Enhanced Nectin-1 Expression and Herpes Oncolytic Sensitivity in Highly Migratory and Invasive Carcinoma

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Abstract

Purpose: Although a variety of malignant tumors are susceptible to therapy with oncolytic herpes simplex viruses, the determinants of tumor sensitivity to these viruses are poorly understood. Nectin-1 is a cell surface adhesion molecule that is a component of intercellular adherens junctions and also functions as a herpes viral receptor. Because highly invasive cells may have decreased intercellular adhesion, we sought to determine if such cells might also have altered availability of cell surface nectin-1 to act as a herpes receptor. Experimental Design and Results: A series of squamous cell carcinoma lines of increasing migratory and invasive potential, termed MG1-MG14, were selected by serial passages of murine SCC7 through Matrigel invasion chambers. Available cell surface nectin-1 was enhanced on the MG11 and MG14 cell lines in comparison to SCC7 as measured by cellular ELISA and immunofluorescence microscopy. A replication-competent, oncolytic herpes virus (NV1023) showed an increased ability to enter MG11 and MG14 cells as compared with SCC7 cells. Furthermore, MG11 and MG14 supported increased herpes viral replication and cytotoxicity over SCC7. For all three of the cell lines, viral entry assays revealed that the actively migrating cells were significantly more susceptible to herpes infection than the nonmigrating cells. Conclusions: These results show that malignant cells with highly migratory and invasive properties may exhibit increased cell surface nectin-1 availability, which may serve as a herpes viral receptor to enhance the efficacy of herpes oncolytic therapy. This finding has implications regarding patient selection for future clinical trials using these promising therapeutic vectors.

Attenuated, replication-competent oncolytic viruses based on herpes simplex type 1 (HSV-1) have potent therapeutic effects in treating a variety of human malignancies. Studies have shown therapeutic efficacy in animal models of brain, breast, colorectal, prostate, ovarian, lung, pancreatic, gastric, esophageal, and head and neck cancers (1–9). Recent phase 1 clinical trials have shown encouraging early results and suggest that these viruses may be safe for clinical application (10–12). Although many different malignancies can be effectively treated with oncolytic HSV in animal models, the specific determinants that define tumor sensitivity to herpes viral therapy remain poorly understood. We examined a series of attenuated, oncolytic HSV and have repeatedly observed that they possess a greater affinity for malignant cells over normal cells in vivo (13–15). We have also noted a degree of variability in the sensitivity of different malignant cell lines to infection, replication, and lysis by these herpes viruses (8, 9). These observations suggest that there are tumor-related factors which determine sensitivity to herpes viral infection, replication, and oncolysis. In this study, we sought to examine the expression of a herpes viral receptor as a potential determinant of herpes viral sensitivity.

In the natural life cycle of HSV-1, a critical step for the successful infection of any cell by a viral particle is its initial attachment and entry into the cell. This process is mediated by glycoproteins that project from the outer viral envelope. HSV glycoproteins B and C initially attach to heparan sulfate proteoglycans that are ubiquitous on cell surfaces. HSV glycoprotein D then forms a critical interaction with cell surface receptors to mediate viral fusion and entry into the cell. There are three described cell membrane receptors that permit HSV-1 fusion and entry: nectin-1, herpes viral entry mediator, and sites in heparan sulfate modified by 3'-O-sulfotransferases (16). A recent study performing a direct comparison of receptors suggested that nectin-1 is more efficient at promoting viral entry than herpes viral entry mediator for a variety of HSV strains (17).

Interestingly, nectin-1 also serves as an important cell surface adhesion molecule. Nectin-1 interacts with afadin to form a component of intercellular adherens junctions that connect adjacent cells with each other (18). E-cadherin and β-catenin form the other main component of adherens junctions. It
is well-established that some malignant cells exhibit impaired E-cadherin activity, leading to dysfunction of intercellular adherens junctions, decreased cell adhesion, and increased invasive and metastatic abilities (19). It is plausible to hypothesize that highly invasive, malignant cells with dysfunctional adherens junctions might have alterations in surface nectin-1 availability. This concept was recently proposed by Yoon and Spear, who elegantly showed that the disruption of adherens junctions of Madin-Darby canine kidney cells through calcium-depletion liberated nectin-1 localized at these junctions and enhanced their availability to serve as herpes receptors (20). In this study, we hypothesize that highly invasive and migratory cells with decreased cell adhesiveness might similarly show enhanced availability of cell surface nectin-1 to serve a herpes viral receptor.

