Specific Overproduction of Very Late Antigen 1 Integrin in Two Human Neuroblastoma Cell Lines Selected for Resistance to Detachment by an Arg-Gly-Asp-containing Synthetic Peptide*

(Received for publication, September 15, 1988)

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Very late antigen (VLA) 1 is a member of the family of integral plasma-membrane glycoproteins known as integrins. It is a heterodimer composed of an α subunit of M, 200,000, noncovalently associated with a β subunit of M, 110,000 which is shared by other VLA molecules (VLA-2–5). Unlike most of the other VLA proteins which have been shown to be receptors for various extracellular matrix proteins, the ligand for VLA-1 is unknown. Utilizing polyclonal antisera against the human fibronectin receptor as well as α subunit-specific monoclonal antibodies and cDNA probes, we have been able to demonstrate that in two human neuroblastoma cell lines, IMR-32 and SK-N-SH, the common β subunit is associated with α1, α2, α5, and α6 subunits. By culturing these two cell lines in the presence of a synthetic peptide, Gly-Arg-Gly-Asp-Ser-Pro, which contains the Arg-Gly-Asp cell attachment promotion tripeptide, we have isolated variant cell lines resistant to the detachment effects of this peptide. Peptide-resistant SK-N-SH and IMR-32 neuroblastoma cells exhibit weaker attachment to type I collagen and laminin, but a similar level of attachment to fibronectin as compared to the parental cells. Although the peptide-resistant variant cell lines proliferate at a rate similar to that of the parental cell lines, they stably overproduce (up to 20-fold) the α1 subunit (VLA-1) specifically; and in the IMR-32 variant cells, the common β1 subunit is also overproduced. The level of expression of α2 and α5 subunits, however, is considerably reduced and that of the α6 subunit is unchanged relative to the parental cells. These data suggest that the expression of integrin α subunits can be regulated differentially and independently of the β subunit and that the VLA-1 heterodimer has an important function in mediating Arg-Gly-Asp-dependent cell adhesion or other phenotypic properties in human neuroblastoma cells.

*This work was supported by a grant from the National Cancer Institute of Canada (to S. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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§The abbreviations used are: VLAs, very late antigens; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Sodium [35]iodide, [3H]methylated molecular weight standards, and [125]I-labeled anti-human fibroactin receptor (18) as well as rabbit anti-human fibronectin and as heterodimers appearing 2–4 weeks after in vitro activation of T cells (1, 3). Subsequently, three additional heterodimers (VLA-3–5) have been described and found to belong to the same protein family (4). The VLA protein heterodimers are each composed of a distinct α subunit noncovalently associated with a common β subunit (4). The VLA family of proteins are part of a superfamily of proteins called integrins (5, 6), which include receptors for extracellular matrix proteins such as fibronectin, vitronectin, collagens, and laminin. Indeed, the ligands for some of the VLA proteins are extracellular matrix proteins, e.g., the ligand for VLA-5 is fibronectin (7), that for VLA-2 is collagen (8), and those for VLA-3 are fibronectin, laminin, and collagen (8). The expression of VLA-1 is rather restricted in that it is weak or absent from most hemopoietic cells (except from very late activated T-cells and human T-cell leukemia virus-1-transformed lymphoblasts) and cell lines (8). VLA-1 is, however, expressed on a human neuroblastoma cell line, SK-N-SH (8). Despite the fact that VLA-1 is a member of the integrin family of protein molecules with significant N-terminal amino acid sequence homology with the other VLAs (9), its ligand is as yet unknown. A role for VLA-1 in cell-matrix adhesion is likely because of its expression of T-cells in the lung and on T cells from the synovium of arthritis patients, but not on peripheral T-cells from the same individual (10, 11). Most of the extracellular matrix proteins have been shown to interact with their respective cellular receptors via the tripeptide Arg-Gly-Asp (6, 12, 13). We have recently shown (14) that exposure of a human osteosarcoma cell line to increasing concentrations of a peptide containing the cell attachment promotion sequence, Arg-Gly-Asp, results in the isolation of cell variants which are resistant to the cell detachment effects of the peptide and overproduce the fibronectin receptor.

