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Anoikis and Metastatic Potential of Cloudman S91 Melanoma Cells[1]

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ABSTRACT

Anoikis is a form of apoptosis induced in normal cells as a result of loss of their adhesion to substrate. In the present study, we have tested whether tumor cells are also sensitive to anoikis and whether selection of tumor cells for resistance to anoikis could increase their metastatic ability. *In vitro* cultured Cloudman S91 melanoma cells are strongly adherent to the plastic. Prevention of their adherence by rocking or by covering culture plates with polyhydroxyethylmethacrylate resulted in induction of anoikis and death of almost all cells. Their death was prevented in the presence of caspase inhibitor Z-Val-Ala-Asp-fluoromethyl ketone. To select anoikis-resistant cells, S91 cells floating in the culture medium were sequentially isolated and transferred for seven generations. As a result, a new subline of S91 cells capable of growing in free cell suspension was selected. These S91 nonadherent (S91Nadh) cells were completely resistant to anoikis and manifested higher metastatic ability than S91Adh cells. Anoikis resistance of S91Nadh cells was not attributable to their resistance to other apoptotic signals *in vitro*, and they showed no increase in their survival *in vivo* in the lungs after i.v. inoculation. Increased metastatic potential of the anoikis-resistant S91Nadh cells was associated with various phenotypic changes, including increased proliferation and loss of VLA-4 integrin expression because of down-regulation of the *VLA-49α* (*CD49d*) gene. In parallel, they showed a reduction in homotypic aggregation and binding to endothelial cells, increased Matrigel invasiveness, and decreased matrix metalloproteinase-2 and matrix metalloproteinase-9 activity that paralleled up-regulation of the *TIMP-1* gene. S91Nadh cells also manifested changes in cell surface carbohydrates, such as appearance of α-galactosyl epitopes as a result of up-regulation of the α1,3-galactosyltransferase gene and concomitant reduction in cell membrane sialylation. Thus, selection of S91 melanoma cells for anoikis resistance resulted in an increase in their metastatic potential in parallel with multiple alterations in their phenotypic properties.

INTRODUCTION

Numerous experimental data indicate that adhesion of various epithelial, endothelial, or muscle cell lines to substrate is crucial for their survival and proliferation. Prevention of their adhesion usually results in rapid cell death, even in the presence of all required nutrients and growth factors (1–3). This death is mediated via induction of apoptosis. Apoptosis induced by prevention of cell adhesion was termed anoikis ("homelessness" in Greek; Ref. 1).

Although these findings are based on the *in vitro* study of cultured cell lines, it is believed that anoikis also occurs *in vivo* and might have important biological significance. Cell adhesion to extracellular matrix provides an important survival signal that also might induce expression of various genes, leading to cell proliferation and differentiation. Cells that have lost such contact and detach from the tissue are programmed to die via induction of anoikis (1). Anoikis was probably an important evolutionary mechanism that emerged in multicellular organisms to maintain the integrity and function of various tissues and organs by elimination of cells that lost contact with the parental tissue or organ, thus preventing their survival and function in new anatomical locations. A basic rule for many cells in the body, then, is "attach or die." However, this is true only for most cells of epithelial origin, whereas migrating cells, such as blood cells, are anoikis resistant.

It is well documented that cell attachment is mostly mediated by integrins, although other cell adhesion molecules can be also involved (3, 4). Extracellular matrix-integrin interactions trigger intracellular signaling and activation of certain genes, leading to cell proliferation and differentiation (4). However, it is unclear how the apoptotic signal is triggered in cells in which integrin-ligand interaction was abrogated. It is believed that anoikis is not activated via ligand activation of CD95 or other death receptors (5, 6). Nonetheless, death receptor activation is somehow involved in anoikis. This conclusion is based on the findings that dominant-negative Fas-associated death domain protein as well as silencers of death domains efficiently inhibited anoikis (5, 6). It was found that anoikis is mediated via activation of caspases, particularly caspase-8 and caspase-3 (5). It was shown that inhibitors of apoptosis, crmA or bcl-2 and bcl-X_L, also inhibit anoikis (7). Some evidence also indicates that the endogenous galactoside-binding lectin, galectin-3, plays a role in inhibition of anoikis (8). It was demonstrated that the cytoplasmic tail of galectin-3 contains a four-amino acid motif (NWGR) that is also present in the BHI domain of the *bcl-2* gene, and it was found to be crucial for the antiapoptotic effect of both bcl-2 as well as galectin-3 (9).

Various protein kinases that are activated/inactivated during cell attachment/deattachment were implicated in the prevention or induction of anoikis. It was shown that integrin-ligand interaction leads to activation of focal adhesion kinase. Some data indicate that activation of focal adhesion kinase might prevent induction of anoikis (10). Similarly, attachment-mediated activation of phosphatidylinositol 3-kinase and protein kinase B/Akt protects cells from anoikis (11). In contrast, inhibition of phosphatidylinositol 3-kinase and protein kinase B/Akt results in an induction of anoikis (11). It was also found that disruption of the cultured cell-matrix interaction leads to activation of JNK and p38, a related stress-activated protein kinase (7). Possible involvement of these kinases in anoikis is suggested by experiments in which transfection and overexpression of JNK[4] and p38 resulted in anoikis (7), although some reports showed no correlation between activation of JNK and induction of anoikis (12). Recently, the importance of mitogen-activated protein kinase kinase kinase in anoikis has been demonstrated (13).

Anoikis has been mostly investigated using normal cultured cell lines. Normal cells usually fail to grow under anchorage-independent

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[4] The abbreviations used are: JNK, c-Jun NH_2-terminal kinase; PolyHema, polyhydroxyethylmethacrylate; PI, propidium iodide; mAb, monoclonal antibody; PE, phycoerythrin; SBA, soybean agglutinin; TIMP, tissue inhibitor of metalloproteinases; RT-PCR, reverse transcription-PCR; α1,3GT, α1,3-galactosyltransferase; Z-VAD-fmk, Z-Val-Ala-Asp-fluoromethyl ketone; WGA, wheat germ agglutinin; MMP, matrix metalloproteinase; VLA, very late activation antigen.

conditions in semisolid agar. In contrast, malignantly transformed cells are able to grow in semisolid agar (14). The ability of the transformed cells to grow anchorage independent in a semisolid agar became a classical assay for *in vitro* evaluation of malignant transformation of various cultured cell lines (14). Analysis of high and low metastatic tumor cell lines showed no correlation between their ability of anchorage-independent growth in semisolid agar and their metastatic potential (15). Anchorage-independent growth of malignant but not normal cells in semisolid agar might indicate that tumor cells are resistant to anoikis. However, the sensitivity of malignant cells to anoikis was not extensively investigated. Some data indicate that tumor cells could be sensitive to anoikis. Human melanoma cells lacking $\alpha_v\beta_3$ integrin showed a high level of apoptotic death when they were cultured in three-dimensional dermal collagen. Transfection of these cells with an α_v cDNA restored $\alpha_v\beta_3$ expression and prevented their deaths (16).

It was shown that prevention of B16F1 melanoma cells adhesion to plastic by culture them on PolyHema-coated flasks affects their cell shape and ability to form metastases (17). Anoikis in these cells was not tested, but melanoma cells survived and proliferated, although their doubling time increased from 16 to 24 h. B16F1 melanoma cells under this condition showed round morphology and grew in clusters as spheroids. B16F1 melanoma cells cultured on PolyHema-coated plates showed an increase in homotypic aggregation and a marked increase in their propensity to establish metastases. However, all of these changes were unstable and reversed 24 h after returning them to uncovered flasks (17).

Numerous studies demonstrated that integrin-substrate interactions are important for tumor cell arrest, migration, and growth in different anatomical locations and formation of distant metastases (3, 18–20). Tumor cells inoculated i.v. are usually eliminated quickly. About 0.1–0.01% of inoculated cells develop metastatic foci in the lungs (21). Although the mechanisms responsible for tumor cell destruction in the blood remain unclear, it was found that natural killer cells play a role in tumor cell elimination. In natural killer cell-depleted mice, tumor cell survival increased, but a vast majority of tumor cells were destroyed (22). During hematogenic migration, metastatic cells are unable to adhere, and under these conditions, they may die as a result of anoikis. Thus, tumor cells that are unable to survive would be unable to form distant metastasis. If so, selection of tumor cells for adhesion independence might increase their resistance to anoikis and their ability to survive in the blood and develop metastatic tumors in distant anatomical locations.