To address this question, a series of progressively more migratory and invasive squamous cell carcinoma lines were generated based on the murine SCC7 squamous carcinoma cell line. After validating their differing migratory and invasive abilities, we selected the most highly migratory and invasive lines for examination of nectin-1 surface expression and sensitivity to herpes cytotoxicity, viral replication, and viral entry using an attenuated, replication-competent, oncolytic herpes virus (NV1023). We have previously described the construction of NV1023 and its related vectors, and have shown its ability to effectively treat a variety of malignancies. The highly migratory and invasive MG11 and MG14 cell lines displayed enhanced nectin-1 availability and increased susceptibility to herpes viral oncolysis by NV1023.

**Materials and Methods**

**Cell lines.** The murine SCC7 cell line (developed by H. Suit, Harvard University, Boston, MA) is a rapidly dividing squamous cell carcinoma line. SCC7 cells were grown in vitro in minimal essential medium (MEM) containing 10% FCS. African green monkey kidney cells (Vero cells, American Type Culture Collection, Manassas, VA) for viral plaque assays were grown in MEM containing 10% FCS and 1% penicillin and streptomycin. Cells were maintained in a 5% CO₂-humidified incubator at 37°C.

**Virus.** NV1023 is an attenuated, replication-competent, oncolytic herpes virus whose construction has been described in detail (21, 22). NV1023 was derived from R7020, an HSV-1 vector originally designed as an HSV-1/2 vaccine candidate (23, 24). NV1023 carries a 5.2 kb fragment of HSV-2 DNA (containing HSV-2 genes US2-2 to US2-5) inserted in the UL5/J junction. NV1023 is attenuated by a 15 kb deletion in the inverted repeat region that extends from the 3' end of UL5/J to the promoter for ICP4, thus deleting UL5/E and one copy of the diploid genes ICP0, ICP4, and the γ234.5 neurovirulence gene. NV1023 also contains the Escherichia coli β-galactosidase gene (LacZ) inserted at the US10-12 locus to serve as a marker of infection. NV1023 was provided by MediGene, Inc. (San Diego, CA).

**Generation of MG1-MG14.** A series of cell lines with increasing invasive abilities were generated by serial passages of SCC7 cells through Matrigel invasion chambers (BD Biosciences, San Jose, CA). SCC7 cells (5 × 10⁴) were added into the chamber insert (8 μm pore, six-well plate size) in 2 mL of MEM without FCS and placed in a six-well plate containing 2.5 mL of MEM with 10% FCS in the bottom chamber to act as a chemoattractant. Cells were allowed to invade through the Matrigel barrier, migrate across the porous polyethylene terephthalate membrane, and detach to fall to the bottom of the well. Cells that were recovered from the well bottom were then trypsinized, expanded in cell culture, and then reseeded into a new invasion chamber for serial passages. Cells completing one passage were termed MG1. Serial passages were done until 14 passages (MG14) were completed.

The thickness of the Matrigel barrier in the insert was progressively increased with later passages, to present a greater challenge for the invasive cells. An additional volume of cold, liquid Matrigel (BD Biosciences) was evenly layered over the Matrigel chamber inserts and allowed to solidify at room temperature. The following increasing volumes of Matrigel were added for each of the MG lines: MG1-2 (no Matrigel added), MG3-5 (15 μL Matrigel added), MG6 (30 μL), MG7 (45 μL), MG8 (60 μL), MG9 (70 μL), MG10 (200 μL), MG11-14 (300 μL).

**Cell line morphology and proliferation.** We selected MG lines at varying passage intervals for characterization. To assess for differences in individual cell morphology and in patterns of growth at confluence, cell lines were grown in T-175 flasks at low and high density for phase contrast microscopy. Proliferation rates were measured for SCC7 and the selected MG lines to determine if the selection process resulted in alterations in replication ability. SCC7, MG3, MG7, MG11, and MG14 were seeded onto six-well plates at 6 × 10⁴ cells per well in 3 mL of MEM with 10% FCS. Wells were trypsinized, stained with trypan blue, and cells were counted at daily intervals to determine the number of viable cells. At day 4, all wells underwent replacement with fresh medium. All samples were assessed in triplicate.