In this report, we demonstrate that a similar exposure of two human neuroblastoma cell lines, SK-N-SH and IMR-32, to an Arg-Gly-Asp-containing synthetic peptide results in the specific overproduction of the VLA-1 α subunit and its associated β subunits and in a decrease in the level of the other α subunits present in these two cell lines. These data suggest that VLA-1 may interact with an as yet unidentified ligand via the Arg-Gly-Asp sequence and that this interaction is important in the cell adhesion or other phenotypic properties of neuroblastoma cells.
vitronectin receptor antisera were kind gifts from Drs. Erkki Ruoslahti and Michael Fierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA). 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-human VLA-2 and VLA-3 (J143) were obtained from Dr. Ken Bachem (University of California, San Diego) and Dr. A. Albino (Memorial Sloan-Kettering Cancer Center, New York, NY), respectively. Monoclonal antibodies against the VLA-5 α subunit and the common β subunit (β1) were kind gifts from Dr. Caroline Damsky (University of California, San Francisco). SK-N-SH and IMR-32 human neuroblastoma and SK-N-MC cell lines were obtained from the American Type Culture Collection (Rockville, MD). Highly purified synthetic peptides with the sequences Gly-Arg-Gly-Asp-Ser-Pro (GRGDS) and Gly-Arg-Gly-Glu-Ser-Pro (GRGESP) were obtained from the La Jolla Cancer Research Foundation.

Cell Lines and Growth Conditions—SK-N-SH and IMR-32 are neuroblastoma cell lines, whereas SK-N-MC is a primitive neuroectodermal cell line (23). All three cell lines were cultured in Dulbecco's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (GIBCO). For routine subculturing, cell monolayers were washed with phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate, pH 7.3) and detached with EDTA (1 mM). Total cell numbers were determined by counting on a Model ZB1 Coulter Counter. All three cell lines were treated with the hexapeptide GRGDSP, and cells resistant to the detachment effects of this peptide were selected as described previously (14), except that the cells were initially treated with 1 mg/ml peptide. The concentration was increased in a stepwise manner to a maximum of 2.0 mg/ml. At this point, some cultures were grown in the absence of peptide. Treatment of cells with a 2 mg/ml concentration of the inactive control peptide, GRGESP, did not inhibit the attachment of the parental neuroblastoma cells. Immunoprecipitations—Parental and peptide-resistant cells were detached from culture with EDTA (1 mM) and resuspended with phosphate-buffered saline containing CaCl2 (1 mM) and MgCl2 (1 mM). The cells (2 × 10⁸) were surface-labeled with 125I and lysed as described previously (14). Immunoprecipitates were prepared utilizing protein A-Sepharose as described before (14), except that a second antibody, rat anti-mouse IgG (monoclonal antibody 187.1), was included during the immunoprecipitation of VLA-1. 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% bromphenol blue). Samples were analyzed by electrophoresis in 7.5% SDS-polyacrylamide gels (14, 15), followed by autoradiography. Isolation and Analysis of Messenger RNAs—Total cellular RNA was prepared from each of the cell lines by the guanidium/cesium chloride method (16, 17). The RNAs were resolved by agarose gel electrophoresis, transferred to nitrocellulose filters, and probed with 32P-labeled probes as described previously (14, 18). Cell Attachment Assays—Attachment of parental and peptide-resistant neuroblastoma cell lines to protein-coated microtiter wells was carried out as described by Ruoslahti et al. (22).

RESULTS AND DISCUSSION

We have previously shown (14) that culturing human osteosarcoma cells in the presence of increasing concentrations of a synthetic peptide containing the cell attachment promotion amino acid sequence, Arg-Gly-Asp, results in the isolation of cell variants which are resistant to the cell detachment activity of this peptide. The variant cells overproduce the common β subunit of the β1-integrin protein family, together with an α subunit most likely to be α5 or α6 (14). On the basis of the increased resistance of these cells to inhibition of cell attachment by GRGDSP peptide on fibronectin-coated surfaces, it was likely that the α/β heterodimer overproduced in these cells was a fibronectin receptor (14). In addition, the peptide-resistant variants of the human osteosarcoma cells exhibited many properties of differentiated, osteoblastic cells not expressed by the parental osteosarcoma cells.