In the present study, we tested our hypothesis. To study the possible role of anoikis in metastasis formation, we selected a stable subline of Cloudman S91 melanoma with high resistance to anoikis. The phenotypic and metastatic properties of anoikis-sensitive and anoikis-resistant S91 melanoma cells have been investigated.

MATERIALS AND METHODS

Mice. Female DBA/2 and B6D2F$_1$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were kept in a specific pathogen-free facility at the University of Pittsburgh that is accredited for animal care by the American Association for Accreditation of Laboratory Animal Care. Mice 2–3 months of age were used in these experiments.

Tumor Cells. Cloudman S91 melanoma cells (a gift from Dr. Vincent Hearing, National Cancer Institute, NIH) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, streptomycin, and penicillin (all from Life Technologies, Inc., Gaithersburg, MD), These cells are strongly adherent to the plastic. Cells were usually transferred after a short trypsin-EDTA (Life Technologies, Inc., Gaithersburg, MD) digestion. To select a nonadherent S91 cell population, cells found floating in the cultured medium were centrifuged, pelleted, resuspended in 5 ml of complete RPMI 1640, and transferred into a T-25 culture flask. When cells in these flasks become saturated, floating cells were again harvested and transferred into a new T-25 culture flask. Such transfers of the floating cells were repeated for seven generations, when a stable subline of nonadherent S91 (S91Nadh) cells was established. These cells were always transferred as nonadherent cells that were found floating in the medium.

Anoikis Induction. To induce anoikis, cells were prevented from adhering to the plastic of the cultured flasks. This was achieved by two methods: rocking of the cells or culturing them in a dish coated with PolyHema. Adherent and nonadherent S91 cells (1×10^6 cells/3 ml of culture medium) were transferred into a Petri dish (60 mm). To prevent cell adhesion, dishes were placed on the platform of the Orbitron Rotator 260250 (Boekel Industries, Inc.) for 18 h at 37°C. The platform was set up at about 10° angle, and it moved at a speed of 30 cycles/min. Under these conditions, the dishes were gently rocked in different directions so that the constant movement of culture medium prevented cell adhesion to the plastic.

PolyHema coating was performed as described (8, 17). PolyHema (Sigma) was dissolved in 95% ethanol at a final concentration 120 mg/ml, diluted 1:10 in ethanol, and pipetted into a Petri dish (60 mm in diameter) at 0. 95 μl/mm^2. After drying, dishes were washed three times with PBS, and 1×10^6 cells in 3 ml of culture medium were transferred into the dish. The PolyHema-coated and uncoated dishes were cultured for 18 h at 37°C.

Apoptosis Assay. Apoptotic cells from PolyHema-coated or rocked dishes were analyzed using the Vybrant Apoptosis Assay kit #4 (Molecular Probes, Eugene, OR). This kit contains the green fluorescent YO-Pro-1 dye that can enter apoptotic but not normal viable cells. Necrotic cells were stained with PI. Cells were washed, resuspended in 0.5 ml of PBS, and 5 μl of the YO-Pro-1 and PI (diluted 1:10) were added. Cells were incubated for 20–30 min on ice and were analyzed by flow cytometry, measuring the fluorescence emission at 530 nm (FL1) and >575 nm (FL3).

Cell Proliferation. To test for tumor cell proliferation under normal and anoikis-inducing conditions, S91 adherent and nonadherent cells (1×10^6 cells/3 ml of medium/well) were plated into six-well plates that were coated or uncoated with PolyHema. The plates were cultured at 37°C under rocking conditions or under regular stationary conditions, and the number of tumor cells/plate was determined during 3–4 days of culture.

Cell Cycle Analysis. Adherent and nonadherent S91 cells (1×10^6) cultured under normal or rocking conditions were fixed in ethanol at −20°C. After washing, the pellet was incubated with RNase A (75 units/ml) at 37°C for 30 min. Cells were washed and resuspended in 0.5 ml of PBS containing 15 μg/ml of PI. Cell cycle distribution analysis was performed by flow cytometry and was analyzed using software Modlif LT (Verity Software House, Topsham, ME).

Cell Adhesion Assay. The murine endothelial cell line was originally established from the lungs by Auerbach *et al.* (23) and was kindly provided by Dr. C. Johnson (University of Pittsburgh Cancer Institute). Endothelial cells were distributed into 96-well plates (2×10^5/well), and 24–48 h later, S91 melanoma cells (4×10^5) were added into each well containing a monolayer of endothelial cells (four wells/group). After 45 min of incubation at 37°C, nonadherent cells were removed by vigorous washing three times with PBS containing 2% of FBS. Removed cells were plated into 24-well plates, incubated at 37°C for 2 h, and their numbers were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (24). The percentage of S91 cells adherent to endothelial cell monolayer was calculated:

$$\%\ \text{adherent S91 cells} = \frac{\text{Absorbance of total number of S91 cells} - \text{absorbance of the removed S91 cells}}{\text{Absorbance of total number of S91 cells}} \times 100$$

Homotypic Aggregation. Single-cell suspensions of S91 melanoma cells were resuspended in 0.5 ml of complete RPMI 1640 supplemented with 10% FCS (2.5×10^6 cells/per tube) and incubated at 37°C for 1 h with intermittent agitation. Some cells were incubated in the presence of PS/2 hybridoma supernatant containing anti-VLA-4α mAb (final dilution, 1:10). Cells were washed by adding 2 ml of complete RPMI 1640, and pellets were resuspended in 1 ml of RPMI 1640 with a large-bore plastic Pasteur pipette (the bulb was squeezed 10 times; Ref. 24). Single, nonaggregating cells were counted in a hemocytometer. The percentage of aggregated cells was calculated as ($1 - Ne$/

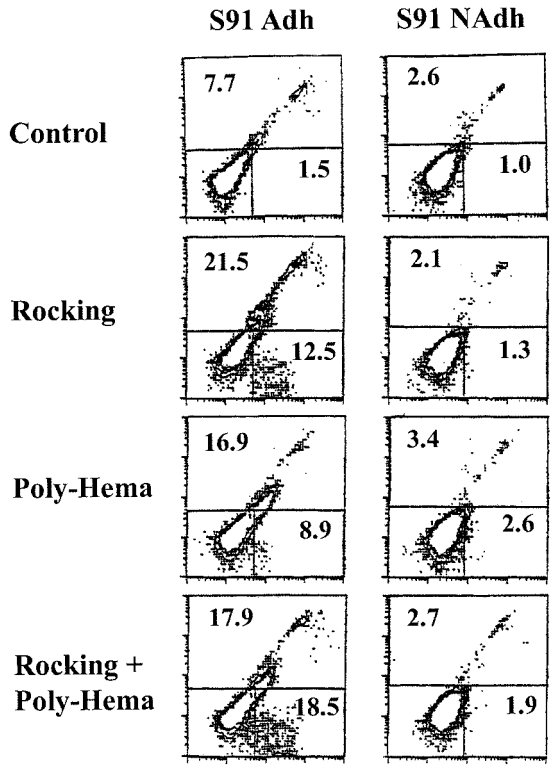

Fig. 1. Sensitivity of S91Adh and S91Nadh melanoma cells to anoikis. S91Adh and S91Nadh cells were cultured in the Petri dishes that were coated with PolyHema or dishes that were placed on the rocking platform. Cells were harvested 16 h later and stained using the Vybrant Apoptosis kit #4 containing YO-PRO-1 (FL1) and PI (FL2). Numbers of apoptotic and necrotic tumor cells were analyzed using flow cytometry.

$Nc \times 100$, where *Ne* is the number of single cells after incubation at 37°C and *Nc* is the number of single cells before incubation (24).