**Migration and invasion assays.** Migration and invasion assays were done on SCC7, MG3, MG7, MG11, and MG14 to validate differences in migratory and invasive function of the cell lines resulting from the selection process. For migration assays, polyethylene terephthalate, 8 μm pore, transparent inserts (Falcon, BD Biosciences) were used in 24-well plates. For invasion assays, Matrigel invasion chambers (BD Biosciences) were used in 24-well plates. Cells (2.5 × 10⁴ for migration, 5 × 10⁴ for invasion) were added into each of the inserts in 0.5 mL volume of MEM without FCS. Below the inserts in each of the wells, 0.75 mL of MEM with 10% FCS was added as a chemoattractant.

For migration experiments, the polyethylene terephthalate inserts were removed at 16 hours. For invasion experiments, the Matrigel inserts were removed at 44 hours. The nonmigrating or noninvading cells from the superior aspect of the membranes were first wiped off using a cotton swab. The circular membranes were then excised using a scalpel. The migrating or invading cells present on the undersurface of the membrane were fixed in 100% alcohol, stained with 1% methylene blue in 1% borax, and mounted on glass slides. Cells were then quantified by counting stained cells in 10 high power fields at predetermined areas on the membrane.

**Nectin-1 immunofluorescence.** SCC7, MG11, and MG14 cells (1 × 10⁴ per mL) were seeded on four-well, slide chamber tissue culture plates. Twelve hours later, cells were washed once with PBS, fixed with 100% methanol at −20°C for 5 minutes, and washed again with PBS. Nonspecific binding was blocked by incubation with 1% bovine serum albumin in PBS for 5 minutes at 37°C. Cells were then washed with PBS for 5 minutes at room temperature. A polyclonal chicken, anti-nectin-1 primary antibody was generously provided by Dr. Patrick Spear (Northwestern University, Chicago, IL). Cells were incubated with primary antibodies or saline as a control at 37°C for 25 minutes at a 1:500 dilution. Cells were washed with PBS for 15 minutes at room temperature and incubated with a green fluorescent, secondary goat anti-chicken IgG antibody (Alexa Fluor 488, Molecular Probes, Eugene, OR) at 37°C for 25 minutes at a 1:1,000 dilution. Cells were washed with PBS for 15 minutes at room temperature, incubated with 4’,6-diamidino-2-phenylindole (5 μg/mL) for 5 minutes and washed with PBS thrice × 5 minutes. Slides were mounted and examined by confocal microscopy (LSM510, Zeiss).

**Nectin-1 cellular ELISA.** SCC7, MG11, and MG14 cells were seeded in 96-well plates at 1 × 10⁴ cells per well in 100 μL of medium. Cells

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were incubated overnight, washed in PBS, and then fixed with 2% formaldehyde and 0.2% glutaraldehyde for 10 minutes. Separate wells were trypsinized and counted to allow for cell number adjustments. Cells were washed and then incubated with chicken anti-nectin-1 primary antibody (1:500) for 30 minutes at 37°C. Cells were washed and then incubated with a biotinylated anti-chicken IgG secondary antibody (1:1,000, Chemicon International, Temecula, CA) on a rocker for 30 minutes at room temperature. Cells were washed and then incubated with Amplex streptavidin-conjugated horseradish peroxidase (1:20,000, Amersham Biosciences, Piscataway, NJ) for 30 minutes at room temperature. Substrate consisting of 3,3',5,5'-tetramethylbenzidine in a phosphate-citrate buffer with sodium perborate was added (T-5525, P4922, Sigma-Aldrich, St. Louis, MO). Plates were read on a spectrophotometer at 370 nm. At least six samples were done for each cell line.