In order to determine whether exposure of other human tumor cell lines to an Arg-Gly-Asp (RGD)-containing peptide would result in similar overproduction of integrin proteins and phenotypic changes, we treated three human neuroblastoma cell lines, SK-N-SH, SK-N-MC, and IMR-32, with a synthetic peptide, GRGDSP, as described previously (14) and under “Materials and Methods.” One reason for choosing neuroblastoma cell lines was that the SK-N-SH cell line has been shown to express VLA-1 (composed of an α2 subunit and the common β1 subunit) (8), which has a restricted cellular expression profile and is poorly characterized and its ligand is as yet unknown. It was therefore of interest to determine whether exposure to an RGD-containing peptide would augment the expression of VLA-1 of these cells. Immunoprecipitation of 125I-surface-labeled parental SK-N-SH cells with a rabbit antiserum prepared against purified human placental fibronectin receptor resulted in the precipitation of three labeled polypeptides of M, 110,000, 140,000–150,000, and 200,000 (Fig. 1A, lane 2). In addition, there is also a very high M, polypeptide precipitated from these cells (see arrow). The rabbit antiserum has previously been shown to immunoprecipitate the integrin common β1 subunit (13) and to immunoblot the integrin β1 and α5 subunits (19). The antibodies present in this antiserum against the common β1 subunit would therefore also be expected to immunoprecipitate all the α subunits associated with it on the surface of a given cell. From the M, of the polypeptides precipitated from SK-N-SH cells, the common β subunit (M, 110,000) appears to be associated with the α5 subunits (M, 140,000–150,000) and the α1 subunits (M, 200,000) (8). Immunoprecipitation of the peptide-treated SK-N-SH cells with this antiserum similarly precipitated three polypeptides. However, the amounts of each of these proteins were found to be consistently different of the peptide-resistant cells. Most surprisingly, these cells overproduced the α5 subunit, but expressed lower amounts of the other α subunits (Fig. 1A, lane 3). In addition, the very high molecular weight protein precipitated from the parental SK-N-SH cells could not be detected on the peptide-treated cells. Subsequent growth of the cells in the absence of the peptide did not alter this pattern of integrin expression. Growth of the parental cell lines in the presence of the inactive

FIG. 1. Immunoprecipitation of integrins from 125I-surface-labeled SK-N-SH and GRGDSP-resistant SK-N-SH neuroblastoma cells. 125I-Surface-labeled cells were extracted as described previously and various integrins were immunoprecipitated and analyzed by SDS-PAGE under nonreducing conditions as described under “Materials and Methods.” A: lane 1, nonimmune serum; lanes 2 and 3, rabbit anti-human fibronectin receptor antiserum; lanes 4 and 5, mouse anti-human VLA-1 monoclonal antibody (TS2/7). Lanes 1, 2, and 4 represent the parental SK-N-SH cells, and lanes 3 and 5 represent the peptide-resistant SK-N-SH cells. B: lanes 1 and 2, mouse anti-human VLA-2 monoclonal antibody; lanes 3 and 4, mouse anti-human VLA-3 monoclonal antibody (J143); lanes 5 and 6, rat anti-human β1 monoclonal antibody. Lanes 1, 3, and 5 represent the parental SK-N-SH cells, and lanes 2, 4, and 6 represent the peptide-resistant SK-N-SH cells. C: rabbit anti-human vitronectin receptor antiserum. Lane 1, parental SK-N-SH cells; lane 2, peptide-resistant SK-N-SH cells. Molecular weight (×10⁵) markers used were: myosin (200,000), phosphorylase b (94,000), bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (30,000).
peptide, GRGESP, did not result in any differences in the pattern of integrin expression as compared to the untreated cells. Immunoprecipitation with a monoclonal antibody against the common $\alpha_2$ subunit gave similar results, i.e., a specific overproduction of $\alpha_3$ subunit and decreased expression of the other $\alpha$ subunits in the GRGDSP-resistant SK-N-SH cells (Fig. 1B, lanes 5 and 6).

Immunoprecipitation with a monoclonal antibody against human VLA-1 demonstrated that the $\alpha_5$ subunit is indeed overproduced in the GRGDSP-resistant SK-N-SH cells (Fig. 1A, lanes 4 and 5). Utilizing monoclonal antibodies against the $\alpha_2$ and $\alpha_3$ subunits, we could demonstrate that both of these are present on SK-N-SH cells and that both are present in significantly lower amounts on the peptide-treated cells (Fig. 1B, lanes 1–4). In addition, immunoprecipitation with an anti-vitronectin receptor antiserum showed a considerable decrease in the amount of vitronectin receptor on the peptide-treated cells. We were unable to demonstrate the presence of the $\alpha_3$ subunit (utilizing an anti-$\alpha_3$ monoclonal antibody) on either of the cell lines.

These data indicate that selection of SK-N-SH cells capable of growing in the presence of an RGD-containing peptide results in an alteration in the amounts of each of the $\alpha$ subunits expressed. Of particular interest is that the $\alpha_3$ subunit is significantly overproduced, suggesting an important Arg-Gly-Asp-dependent function for this integrin in cell adhesion or other cellular phenotypes. To determine whether other GRGDSP-resistant neuroblastoma cell lines would also express this altered pattern of integrin expression, we carried out a similar selection process with IMR-32 and SK-N-MC neuroblastoma cell lines. Fig. 2A shows that the IMR-32 cells responded in a similar manner, with the peptide-treated cells specifically overproducing the $\alpha_1 \beta_1$ (VLA-1) complex as shown by immunoprecipitation of $^{125}$I-surface-labeled parental and peptide-treated IMR-32 cells with the anti-fibronectin receptor antiserum (Fig. 2A, lanes 1 and 2) and VLA-1 $\alpha$ monoclonal antibody (Fig. 2A, lanes 3 and 4).

