Factors Influencing the Mechanism of Neural Progenitor Cell-Tumor Cell Binding

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Background and Significance

Every year, over 17,000 people are diagnosed with malignant primary brain tumors and over 13,000 people die from them in the United States (Entremed). A large part of this high mortality rate is due to the lack of cell-based therapies that target and eliminate invasive tumor cells and therefore would lower the 98% recurrence rate of glioma (Castro et al., 2003). However, stem cell therapies are a newer avenue that has yet to be fully explored and hold much promise to ameliorating this condition.

In rodent models, neuroprogenitor cells (NPCs) injected into the animal, regardless of location of injection, migrate specifically to the location of the tumor in the brain (Aboody et al., 2000). This phenomenon may allow us to use stem cells to target infiltrating cells and deliver anti-cancer agents to the precise tumor locations. But first, the morphological and mechanistic properties of the stem cell-tumor cell interaction must be understood. In our experiment, we used HB1.F3 neuroprogenitor cells and allowed them to interact with tumor cells in order to observe their interaction. By varying tumor cell lines, immunocytochemistry, antibody inhibition, and Calcein Blue staining, we can begin to determine what is occurring when these two cells come into contact with each other.

Knowledge of the factors involved in this interaction may help in developing methods to increase NPC tumor targeting and specificity and therefore the effectiveness of stem cell therapies. We hypothesize that the HB1.F3 progenitor cells are interacting with the U251 glioma cells similar to how immature neurons interact with radial glia in inside-out development. The immature neuron (F3) migrates up the radial glia (U251), which acts as the scaffolding for the brain, and differentiates during development.

Materials and Methods

Cell culture. HB1.F3 is an immortalized human neurostem cell line derived from the human fetal brain (gift of K. Aboody and S.Y. Kim). These cells express EGFP (by lintiviral transfection). Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 100 units/ml penicillin, 100 µg/ml streptomycin. U251 glioma cells express dsRed 2. All tumor cell lines were received from K. Aboody.

Cells were trypsinized and made to be at a $2x10^6$ cells/ml concentration. Then 37.5 µl of HB1.F3 and 37.5 µl of a tumor cell line were mixed with 75 µl of .3% Puramatrix (3DM Inc.). Then 150 µl of cell Puramatrix mixture was pipetted into 1 ml of DMEM media in a well plate. The Puramatrix was allowed 24 hours for incubation before being fixed for 24 hours with 4% paraformaldehyde in Tris-buffered Saline (TBS). After fixation, the Puramatrix was washed with TBS with 0.1% Triton-X-100 (Invitrogen, Carlsbad, CA) and then mounted.

Image Acquisition. Images were taken using an inverted Zeiss 510/NLO confocal microscope. Images were taken with a Zeiss Fluar 10x/0.5 NA objective in 30 µm stacks, typically 11 slices 3 µm apart. Fields were scored using z-axis vertical projection with individual slices examined to confirm cell pairs.

Varying tumor cell lines. D566 glioma (invasive [Aboody, unpublished data]), SKBR3 breast cancer, and MDA-MB-231 breast cancer (invasive [Aboody, unpublished data]) were labeled with CM-DiI, Chloromethylbenzamide (Molecular Probes Cat # C-7000) and then the Puramatrix protocol outlined above was followed.

Immunocytochemistry. After fixation of the Puramatrix with U251 and HB1.F3 cells, the mixture was left in blocking solution (50% Western Blocking Solution (Roche), 50% Block-Aid (Molecular Probes), 1% Triton-X-100) overnight at 4ºC. One primary antibody was added to each Puramatrix to set overnight. Primary antibodies used were: Connexin 43 (MAB3068, Chemicon, Temecula, CA), a protein found in gap junctions, Actin (A2066, Sigma), a protein necessary in motility, Alpha V integrin (MAB1980, Chemicon), a surface protein responsible for radial glial adhesion, and Alpha 3 integrin (MAB1952, Chemicon), a surface protein integral in neuronal recognition. Secondary antibody tagged with Alexa 647 was applied for 2 hours at room temperature before imaging.

Antibody Inhibition. U251 glioma cells and HB1.F3 neuroprogenitor cells were mixed with Alpha 3 integrin primary antibody (Chemicon) at a 1:50 dilution and allowed to set for 1 minute before being mixed with each other and the Puramatrix. The Puramatrix was incubated in media with the antibody in a 1:100 dilution for 24 hours. After fixation for 24 hours, the Puramatrix was placed in blocking solution for 12 hours then secondary antibody (Molecular Probes) for 2 hours before imaging in order to ensure antibody application and thus inhibition.

Calcein Blue in HB1.F3 cells. HB1.F3 cells were loaded with Calcein Blue (Molecular Probes) by given protocol. The loaded cells were immediately mixed with U251s and placed in .3% Puramatrix. Live cultures were imaged 15 minutes after Puramatrix formation.