**Viral entry assays.** NV1023 expresses β-galactosidase, permitting the detection of successfully infected cells using lacZ assay. SCC7, MG11, and MG14 cell lines were plated at a density of 2 × 10^4 cells per well in 12-well plates in 2 mL medium. After incubation for 6 hours, NV1023 (100 µL) was added to each well at a multiplicity of infection (MOI) of 1 or 5. At varying intervals, cells were washed with PBS, fixed with 1% glutaraldehyde for 5 minutes, and again washed thrice with PBS. Cells were then stained for 4 hours in 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (1 mg/mL) in an iron solution of 5 mmol/L K₄Fe(CN)₆, 5 mmol/L K₃Fe(CN)₆, and 2 mmol/L MgCl₂. Cells were washed and plates were examined and photographed by light microscopy. The percentage of blue staining cells for each cell line was calculated by counting stained and unstained cells for a minimum of nine high-power fields per well.

**Viral proliferation.** For viral proliferation studies, SCC7, MG11, and MG14 cells were plated at 2 × 10^4 cells per well in 12-well plates in 2 mL medium. After incubation for 6 hours, NV1023 (100 µL) was added to each well at a multiplicity of infection (MOI) of 0.1 [2 × 10^3 plaque-forming units (pfu)]. Supernatant samples from each of the wells were collected daily and frozen. Vero cells were grown to confluence on six-well plates. Supernatant samples were then thawed, and serial 10-fold dilutions of the samples were prepared. Varying dilutions of supernatant were then incubated on confluent Vero cells for 4 hours. Wells were gently washed with medium and covered with 1% agarose with medium. After 48 hours of incubation, 2 mL of neutral red solution (2% by volume) was added and viral plaques were counted after an additional 24 hours of incubation. Resulting viral plaques were counted. All samples were analyzed in triplicate.

**Cytotoxicity assays.** SCC7, MG11, and MG14 cells were plated at 2 × 10^4 cells per well in 12-well plates in 2 mL medium. After incubation for 6 hours, NV1023 (100 µL) was added to each well at an MOI of 0, 1, and 5. Viral cytotoxicity was tested at daily intervals. On day 4, 1 mL of fresh medium was added to wells to allow viable cells to grow. Cells were washed with PBS and lysed with Triton X 1% to release intracellular lactate dehydrogenase, which was quantified from these cell lysates with a Cytotoxicity 96 kit (Promega, Madison, WI) at 450 nm on a spectrophotometer (EL321e, Bio-Tek Instruments, Winooski, VT). With this assay, lactate dehydrogenase is detected by the conversion of a tetrazolium salt into a red formazan product that is measured with a spectrophotometer (25, 26). The amount of color detected is directly proportional to the number of viable cells that were lysed by Triton X. Results are expressed as the percentage of surviving cells. This percentage was determined by comparing the measured lactate dehydrogenase of each infected sample relative to control, untreated, cell samples which were considered 100% viable. All samples were analyzed in triplicate.

**Viral entry assays of actively migrating cells.** Combined migration and viral entry assays were done on SCC7, MG11, and MG14 to examine differences in viral susceptibility for actively migrating cell lines. Polyethylene terephthalate, 8 µm pore transwell inserts (Falcon, Becton Dickinson) were used in 24-well plates. Cells (2.5 × 10^5) were added into each of the inserts in 0.5 mL volume of MEM without FCS. Below the inserts in each of the wells, 0.75 mL of MEM with 10% FCS was added as a chemoattractant. NV1023 (100 µL) was added into each insert at a MOI of 5. The NV1023 virus is able to freely pass through the 8-µm membrane pores and infect either nonmigrating cells on the superior surface, or migrating cells on the inferior surface of the insert membrane.

At 12 hours, all inserts were removed. To determine migrating cell sensitivity to viral entry, the nonmigrating cells were removed from the superior surface of the polyethylene terephthalate insert using a cotton swab and the membrane was then excised with a scalpel. To determine nonmigrating cell sensitivity to viral entry, the migrating cells were removed from the inferior surface of the polyethylene terephthalate insert using a cotton swab and the membrane was excised. Excised membranes were then placed in 24-well plates and fixed with 1% glutaraldehyde for 5 minutes, and again washed thrice with PBS. Membranes were then stained for 4 hours in 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (1 mg/mL) in an iron solution of 5 mmol/L K₄Fe(CN)₆, 5 mmol/L K₃Fe(CN)₆, and 2 mmol/L MgCl₂, as previously described. A minimum of nine high-powered fields per well were examined by microscopy and the percentage of blue staining cells was calculated by counting stained and unstained cells for both migrating and nonmigrating cell populations. All samples were done in triplicate.