The SK-N-MC neuroblastoma cells responded differently, however, in that peptide selection did not result in an overproduction of the $\alpha_3$ subunit, but rather of the heterogeneous $\alpha$ subunits with $M_r$ values between 140,000 and 150,000, together with an overproduction of the $\beta_1$ subunit (Fig. 2B).

One of the $\alpha$ subunits overproduced in these cells is $\alpha_5$ as determined by Northern blot analysis of total RNA utilizing $^{32}$P-labeled $\alpha_5$ cDNA (Fig. 3A, lanes 1 and 2), indicating an increase in the amount of $\alpha_5$ mRNA in these cells. A similar analysis of RNA from SK-N-SH and IMR-32 parental and peptide-treated cells demonstrated the presence of either low levels of $\alpha_5$ mRNA in these cells with a slight decrease or similar levels of this mRNA present in the peptide-resistant SK-N-SH and IMR-32 cells (Fig. 3A, lanes 3–6). Northern blot analysis of mRNA from IMR-32 parental and peptide-resistant cell lines utilizing $^{32}$P-labeled $\alpha_5$ cDNA indicated an increase in the level of $\beta_1$ mRNA in the peptide-resistant variants of this cell line (Fig. 3B), confirming the immunoprecipitation data (Fig. 2A). A similar increase in $\beta_1$ mRNA could not be detected in the SK-N-SH peptide-resistant cells, agreeing with the immunoprecipitation data which show that although there is an overproduction of the VLA-1 $\alpha$ subunit in the peptide-resistant SK-N-SH cells, the level of the $\beta_1$ subunit appears to be unchanged.

Fingermann and Hemler (20) have recently reported that the expression of VLA-1 is low and that of VLA-2 and VLA-3 is high in rapidly proliferating fibroblasts. Upon prolonged quiescence, this pattern of expression is reversed, whereby VLA-1 expression is increased and that of VLA-2 and VLA-3 is decreased. The RGD-resistant IMR-32 and SK-N-SH cells expressing high levels of VLA-1 and low levels of VLA-2 and VLA-3, however, are by no means quiescent and proliferate at a rate similar to that of the parental cell lines.
It would therefore appear that the inverse pattern of expression of VLA-1 and VLA-2/3 is not solely related to the proliferative status of the cells and that cell populations exist in which this pattern of integrin expression is a stable phenotype. It will be of interest to determine whether these variant cell lines express altered phenotypes, especially those associated with greater degrees of neuronal differentiation such as responsiveness to nerve growth factor and expression of neurotransmitters. We have observed that the RGD-resistant SK-N-SH cells have a different cellular morphology from that of the parental cells in that they are entirely composed of neuronal cells which express an extensive network of neurites. In addition, preliminary data indicate that both the SK-N-SH and IMR-32 RGD-resistant variants express higher amounts of transforming growth factor-β mRNA than their parental counterparts, an interesting observation since transforming growth factor-β has been shown to induce the expression of integrins in a wide variety of cell lines (21).

Both the parental and peptide-resistant SK-N-SH cells attached to type I collagen, fibronectin, and laminin (Fig. 4). However, the peptide-resistant SK-N-SH cells exhibited a weaker attachment (at least in the number of cells attached at a given ligand concentration) to laminin and type I collagen and a similar level of attachment to fibronectin (Fig. 4). The weaker attachment of the peptide-resistant SK-N-SH cells to type I collagen is consistent with the decreased expression of VLA-2, the putative collagen receptor (8), on these cells. The similar level of attachment of the variant cells to fibronectin correlates with the similar level of VLA-5 (fibronectin receptor) expression in the two cell lines (Fig. 3). Peptide-resistant IMR-32 cells also exhibited a similar decrease in the attachment to type I collagen as compared to the parental cells (data not shown). Consistent with the increased resistance of these cell lines to the GRGDSP peptide when plated onto tissue culture plastic, a higher concentration of GRGDSP peptide is required to inhibit attachment of the peptide-resistant cell lines than the corresponding parental cells.

The specific overproduction of VLA-1 in response to the GRGDSP peptide without an obvious increase in the attachment of the variant cells to any of the known integrin extra-
VLA-1 Overproduction