Flow Cytometric Analysis. To test MHC class I expression, S91 melanoma cells (5×10^5 cells/tube) were incubated with anti-H-2K^d (hybridoma 31-3-4) and anti-H-2D^d (hybridoma 34-5-8) for 30 min at 4°C, washed, and stained with antimouse IgG-PE as a second antibody (25). To analyze expression of α-galactosyl epitopes and SBA binding carbohydrates, S91 melanoma cells were incubated with the biotinylated GS1B4 or SBA lectin (10 μg/ml) for 30 min at 4°C, washed, and stained with avidin-PE. To evaluate the masking effect of sialic acid on expression of SBA binding carbohydrates, S91 melanoma cells were treated with 0.1unit/ml of neuraminidase type V from *Clostridium perfingens* (Sigma Chemical Co., St. Louis, MO) for 30 min, washed, and stained with biotinylated SBA lectin and avidin-PE (26, 27). Integrin expression by S91 melanoma cells was analyzed using the following anti-integrin antibodies: hamster antimouse VLA-1α (CD49a), hamster antimouse VLA-2α (CD49b; PharMingen, San Diego, CA), rat antimouse VLA-4α (CD49d) PS/2 mAb (American Type Culture Collection, Rockville, MD), and rat antihuman VLA-6α (CD49f; Amac, Inc., Westbrook, ME). After washing, cells were stained with species-specific secondary FITC-labeled antibodies. Analysis of fluorescence was performed on at least 5000 cells using a FACStar or FACStar-Plus flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA; Refs. 26 and 27).

Northern Blot Analysis. To test *TIMP-1* gene expression, total cellular RNA was prepared from cell lines using a RNA extraction kit (RNAzol B; Rel-Test, Inc., Friendswood, TX). The extracted RNA (10 μg/lane) was electrophoresed on 0.7% agarose-formaldehyde gel and transferred to nylon membranes and UV fixed before hybridization. The ^{32}P-labeled probe used for hybridization was the cDNA of TIMP-1 prepared by random priming (Life Technologies, Inc., Grand Island, NY) as described previously (24).

RT-PCR Analysis. To perform RT-PCR analysis of VLA-4 and α1,3GT message in S91 melanoma cells, total RNA was extracted as described (24). Four μg were subjected to a one-step RT-PCR reaction (Life Technologies, Inc., Grand Island, NY) using the VLA-4α specific primers and Taq polymerase (Life Technologies, Inc., Grand Island, NY). The primers used were: upstream 5′-GTCTTCATGCTCCCAACAGC-3′; and downstream 5′-ACTTCTGACGTGATTACAGGAAGC-3′ (19). PCR was run in the sequence of 94°C l min for denaturation, 55°C 1 min for annealing, and 72°C 1 min for extension with up to 30 cycles in total. Similarly, RT-PCR analysis of α1,3GT message was performed using the following primers: upstream 5′-GTGGTTGTCGTGTTTTGCGAATAT-3′; and downstream 5′-ATCTGAAGGCAGGCCTATCTGATA-3′. RT-PCR was also performed with the primers specific for the *β-actin* gene under the same conditions. After amplification, PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized with UV light (26).

***In Vitro* Invasion Assay.** Tumor cell invasiveness was tested using a modification of the Matrigel method (24, 28). Melanoma cells (5×10^4 in 0.2 ml of medium) were loaded on the Matrigel-coated polycarbonate membrane in the upper compartment of Transwells (Collaborative Research, Inc., Waltham, MA) in a 24-well plate. In the control group, melanoma cells were loaded into upper part of the inserts not coated with Matrigel. After 48 h, the nonpenetrating cells on the top of the filter were removed by "scrubbing" with a cotton-tipped swab. The membranes were cutoff and stained with the Diff-Quik stain, and the total number of invaded cells on the back of the filter was counted under the microscope. After removal of the inserts, cells that had penetrated the filters and sedimented to the bottom of the well were cultured

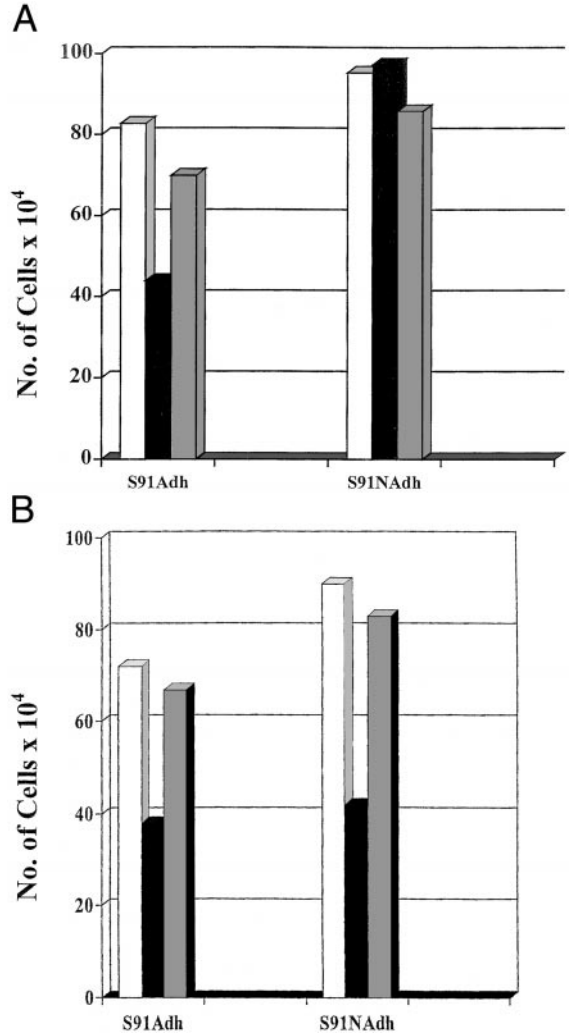

Fig. 2. *A*, inhibition of anoikis in S91Adh cells by the caspase inhibitor Z-VAD-fmk. S91Adh and S91Nadh melanoma cells ($5 \times l0^5$) were cultured for 16 h under normal stationary (□) or under rocking conditions in the presence (▨) or absence (■) of Z-VAD-fmk (100 nM). The numbers of viable melanoma cells/dish were determined. *B*, sensitivity of S91Adh and S91Nadh cells to WGA lectin-induced apoptosis. S91Adh and S91Nadh cells were cultured alone (□) or in the presence of WGA lectin (10 μg/ml; ■) under stationary conditions. Some cells were incubated with WGA and the caspase inhibitor Z-VAD-fmk (100 nM; ▨). After 16 h of incubation, the numbers of viable melanoma cells/dish were determined.

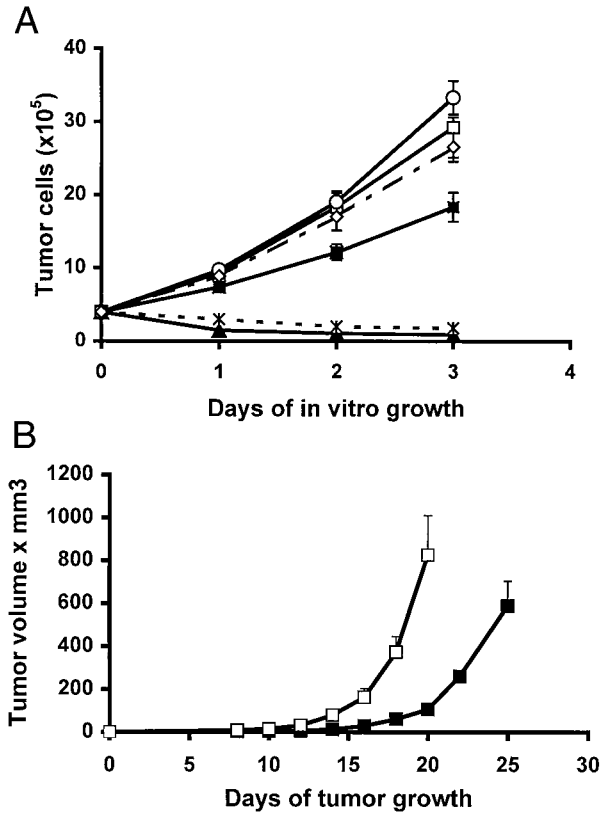

Fig. 3. *In vitro* and *in vivo* growth of S91Adh and S91Nadh melanoma cells. *A, in vitro* growth of S91Adh and S91Nadh melanoma cells under normal and anoikis-induced conditions. S91Adh and S91Nadh melanoma cells ($4 \times l0^5$) were distributed into six-well plates that were uncoated or coated with PolyHema. S91Adh (■, ▲) and S91Nadh (□, ◇) cells were cultured for 3 days under stationary or rocking conditions, respectively. S91Adh (×) and S91Nadh (○) cells were also cultured in PolyHema-coated plates. *Bars*, SE. *B, in vivo* growth of S91Adh and S91Nadh melanoma cells. S91Adh (■) and S91Nadh (□) cells were inoculated s.c. into DBA/2 mice (1×10^6 cells/mouse; five mice/group). Tumor growth was evaluated by measurement of tumor diameters three times a week, and total tumor volume was calculated as width^2 × length × 0.52. Differences in tumor diameters are significant ($P < 0.01$). *Bars*, SE.