**Results**

**Cell line morphology and proliferation.** The morphology of the MG lines showed a gradually increasing spindle-cell appearance with higher MG passage. Whereas SCC7 cells appeared mostly oval or round (Fig. 1A), MG11 and MG14 cells exhibited a progressively more narrowed, elongated, and spindle-shaped appearance (Fig. 1B and C). When grown in tissue culture flasks to maximal confluence, SCC7 cells grew together densely, with adjacent cells crowded tight together (Fig. 1D). In contrast, confluent MG11 and MG14 cells grew in more open patterns, leaving small circular areas and gaps between cells, and showing a decreased ability to come into full contact with each other (Fig. 1E and F).

Proliferation rates for SCC7, MG3, and MG7 were similar, without measurable statistical differences. By day 5, SCC7 showed a 91-fold increase from the starting cell number (6 × 10⁴ cells), and both MG3 and MG7 exhibited an 85-fold increase. MG11 showed only a slightly lower proliferation rate as compared with SCC7, with a 73-fold increase in cell number (P < 0.05, t-test). MG14 showed the lowest proliferation rate with a 60-fold increase (P < 0.05, t-test; Fig. 1G).

**Migration and invasion assays.** Migration and invasion assays were done to validate anticipated functional differences across the selected MG cell lines. Experiments were done in triplicate, and one representative experiment is shown. Data are presented as the average number of migrating or invading cells per high-powered microscopic field, with the SE. Migratory function was measured by the ability of cells to cross a porous polyethylene terephthalate membrane under attraction using FCS as an attractant. SCC7 (48.2 ± 6.5), MG3 (79.1 ± 7.5), MG7 (100.7 ± 10.5), MG11 (167.7 ± 18.5), and MG14 (284.4 ± 21.4) all showed a smooth, gradual trend towards an enhanced migratory ability with higher MG passage (Fig. 2A and C).

Invasive function was measured by the ability of cells to cross a thin Matrigel barrier and a porous polyethylene terephthalate membrane using FCS as an attractant. There...
was a trend towards increasing invasive ability with increasing MG passage. SCC7 (98.9 ± 9.0), MG3 (133.8 ± 11.9), MG7 (282.3 ± 27.8), and MG11 (305.2 ± 23.1) all show increasing invasiveness, with a mild decline by MG14 (207 ± 16.1; Fig. 2B and D). The greatest functional increase from SCC7 in both migration and invasion seemed to exist for MG11 and MG14. We therefore selected these three cell lines for further investigation.

**Nectin-1 immunofluorescence and cellular ELISA.** Nectin-1 immunofluorescence microscopy revealed sporadic, relatively mild expression by the parent SCC7 cell line. In contrast, the MG11 and MG14 cell lines showed a more consistent, homogeneous, and more intense level of Nectin-1 expression on cell surfaces (Fig. 3A-D). We noted that the spindle morphology of MG11 and MG14 was lost and cells became round during the fixation process. To quantitate differences in surface nectin-1 expression between these cell lines, we did cellular ELISA studies. This confirmed significant differences in the expression of nectin-1. The mean absorbance of SCC7 (0.538 ± 0.007) was significantly lower than MG11 (1.064 ± 0.018) and MG14 (1.087 ± 0.019), \( P < 0.05 \) for both comparisons (Fig. 3E). These studies show that surface nectin-1 expression was significantly increased in the MG11 and MG14 cell lines.

**Viral entry assays.** Because nectin-1 functions as a herpes viral receptor, we sought to determine if the herpes oncolytic vector NV1023 would show differences in its ability to enter SCC7, MG3, MG7, MG11, and MG14. LacZ assays were done to assess for successful viral entry. At an MOI of 1, only 19% of cells stained blue by day 6, in contrast to 100% of MG11 and 99% of MG14 cells. Similarly, at an MOI of 5, only 57% of cells stained blue by day 4, in contrast to 100% of MG11 and 96% of MG14 cells. MG11 and MG14 supported significantly more efficient viral entry as compared with SCC7 (Fig. 4).

