invasion. In addition, decreased expression of vitronectin receptors and low levels of high affinity fibronectin receptor expression would render these cells less adhesive to stromal components immediately surrounding them.

Similar studies with other chemically and virally transformed human cells should provide interesting and important

data about the role of integrins in the expression of the malignant phenotype.

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