for an additional 48 h, and then they were stained and photographed. The percentage of Matrigel invasion was:

$$\% \text{ invasion} = \frac{\text{Number of cells that traverse Matrigel-covered filter}}{\text{Number of cells that traverse uncovered filers}} \times 100$$

Gelatinase Activity of S91Adh and S91Nadh Melanoma Cells. Melanoma cells were cultured to near-confluence in complete RPMI 1640. The cell monolayers were rinsed with PBS and cultured with serum-free RPMI 1640 for 24 h. Supernatants were collected and centrifuged to remove floating cells and cell debris. Gelatinase activity of tumor cell supernatants was tested as described previously (24). The protein concentration in each conditioned medium was determined by Bradford's protein assay (Bio-Rad, Richmond, CA). Protein-standardized samples were resolved in 10% SDS-PAGE containing 1 mg/ml gelatin (Sigma). For this purpose, gelatin was solubilized in water by warming to 60°C while stirring. After gel electrophoresis, incubation with 2.5% Triton X-100 for 30 min was followed by enzymatic degradation of the substrate in a buffer containing 0. 1 M Tris-HCl (pH 8.0), 5 mM $CaCl_2$, and 0.04% NaN_3 for 48 h at 37°C. Gels were stained with 2.5% Coomassie Blue for 30 min and destained with methanol:acetic acid:water (40:10:50). To verify equal protein loading in each lane, parallel gels were silver stained (Silver Stain Plus kit; Bio-Rad, Hercules, CA).

***In Vivo* Survival of S91 Melanoma Cells.** S91Adh and S91Nadh melanoma cells were labeled with [^{111}In]Ox (Amersham Life Science Inc., Arlington Heights, IL) as described previously (29). Tumor cells (10×10^6/ml) were incubated with 10 μCi of [^{111}In]Ox for 10 min at room temperature in complete RPMI 1640. The cells were then washed three times in 15 ml of medium, counted, and resuspended at 1×10^6 cells/ml. Radiolabeled cells were inoculated i.v. into DBA/2 mice (2.5×10^5 cells/mouse); 5 min, 5 h, and 24 h after tumor cell inoculation, five mice/time point were sacrificed, and lungs were removed. The level of radioactivity remaining in the lungs was detected using the gamma counter (29).

Metastatic Properties of Melanoma Cells. DBA/2 or B6D2F_1 mice were inoculated i.v. with 1×10^6 S91 adherent and nonadherent cells (five mice/group). Lungs were harvested 30 days later, and metastatic foci in the lungs were counted under a dissecting microscope (26). The experiments were repeated twice. The significance of the differences between numbers of metastases was determined by the Mann-Whitney *U* test.

RESULTS

Cloudman S91 melanoma cells growing *in vitro* are strongly adherent to plastic with very few floating nonadherent cells. A population of nonadherent S91 cells capable of growing in suspension was selected by sequential transfer of floating S91 melanoma cells. Although in the first passages the vast majority of cells grew again as strongly adherent cells with only a few floating cells, after seven rounds of transfer of nonadherent cells, a new cell subline had been established that was able to grow in suspension. Plastic nonadherent S91 cells were round, and only a minority of cells were lightly attached to the plastic, and these cells were easily removed by shaking of the flask without treatment with trypsin-EDTA. The new subline was termed S91 nonadherent (S91Nadh) and was maintained continuously by transferring only the floating cells. The parental adherent S91 cell line (hereafter termed S91Adh) was transferred after trypsin-EDTA digestion. We compared the ability of S91Adh and S91Nadh cells to survive under conditions when their adhesion was prevented. Culture of S91Adh cells on a rocking platform for 18 h resulted in induction of apoptosis in 12.5% cells, whereas control (nonrocking) cells had an incidence of only 1.5% apoptotic cells. Some apoptotic cells lost membrane integrity and were stained with PI; therefore, the number of PI-positive cells also increased from 7.7 to 21.5% (Fig. 1). Similarly, prevention of cell adhesion in PolyHema-coated, six-well plates also resulted in an increase of apoptosis of S91Adh cells. However, some cells growing on PolyHema formed clumps and survived in these clumps. When PolyHema-coated plates were rocked, clumping of cells was prevented, and the number of apoptotic S91Adh cells increased further (Fig. 1). In contrast, no changes in survival of S91Nadh cells were observed when these cells were cultured under rocking conditions and/or on PolyHema (Fig. 1).

These data indicate that S91Adh cells are sensitive to anoikis and S91Nadh cells are resistant to anoikis. The survival of rocked S91Adh cells substantially increased in the presence of caspase inhibitor Z-VAD-fmk (100 μM; Fig. 2*A*), indicating the involvement of caspases in the death of these cells. We next tested whether selection of S91Nadh cells for resistance to anoikis was associated with resistance to other apoptotic signals. We showed previously that WGA lectin is highly efficient for inducing apoptosis in various normal and malignant cells (17). S91Adh and S91Nadh cells showed equal sensitivity to the apoptotic effects of WGA (10 μg/ml). Survival of these cells equally increased in the presence of the caspase inhibitor Z-VAD-fmk (Fig. 2*B*). Similarly, both sublines were equally sensitive to the apoptotic effect of VP16 (data not shown). Thus, resistance of S91Nadh cells to anoikis was not a result of a general blockage of the apoptotic cascade.

It is possible that anoikis resistance of S91Nadh cells is associated with an increase in the proliferative survival signal. To test this, we cultured S91Adh and S91Nadh for 3 days under normal or anoikis-induced conditions. As shown in Fig. 3*A*, under normal culture conditions S91Nadh cells manifested a significantly higher rate of proliferation than S91Adh cells. Similarly, S91Nadh cells inoculated into

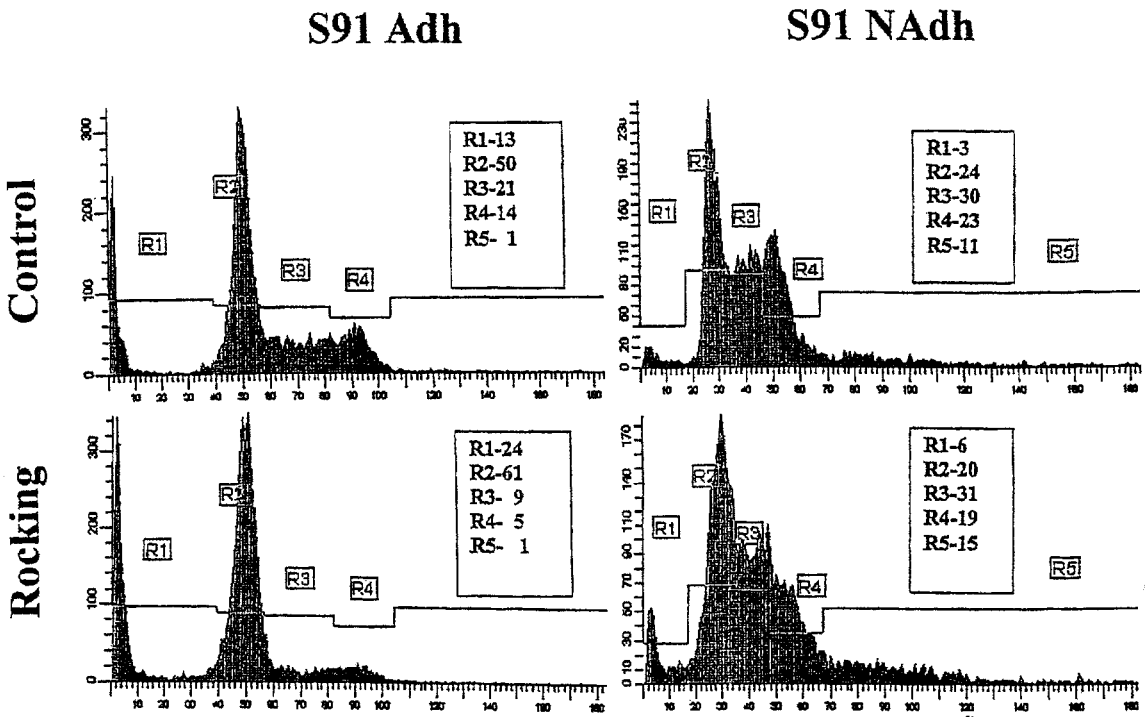

Fig. 4. Cell cycle analysis of S91Adh and S91Nadh melanoma cells under normal and anoikis-induced conditions. S91Adh and S91Nadh cells were cultured under rocking and stationary conditions for 16 h. Cells were fixed and stained with PI. Flow cytometric analysis of DNA contents was performed, and the percentage of cells with different DNA contents was determined using Winlist software (Verity Software House; see *insets*). The percentage of cells in the different phases of the cell cycle was determined using Modfit software (Verity Software House).