**Viral proliferation assays.** Viral titer experiments showed significantly enhanced viral proliferation within the MG11 and MG14 cell lines as compared with SCC7. At day 6 following viral infection, NV1023 titers in MG11 cells reached an average of \( 8.5 \times 10^5 \) pfu, and in MG14 reached \( 6.8 \times 10^5 \) pfu. These represent a 425-fold and 340-fold increase in viral titer from the initial starting NV1023 dose of...
2 × 10³ pfu. In contrast, SCC7 cells supported viral proliferation to just 2.2 × 10⁴ pfu, representing only an 11-fold increase (Fig. 5A).

**Cytotoxicity assays.** Viral cytotoxicity assays were done to assess the sensitivity of SCC7, MG11, and MG14 to NV1023-induced oncolysis. At an MOI of 5 on day 3, only 9.4% of MG11 and 10.5% of MG14 cells remained viable. In contrast, 68.3% of SCC7 cells remained viable at these conditions (Fig. 5B). At later time points, SCC7 cells did eventually succumb to viral cytotoxicity at MOI 5 by day 5. Similarly, at an MOI of 1 on day 6, only 8.5% of MG11 and 4.3% of MG14 cells remained viable. In contrast, 89% of SCC7 cells remained viable at these conditions.

**Viral entry assays of actively migrating cells.** Actively migrating SCC7, MG11, and MG14 underwent lacZ staining to determine differences in susceptibility to herpes viral entry. As expected, the MG11 and MG14 lines showed a higher overall proportion of migrating cells in comparison to SCC7, as previously shown. Interestingly, the proportions of migrating or nonmigrating cells that permitted viral entry were similar for all three of the cell lines. Migrating cells displayed nearly complete viral infection (Fig. 6). Migrating SCC7 showed 91% successful viral entry, whereas migrating MG11 and MG14 showed 100% successful viral entry. In contrast, the nonmigrating cells displayed a significantly lower susceptibility to viral entry. Nonmigrating SCC7 showed just 33% viral entry, whereas non-migrating MG11 and MG14 showed 35% and 28% viral entry, respectively.

**Discussion**

HSV have long been known to possess a natural ability to infect neoplasms. A French study published in 1922 reported on the ability of herpes virus to naturally propagate within epithelial tumors (27). In 1949, Moore described the ability of HSV to infect and reduce the size of malignant tumors implanted in mice (28). Initial interest in applying HSV as a cancer therapy was diminished by observations of viral neurotoxic effects in animal studies. However, in recent years, techniques allowing for the development of attenuated HSV have offered the possibility of safer clinical application and have generated renewed interest in HSV as a form of cancer therapy. Attenuated, replication-competent, oncolytic herpes viruses have shown an ability to infect and lyse a wide variety of different malignant tumors in preclinical studies. Several early clinical trials using oncolytic HSV for brain and colorectal cancers have recently reported encouraging safety data (10–12).

These oncolytic HSV are attenuated by specific deletions within the viral genome. Some of these deletions may promote viral selectivity for malignant cells over normal cells. For example, the G207 virus has a deletion in HSV ribonucleotide reductase and therefore relies on host cell ribonucleotide reductase for viral replication (1). Because ribonucleotide reductase is generally present in higher amounts in rapidly dividing cells, HSV replication may occur preferentially in malignant cells. Both G207 and the HSV1716 virus have deletions in both copies of the HSV

![Fig. 2. Differences in migration (A) and invasion (B) abilities for SCC7, MG3, MG7, MG11, and MG14 were measured to validate the selection process for the MG cell lines. Representative photographs are shown of stained cells that successfully migrated across a porous polyethylene terephthalate membrane (C) or invaded across a Matrigel barrier and then crossed a polyethylene terephthalate membrane (D). There was a strong trend towards increased migratory and invasive ability with higher MG passage.](image-url)
The γ34.5 gene, which facilitates viral utilization of host cell protein production (10, 11). This double deletion decreases the neurovirulence of these vectors, and may also enable preferential replication of herpes virus within malignant cells.