DBA/2 mice showed a higher rate of tumor growth than S91Adh cells (Fig. 3*B*). Prevention of cell adhesion by rocking or PolyHema-coated culture dishes did not significantly affect growth of S91Nadh cells, whereas growth of S91Adh cells was prevented completely. In fact, their numbers actually reduced, and very few survived (Fig. 3*A*).

These data indicate that resistance to anoikis in S91Nadh cells was associated with increase of the proliferative signals and probably attributable to changes in their cell cycles. Several studies suggest that cell cycle regulation is associated with sensitivity to anoikis (8, 30, 31). Therefore, we performed cell cycle analysis of S91Adh and S91Nadh cells under normal or anoikis-induced conditions (Fig. 4). When adhesion of cells was prevented by constant rocking of the plates, the percentage of apoptotic (hypodiploid cells, R1 region) in S91Adh cells increased from 13 to 24%. Analysis of the cell cycle status of the remaining nonapoptotic cells was performed using Modfit software (Verity Software House). The results indicate that S91Adh cells have more cells in G_0-G_1 phase than S91Nadh cells (53.7 and 29.6%, respectively), whereas S91Nadh cells had higher proportion of cells in G_2 + M phase (33.3%) than S91Adh cells (10.16%). These differences in cell cycle might be responsible for the observed differences in their cell proliferation. Under anoikis-induced conditions, the vast majority (82.5%) of rocking S91Adh cells was blocked in G_0-G_1 phase, with concomitant reduction of cells in S (from 36.2 to 12%) and G_2 + M phase (from 10.2 to 5.5.%). Rocking of S91Nadh cells showed no change in the proportion of cells in G_0-G_1 phase and an increase up to 65.1% of cells in the S-phase. Thus, disruption of S91Adh cell adhesion to the substrate induced blockage of transition from the G_1 to S-phase that was not observed in anoikis resistance of S91Nadh cells.

We next investigated whether selection of S91 melanoma cells for anoikis resistance affected their metastatic potential. We found that S91 melanoma cells have a very low metastatic ability. To produce experimental metastases, a relatively high number of cells needed to be injected i.v. Even after inoculation of 1×10^6 S91Adh cells, three of five mice still did not develop grossly visible metastatic tumors in the lungs, whereas the remaining two mice had three and nine nodules/lung. In contrast, S91Nadh cells induced significantly more ($P < 0.05$) metastatic nodules in all inoculated mice, ranging from 6 to 36 nodules/lung. No metastases have been found in other organs (Table 1). These data demonstrate that selection of S91 for resistance to anoikis was associated with a significant increase in their ability to form metastasis.

This increase in the metastatic ability of anoikis-resistant S91Nadh cells might be attributable to increase in their ability to survive in the blood. To test this hypothesis, melanoma cells were labeled with [^{111}In]Ox because these cells showed low labeling with [^{125}I]UDR. Previously, we showed that labeling of tumor cells with [^{111}In]Ox or [^{125}I]UDR gives similar results in testing tumor cell clearance or tumor cell survival in the lungs (29). After i.v. inoculation of 2.5×10^5 radiolabeled S91Adh and S91Nadh cells (80,333 and 77,179 cpm, respectively), very fast elimination of these cells was observed, and no differences in survival of S91Adh and S91Nadh cells in the lungs were found. After 5 h of inoculation, both S91Adh and S91Nadh lines had ~4% of cells in the lungs (3276 ± 430 and

Table 1 *Metastasis formation by anoikis-sensitive S91Adh and anoikis-resistant S91Nadh melanoma cells*

Tumor cells[a]	Median no. of metastases (range)	% metastasis free mice
S91Adh	0 (0–9)	60
S91Nadh	19 (6–36)[b]	0

[a] S91Adh and S91Nadh 1×10^6 cells were inoculated i.v. into B6D2F$_1$ mice (five mice/group). Lungs were harvested 30 days later, and metastatic foci were counted under a dissecting microscope. The results of one of two repeated experiments are included in the Table.

[b] Differences between groups are significant ($P < 0.05$) according the Mann-Whitney test.

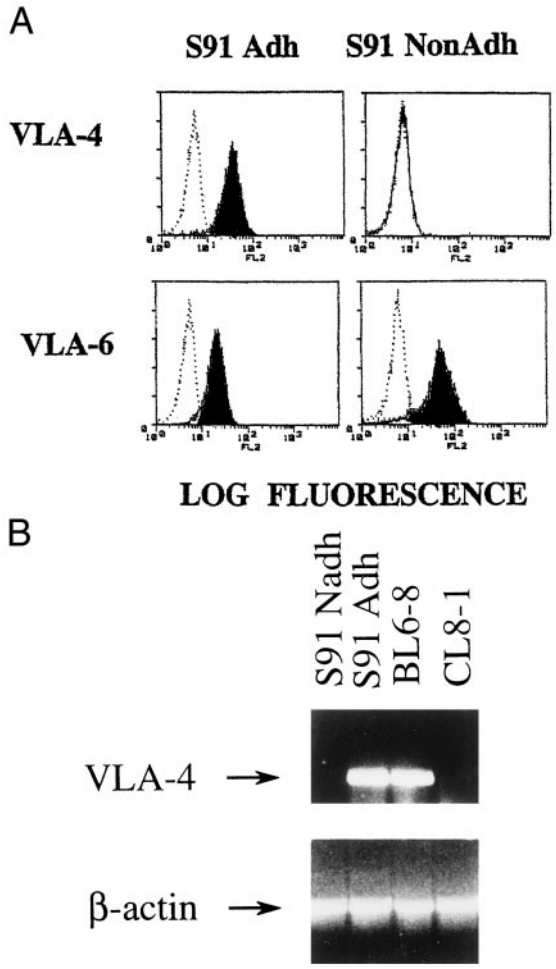

Fig. 5. Loss of VLA-4 expression by S91Nadh melanoma cells. *A*, S91Adh and S91Nadh melanoma cells were incubated with anti-VLA-4α and anti-VLA-6α mAb and washed and stained with anti-rat-IgG-PE-second antibody. Cells were analyzed using a FACStar-Plus flow cytometer. *B*, RT-PCR analysis of the VLA-4α message in S91Adh and S91Nadh melanoma cells. RNA was extracted from S91Adh and S91Nadh cells. RNAs from BL6-8 and CL8-1 melanoma cells were used as a positive and negative control, respectively. After amplification, PCR products were separated by 1.5% agarose electrophoresis and stained with ethidium bromide.

3164 $\pm$ 178 cpm, respectively). At the 24-h time point, only 1% of inoculated S91Adh or S91Nadh remained in the lungs (1200 $\pm$ 304 and 939 $\pm$ 206 cpm, respectively). Because these cells are different in their susceptibility to anoikis, these results indicate that resistance to anoikis did not increase survival of S91Nadh cells in the blood. It is most likely that anoikis was not induced, because shortly after entering the blood, S91Adh cells were trapped in the capillary bed of the lungs and quickly adhered to the endothelial cells that prevented anoikis in these cells.

Thus, cell survival in the blood cannot be responsible for the observed increase in metastatic ability of S91Nadh cells. Therefore, we investigated other mechanisms that might be responsible for the observed differences in the metastatic potentials of these cells. Because anoikis resistance of S91Nadh cells was associated with a loss of cell adhesion and because integrins play a key role in cell adhesion to various substrates as well as in metastasis formation (2, 3), we analyzed the expression of various integrins by S91Adh and S91Nadh cells. Neither S91Adh nor S91Nadh cells expressed VLA-1α and VLA-2α (data not shown). Both sublines expressed VLA-6, and the only detectable difference in expression of integrins was found for VLA-4, where S91Nadh cells lost expression of VLA- 4α (Fig. 5*A*). RT-PCR analysis revealed that S91Adh cells express VLA-4α (CD49d), whereas no message for VLA-4α was found in S91Nadh cells (Fig. 5*B*). Thus, loss of adhesion of S91Nadh cells was associated with a loss of VLA-4α (CD49d) that was attributable to down-regulation of *VLA-4α* gene expression.

Some experimental data indicate that VLA-4-ligand interactions might be involved in metastasis formation (28). Several VLA-4 ligands have been identified such as fibronectin, vascular cell adhesion molecule-1 as well as VLA-4α (CD49d) subunit. VLA-4 is a heterodimer consisting of α4 (CD49d) and β1 (CD29) units (32). Therefore, VLA-4-positive cells could adhere to each other via VLA-4α/β1 interaction with VLA-4α on another cells, leading to homotypic and heterotypic adhesion or aggregation. Such aggregation and formation of the clumps in the blood stream might increase tumor cell survival, capillary embolization, and metastasis formation (33). Therefore, we compared the ability of S91Adh and S91Nadh cells to form homotypic aggregation. S91Adh cells showed a high level of homotypic aggregation that was significantly blocked by anti-VLA-4α mAb. S91Nadh cells showed minimal aggregation, and this was not significantly affected by anti-VLA-4 mAb (Fig. 6*A*). When the ability of S91 sublines to adhere to endothelial cells was analyzed, it was found that S91Adh cells effectively bind to endothelial cells, and this bind-

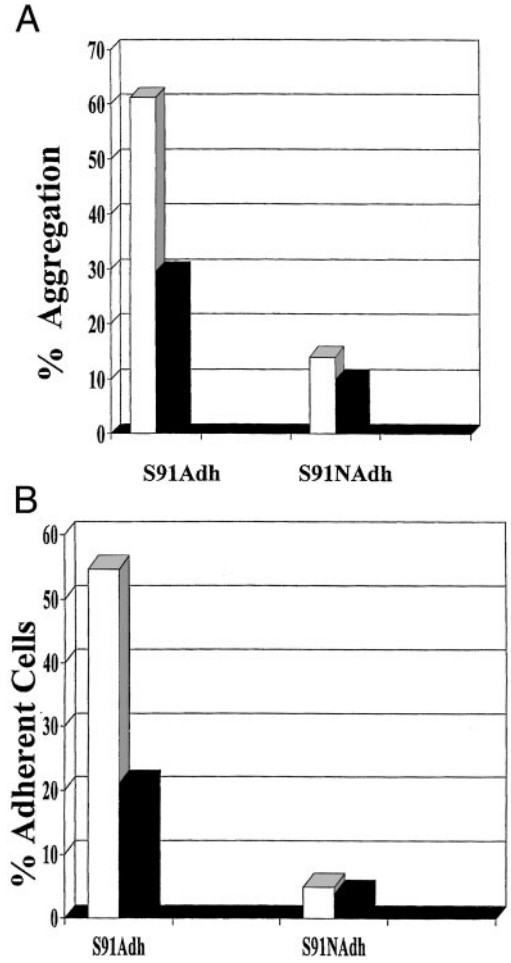

Fig. 6. Homotypic aggregation (*A*) and endothelial cell adhesion property (*B*) of S91Adh and S91Nadh melanoma cells. *A*, single cell suspensions of S91Adh and S91Nadh cells were incubated at 37°C for 1 h in the absence (□) or presence (■) of PS/2 hybridoma supernatant containing anti-VLA-4 mAb (final dilution, 1:10). After centrifugation, cells were resuspended, and the percentage of cells in aggregates was calculated. *B*, S91Adh and S91Nadh cells were added to the monolayer of murine endothelial cells in the absence (□) or presence (■) of anti-VLA-4 mAb. After 45 min of incubation at 37°C, nonadherent cells were removed, and the percentage of melanoma cells adherent to endothelial cells was determined. Results represent a summary of three repeatable experiments.

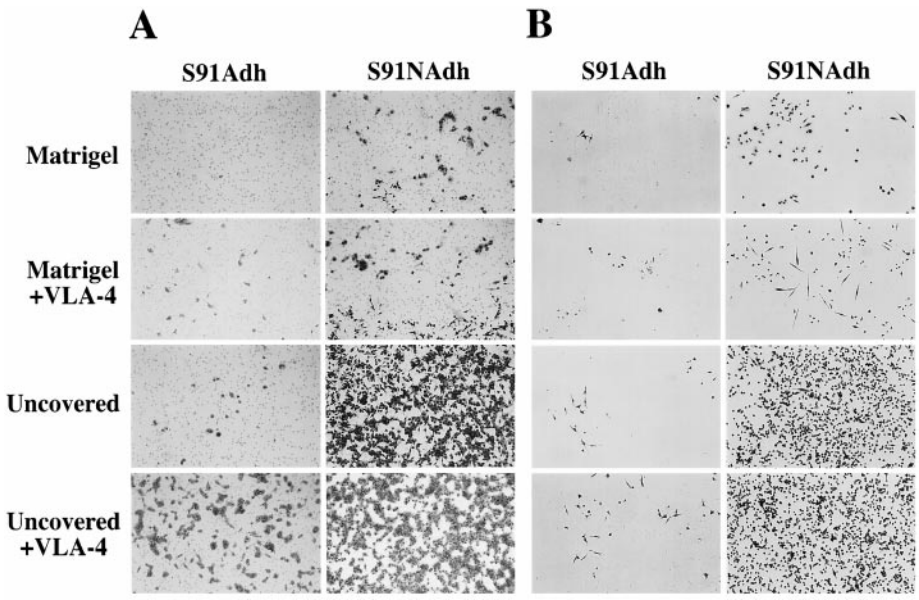

Fig. 7. Invasive properties of S91Adh and S91Nadh melanoma cells. *A*, S91Adh and S91Nadh cells were loaded on the Matrigel coated and uncoated polycarbonate membrane in the upper compartment of Transwell 24-well plates. The cells were cultured in the presence or absence of anti-VLA-4 mAb. After 48 h of culture, noninvading cells on the top of the filter were removed, the membranes were cutoff, and cells on the back of the membrane were stained with Diff-Quik stain. *B*, after removal of the inserts, cells that penetrated the filters and sedimented to the bottom of the well were cultured for additional 48 h and then were stained.

ing was reduced in the presence of anti-VLA-4α mAb. In contrast, S91Nadh cells showed a low property for adhesion to endothelial cells (Fig. 6*B*).

Adhesion of tumor cells to endothelial cells and extravasation are important steps in metastasis development (33). Therefore, we compared the invasive properties of S91Adh and S91Nadh melanoma cells using the Matrigel invasive assay. Melanoma cells were placed on the top of uncoated membranes and membranes coated with Matrigel Basement Membrane Matrix. After 48 h of culture in 24-well plates, cells that penetrated Matrigel and traversed the pores in the membrane were stained and counted. Very few S91Adh cells were able to invade Matrigel and cross the membrane, whereas S91Nadh cells invaded the Matrigel in higher numbers (Fig. 7*A*; Table 2). S91Nadh cells were also more efficient in traversing uncoated membranes. Calculation of the percentage of invasion revealed that only 6.5% of S91Adh *versus* 16% of S91Nadh cells were able to invade Matrigel-coated membranes. Anti-VLA-4 mAb significantly increased the ability of S91Adh cells to traverse Matrigel-coated and uncoated membranes. As a result, 33% of S91Adh cells were able to invade Matrigel. Anti-VLA-4 mAb had no significant effect on invasive properties of S91Nadh cells (Fig. 7*A*; Table 2). Some of the invaded cells detached from the back of the filter into the bottom of the well. After removal of the inserts, these cells were allowed to grow for an additional 48 h. The number of S91Nadh cells that reached the bottoms of the wells was higher than for S91Adh cells (Fig. 7*B*). Therefore, it seems that invasion of the Matrigel-coated filters depends not only on the ability of tumor cells to digest Matrigel but also on their motility, particularly their ability to move and cross the pores in the membrane. Thus, the observed differences in the Matrigel invasion could be explained, at least in part, by the higher ability of S91Adh cells to adhere to Matrigel, and their adherence reduced their motility and ability to traverse the membrane. Incubation of S91Adh cells with anti-VLA-4 mAb prevented their adhesion and thus increased their ability to traverse Matrigel-coated and uncoated membranes.