Our group has studied a series of herpes viruses attenuated by the deletion of one copy of the γ34.5 gene (21). These viruses are based on the R7020 (NV1020) virus that was originally designed as a potential vaccine and was found to be tolerated in owl monkeys at doses 10,000-fold higher than lethal doses of wild-type HSV (24). We have repeatedly observed the ability of these viruses to selectively infect and lyse malignant tumor cells while sparing normal adjacent cells (13, 14). When injected i.v. in mice, these viruses infect pulmonary carcinoma nodules while sparing adjacent normal alveolar cells (13). No toxic effects were noted in these animals, and subsequent organ sections failed to show any cytopathic effects 2 weeks after high dose treatment.

Because the ribonucleotide reductase gene and one copy of the γ34.5 gene remain intact in these HSV, these observations have led us to consider the possibility that there might be factors other than viral attenuation to account for their selectivity for malignant tumors. We hypothesize that there may be innate factors to malignant tumors that make them susceptible to HSV. Such an idea would also support historical observations of natural tumor affinity by wild-type HSV. The current study offers one theory by demonstrating that nectin-1 expression may be enhanced on cells with greater invasive and migratory capabilities, leading to enhanced viral susceptibility.

Cell surface nectin-1 expression forms an intriguing link between malignancy and herpes sensitivity. Nectins are cell surface glycoproteins and members of the immunoglobulin superfamily. These cell adhesion molecules interact with each other at intercellular adherens junctions to engage in cell-cell interactions.
adhesion (18). The loss of function by intercellular adherens junctions is associated with an invasive phenotype in cells with dysfunctional E-cadherin (19). Interestingly, nectin-1 also serves as a cellular HSV receptor that permits viral fusion and entry. A recent study by Yoon and Spear elegantly demonstrated that cell surface nectin-1 availability is dependent on the status of intercellular adherens junctions (20). Although herpes viral particles are not normally able to access the nectin-1 engaged within intact adherens junctions, the disruption of intercellular adherens junctions led to an enhanced surface availability of nectin-1, and an enhanced susceptibility to herpes viral infection. Based on these findings, our hypothesis was that highly invasive and migratory cells might also display enhanced nectin-1 availability, which may serve as a receptor for herpes viral oncolysis.

There is evidence linking the function of other nectins with cell motility. Takai and his colleagues have shown that complex interactions between nectins on adjacent cells can signal a variety of intracellular events. Secondary signaling molecules (Cdc42 and Rac) induced by nectin interactions can regulate the dynamic formation or disruption of cell adherens junctions, reorganize the actin cytoskeleton, and affect cell motility (29). In addition, nectin-like molecules such as necl-5 have been found to directly regulate cell migration and cell adhesion (30). Nectins and nectin-like molecules therefore engage in complex interactions that are linked to cell motility.

We generated a family of squamous cell carcinoma lines by serial selection through Matrigel invasion chambers of increasing barrier thickness. This resulted in a series of cell lines with a spectrum of increasing migratory and invasive abilities. Migratory and invasive function was noted to be greatest for MG11 and MG14. A higher proportion of cells were migratory for the MG11 and MG14 cell lines as compared with SCC7, as previously demonstrated (data not shown).

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**Fig. 5.** Viral proliferation (A) was measured by plaque assays after infecting cells with NV1023 at an MOI of 0.1. Viral titers at day 6 were significantly higher for the MG11 and MG14 cell lines (425-fold and 340-fold increases, respectively) in comparison to SCC7 (11-fold increase, P < 0.05 for both comparisons, t test). Lactate dehydrogenase cytotoxicity assays (B) were done daily over a 6-day period at an MOI of 5, 1, and 0.1. For all conditions, MG11 and MG14 cell lines consistently showed a significantly increased susceptibility to oncolysis with NV1023 as compared with SCC7.

**Fig. 6.** NV1023 was added to cells that were migrating across a porous polyethylene membrane. The NV1023 virus is able to pass freely across the membrane. Of the cells that had successfully migrated across the membrane (Mig), nearly all of the cells were susceptible to viral entry, regardless of the cell line type. For the nonmigrating cells remaining on the original side of the membrane (Non-Mig), ~30% were susceptible to viral entry. These results were similar for SCC7, MG11, and MG14. A higher proportion of cells were migratory for the MG11 and MG14 cell lines as compared with SCC7, as previously demonstrated (data not shown).
pass across. In contrast, the invasion assay measures the ability to cross a thin Matrigel barrier in just 44 hours. Therefore, although MG14 may be uniquely able to invade across the thickest barrier, it might have less speed to account for its lower results than MG11 on the invasion assay. This serial selection process might also account for the decreased proliferation rate observed in the MG14 cell line. Cells with lower metabolic and proliferation rates might have been better able to survive the long transit through the Matrigel without requiring nutrient-rich medium.