The observed differences in Matrigel invasion between S91Adh and S91Nadh cells might also be attributed to the differences in their proteolytic activity. The ability of tumor cells to digest basement membrane matrix depends on the production of MMPs (34). We analyzed MMP activity in the supernatants from S91Adh and S91Nadh cells using gelatin as a substrate in the zymograms. S91Nadh cells, when compared with their adherent counterparts (S91Adh cells), showed a marked decrease in their enzymographic profiles (Fig. 8). Specifically, reduction in the bands corresponding to gelatinase A (MMP-2) at M_r 70,000, gelatinase B (MMP-9) at M_r 93,000, Matrilysin (MMP-7) at M_r 28,000, and collagenase 3 (MMP-13) or stromelysins (MMP-3 or MMP- 10) at M_r 55,000 was found. Taking into account the results of the zymogram, it is possible to conclude that low invasiveness of S91Adh cells assessed by Matrigel assay was not attributable to their low MMP enzymatic activity but probably was a result of their high adhesiveness and low mobility. In contrast, S91Nadh cells had lower MMP enzymatic activity but higher mobility that resulted in their higher invasiveness of the Matrigel-coated membranes.

The activity of MMP-2 and MMP-9 is largely controlled by TIMP-1 (35). Therefore, we investigated whether the differences in MMP activity in S91Adh and S91Nadh cells are attributable to the differences in their expression of TIMP-1. Previously, we showed that the *TIMP-1* gene is not expressed in BL6 melanoma cells (24). Using Northern blot analysis, we found that S91Adh melanoma cells similar to BL6 melanoma cells, do not express *TIMP-1* gene. In contrast, S91Nadh cells showed a high level of TIMP-1 expression (Fig. 9). These results suggest that lower gelanolytic activity in the supernatant of S91Nadh cells might be attributed to TIMP-1 expression in these cells.

We have found previously that various melanomas (BL6, JB/RH,

Table 2 *Invasion of Matrigel-coated and Uncoated Membranes by S91Adh and S91Nadh melanoma cells*[a]

Tumor cells	No. of traversed cells (+SE)		% invasion
	Matrigel coated	Matrigel uncoated	
S91Adh	6.3 ± 1.3[b]	98.5 ± 4.8[b]	6.5[b]
S91Adh + αVLA-4	42.4 ± 2.9	127.6 ± 3.8	33.0
S91Nadh	77.7 ± 1.6	485.7 ± 94.6	16.0
S91Nadh + αVLA-4	90.0 ± 4.0	575.9 ± 111.3	17.0

[a] Melanoma cells (5×10^4/0.2 ml) were loaded on the uncoated or Matrigel-coated polycarbonate membrane in the upper compartments in Transwell 24-well plates (three wells/group). Cells were cultured 48 h. In some groups, cells were cultured with anti-VLA-4 mAb (final dilution of PS/2 hybridoma supernatant, 1:10). The noninvaded cells on the top of the filter were removed by "scrubbing" with a cotton-tipped swab. The membranes were cut off and stained with Diff-Quik stain, and the total number of invaded cells on the back of the filter was counted under the microscope. The percentage of invasion was calculated as described (see "Materials and Methods").

[b] Significant difference ($P < 0.05$) from other groups.

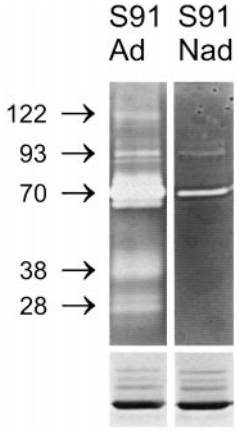

Fig. 8. Enzymatic activity of S91Adh and S91Nadh melanoma cells. Serum-free supernatants of S91Adh (*Ad*) and S91Nadh (*Nad*) cells were resolved in 10% SDS-PAGE containing 1 mg/ml gelatin. After gel electrophoresis, enzymatic degradation of the substrate was performed for 48 h at 37°C. *Lower gel*, supernatants were electrophoresed in 10% SDS-PAGE and then silver stained to verify equal protein loading in the zymogram.

and JB/MS) express a low level of MHC class I molecules and do not express SBA and $GSIB_4$ lectin-binding carbohydrates that might affect the metastatic ability of tumor cells (26, 36). Therefore, we compared the expression of MHC class I molecules and cell surface carbohydrates in S91Adh and S91Nadh melanoma cells. The data presented in Fig. 10*A* show that S91Adh cells express a very low level of H-$2K^d$ and a higher level of H-$2D^d$ molecules. The expression of these molecules was lower in S91Nadh cells. Similar to other murine melanomas, S91Adh melanoma does not express SBA and $GSIB_4$ lectin-binding carbohydrates. However, in S91Nadh cells, high levels of these carbohydrates were found (Fig. 10*A*). The ligand for $GSlB_4$ lectin has been shown previously to be the carbohydrate structure Galα1–3Galβ1–4GlcNAc-R (termed the α-galactosyl epitope; Ref. 37). This epitope, which is abundant on mouse normal and malignant cells, is synthesized in the Golgi apparatus by the enzyme α1,3GT (38). It seems that differences in expression of α-galactosyl epitopes in S91Adh and S91Nadh cells might be attributable to the differences in expression of the *α1,3GT* gene. Indeed, our RT-PCR analysis showed that S91Adh cells do not contain message for *α1,3GT*, whereas in S91Nadh cells selected for resistance to anoikis in the *α1,3GT* gene was up-regulated (Fig. 10*B*).

We have found previously that in BL6 melanoma cells, the SBA binding sites are actually present, but they are blocked by sialic acid and can be detected after neuraminidase treatment (26). When S91Adh cells were treated with neuraminidase, it resulted in unmasking of SBA binding carbohydrates (Fig. 10*C*). S91Nadh cells express SBA-binding carbohydrates, and their expression was further increased after neuraminidase treatment. These data indicate that S91Adh cells are heavily sialylated, and sialic acid completely masks SBA lectin-binding carbohydrates in these cells. In contrast, S91Nadh cells are less sialylated and express SBA binding carbohydrates (Fig. 10*C*).

DISCUSSION

Our data indicate that S91 melanoma cells are sensitive to anoikis. We were able to select a permanent subline, S91Nadh, that is resistant to anoikis. The resistance to anoikis of these cells was not attributable to a blockage in the apoptotic pathway but was associated with an increase in proliferation signal and the percentage of cells in S and G_2 + M phases of the cell cycle. It was demonstrated previously that prevention of adherence of normal cells leads to a block in cell cycle progression from late G_1 to S that was attributable to down-regulation of cyclin A (30). Under anoikis-induced conditions, similar blockage was found in S91Adh but not in anoikis-resistant S91Nadh cells.

Selection of S91 melanoma cells for anoikis resistance was associated with an increased metastatic potential. It is of note that this increase in metastasis formation was not directly attributable to the increase in survival of anoikis-resistant S91Nadh cells after i.v. inoculation. Tumor cells entering into the blood trigger hemostatic cascade, resulting in a fibrin deposition, platelet aggregation around tumor cells, and embolization of the small blood vessels that allow tumor cell adhesion to the endothelium (21). All of these events could prevent anoikis. Indeed, our data showed that anoikis-sensitive S91Adh and anoikis-resistant S91Nadh melanoma cells had a similar rate of elimination from the blood, suggesting that anoikis is not responsible for tumor cell elimination. Tumor cell survival in the blood is an important step in the metastatic cascade, but their further ability to extravasate and proliferate in a new environment is also important for metastasis formation (21). Thus, the increased metastatic potential of S91Nadh cells could be attributed to some phenotypic changes that emerged in these cells as a result of selection for anoikis resistance. Selection of S91 cells for nonadherence resulted in a loss of VLA-4 integrin expression, decrease in homotypic aggregation, adhesion to endothelial cells, MMP-2 and MMP-9 activities, increase in Matrigel invasiveness, TIMP-1 expression, up-regulation of the *α1,3GT* gene and α-galactosyl epitopes, and reduction in cell membrane sialylation. Numerous experimental data showed that these phenotypic changes are very important for the metastatic ability of tumor cells (17, 28, 34–36, 39).