We noticed differences in both cell morphology and patterns of growth for SCC7, MG11, and MG14. Although confluent SCC7 cells grew densely packed together, MG11 and MG14 grew in looser patterns with gaps persisting between cells even at maximal confluence. This loose pattern suggests that MG11 and MG14 have less cohesiveness between adjacent cells, and supports the idea of impaired adherens junctions in these lines. Consistent with this idea was our finding that MG11 and MG14 showed enhanced nectin-1 surface availability as compared with SCC7. Furthermore, this enhanced nectin-1 seemed able to function as a herpes viral receptor in these cell lines. MG11 and MG14 showed an enhanced susceptibility to NV1023 viral entry, viral replication, and cytotoxicity. Enhanced cellular migratory and invasive abilities were therefore associated with (a) decreased adjacent cell cohesiveness in culture, (b) enhancement in cell surface nectin-1 availability, and (c) increased sensitivity to herpes viral entry, replication, and cytotoxicity.

In some studies, tumor cell proliferation rate has been associated with herpes viral sensitivity. As an example, the G207 oncolytic herpes virus is deficient in ribonucleotide reductase and has been shown to have greater efficacy in tumor cells with elevated rates of proliferation and S phase fractions (3). In contrast, the NV1023 virus has an intact ribonucleotide reductase gene and therefore may not have a similar predilection. To investigate this possibility, we measured proliferation rates and found that the more sensitive MG11 and MG14 cell lines had mildly decreased proliferation rates as compared with SCC7. Therefore, enhanced cellular proliferation did not account for our observed differences in viral sensitivity.

Interestingly, we also found that cells that were actively migrating were significantly more susceptible to herpes viral entry than nonmigrating cells. As expected, a higher proportion of cells were migratory for MG11 and MG14 than SCC7. Of the actively migrating cells, there was nearly complete viral entry for all three lines, with only 30% of nonmigrating cells allowing viral entry. This finding suggests that the active, dynamic state of the cell may also be an important determinant of nectin-1 availability. The integral association of nectin-1 to its partner afadin, a protein that is anchored to cytoskeletal actin, provides one possible link between these two functions. Nectin-1 has extracellular, transmembrane, and intracellular components. The intracellular portion of nectin-1 connects to afadin, which in turn connects to filamental-actin bundles (31). It is conceivable that structural changes during cell motility might potentially lead to dynamic changes of cytoskeletal actin, afadin, nectin-1, and HSV receptiveness.

In summary, squamous cell carcinoma lines selected for increasing migratory and invasive function had decreased cell cohesiveness at confluence and increased nectin-1 surface availability. In comparison to the parent SCC7 line, these cells showed increased susceptibility to herpes viral entry, replication, and cell lysis by NV1023, an attenuated oncolytic herpes virus. The finding that nectin-1 expression may be enhanced on invasive cells and is a determinant of tumor HSV sensitivity has several clinical implications. First, it provides one potential explanation for the observation that malignant cells frequently support selective HSV infection and proliferation. Second, it implies that the most aggressive, highly invasive tumors might also represent the most appropriate targets for herpes oncolytic therapy. Patients with such highly invasive tumors are most often the ones failing conventional treatments, and therefore have the greatest need for novel, alternative therapies. And third, it suggests that an assessment of tumor nectin-1 expression might be a useful method of predicting tumor sensitivity to oncolytic HSV. An understanding of the determinants of tumor susceptibility to HSV might allow us to someday select patients with the most sensitive tumors for entry into future clinical trials with these promising new vectors.

References


Acknowledgments

We thank Drs. Patricia Spear and Mi Yi Yoon for their generous provision of the chicken anti-nectin-1 antibody, and for their helpful advice regarding the immunofluorescence and cellular ELISA experiments.

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Zhenkun Yu, Mei-Ki Chan, Pornchai O-charoenrat, et al.