It is unclear whether the anoikis-resistant cells with altered phenotypic properties preexisted or were induced during selection for independence on substrate adhesion. It is difficult to completely prove or disprove this. However, we were unable to isolate VLA-4-negative and α-galactosyl-positive cells from S91Adh cells using flow cytometric cell sorting. In addition, RT-PCR analysis of S91Adh cells showed no message for the *α1,3GT* gene that could be detectable if α1,3GT-positive cells preexisted. These results support the possibility that the genetic and phenotypic changes were induced in cells selected for resistance to anoikis. It was shown that cell adhesion triggers the expression of various genes (2–4). It is possible that in the cells capable of survival without adhesion to the substrate, another set of genes is activated. Therefore, numerous phenotypic changes have

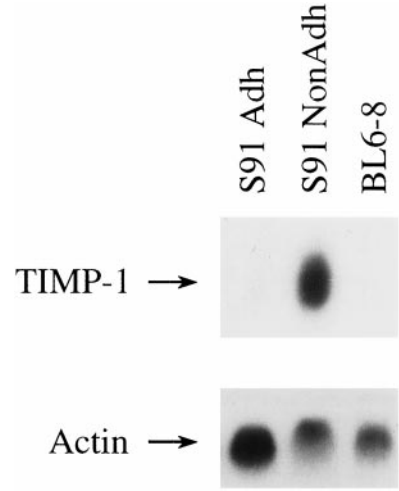

Fig. 9. Northern blot analysis of TIMP-1 expression in S91Adh and S91Nadh cells. Total RNA was extracted from S91Adh, S91Nadh, and BL6-8 melanoma cells. The extracted RNAs were electrophoresed, transferred to nylon membranes, and hybridized with the ^{32}P-labeled TIMP-1 cDNA probe.

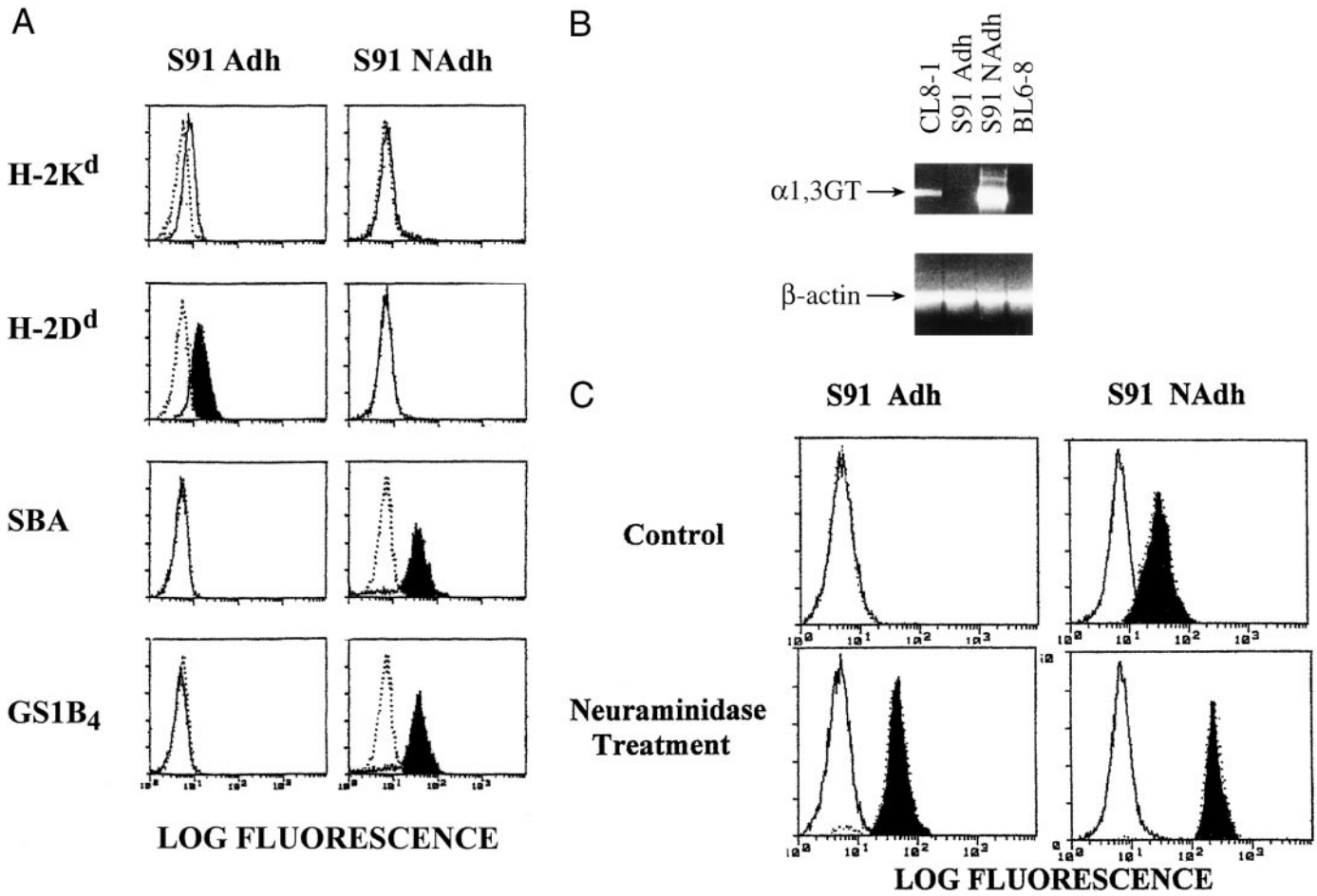

Fig. 10. MHC class I and cell surface carbohydrate expression by S91Nadh cells selected for resistance to anoikis. *A*, S91Adh and S91Nadh melanoma cells were incubated with anti-H-2K^d and anti-H-2D^d mAb and washed and stained with antimouse IgG-PE second antibody. To analyze cell surface carbohydrate expression, cells were incubated with biotinylated GS1B4 and SBA lectins and washed and stained with avidin-PE. *B*, RT-PCR analysis of the α1,3GT message in S91Adh and S91Nadh cells. RNAs from BL6-8 and CL8-1 melanoma cells were used as a negative and positive control, respectively. *C*, expression of SBA-binding carbohydrates after neuraminidase treatment of S91Adh and S91Nadh melanoma cells.

been found in anoikis-resistant cells. Some of these changes affected the metastatic potential of anoikis-resistant S91Nadh cells.

It remains to be determined whether loss of VLA-4 is crucial for loss of adherence and prevention of anoikis. However, it is likely that the loss of VLA-4 is probably responsible for the observed decrease in homotypic aggregation and adhesion to endothelial cells. Consistent with our findings, it was found that B16a melanoma cells that do not express VLA-4 also showed higher Matrigel invasiveness than VLA-4-positive B16 melanoma cells (18). Transfection of B16a cells with the *VLA-4α* gene reduced their invasiveness, and blocking VLA-4 by anti-VLA-4 mAb increased invasiveness of B16 melanoma cells (18). We believe that the increased *in vitro* invasiveness of the VLA-4-S91Nadh cells is most likely based on their low adhesion and increased mobility rather than a result of their increased proteolytic activities. In fact, poorly invasive VLA-4-positive S91Adh cells have higher proteolytic activity and produced more MMP-2 and MMP-9 as well as other MMPs than did highly invasive VLA-4-S91Nadh cells.

Previous analysis of the possible involvement of VLA-4 in metastasis formation gave quite controversial results. Depending on the experimental model, VLA-4 expression decreased or increased metastasis formation (28, 40, 41). Although in our study higher metastatic ability of S91Nadh cells was associated with loss of VLA-4 expression, we found concomitant changes in other phenotypic properties in these cells that might affect their metastatic ability.

In summary, our data indicate that anoikis resistance of S91Nadh melanoma cells did not provide them with an increased survival in the blood. It might be that time of free circulation of tumor cells in the blood as nonadherent cells is too short to trigger anoikis. Tumor cells adhere quickly to the endothelium, which could prevent anoikis. Selection of S91 cells for anoikis was associated with various phenotypic changes. Most of these changes coincide with rather than directly determine the anoikis resistance. However, some of these changes might be responsible for the observed increased metastatic ability of S91Nadh melanoma cells.

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