Results

Varying tumor cell lines. HB1.F3 neuroprogenitor cells were mixed in Puramatrix with four different tumor lines: D566 glioma, U251 glioma, SKBR3 breast cancer, and MDA-MB-231 breast cancer. D566s and SKBR3s prominently, and MDA-MB-231s to a lesser degree, displayed a morphologically different type of binding than the U251s. HB1.F3 progenitor cells mixed with D566, SKBR3 or MDA-MB-231 often displayed a sort of burst when bound to each other (see Figure 1) as well as capping (see Figure 2) while U251 only showed various stages of capping. In some experiments, the burst effect accounted for as much as 25% of the total interactions.

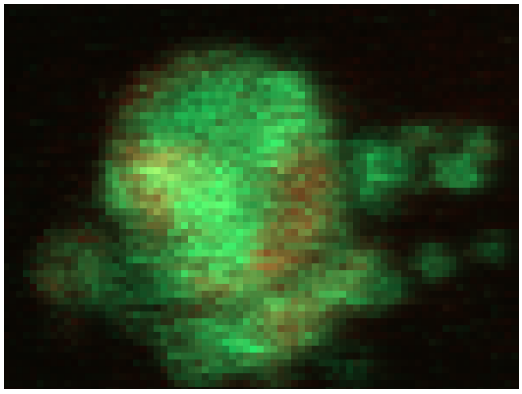

Figure 1. The Burst Effect: CM-DiI labeledD566 glioma (red) with HB1.F3 neuroprogenitor (green)

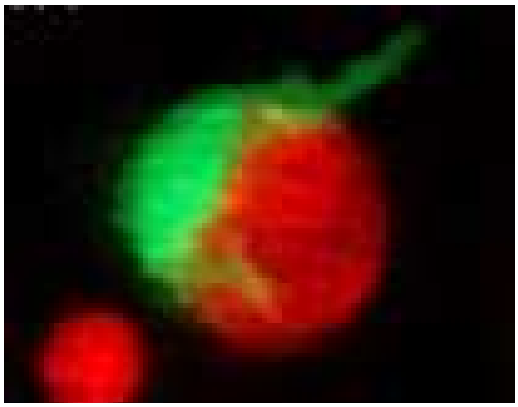

Figure 2. Capping: U251 glioma (red) seemingly being engulfed by HB1.F3 neuroprogenitor (green)

Table 1 displays the average percent of HB1.F3 neuroprogenitor cells bound to each type of cell.

Tumor Line	Percent of HB1.F3 cells bound to Tumor Cells	Percent of HB1.F3 cells in self-pairs
U251 glioma cells	8-15%	8-13%
D566 glioma cells	31-50%	8-9%
MDA-MB-231 breast cancer cells	11-25%	7-21%
SKBR3 breast cancer cells	17-45%	9-14%

Table 1. Percent of total HB1.F3 neuroprogenitor cells in each pair type from 2 experiments performed.

Immunocytochemistry. Cell-cell interactions were observed in a Puramatrix with HB1.F3 neuroprogenitor cells and U251 glioma cells with antibody staining to determine where and how much of each protein was present. Figures 3-6 illustrate the most common types of images found for each type of antibody.

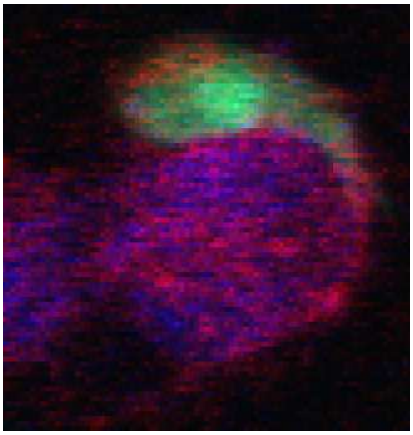

Figure 3. Connexin 43 (red) was slightly more prevalent on the U251 (blue) glioma than the F3 (green)

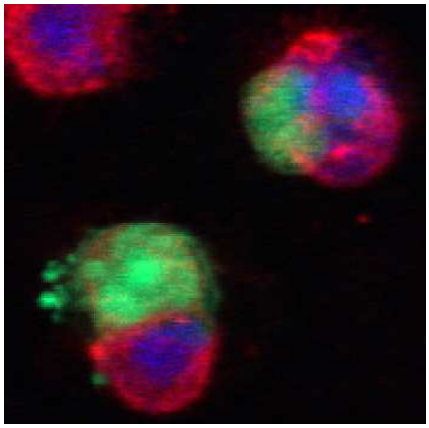

Figure 4. Alpha 3 integrin (red) was much more prevalent on the U251 (blue) glioma than the F3 (green)

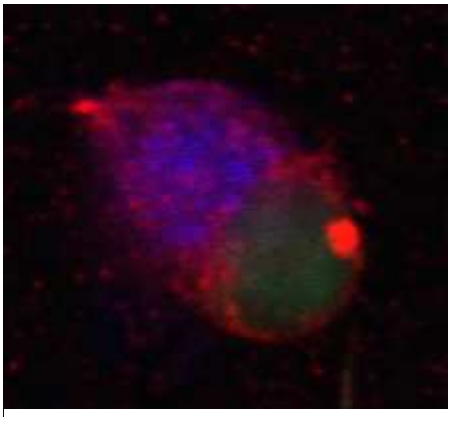

Figure 5. Actin (red) was notably bunched around the point of interaction, probably where F3 (green) podia are beginning to move to cap the U251 (blue)

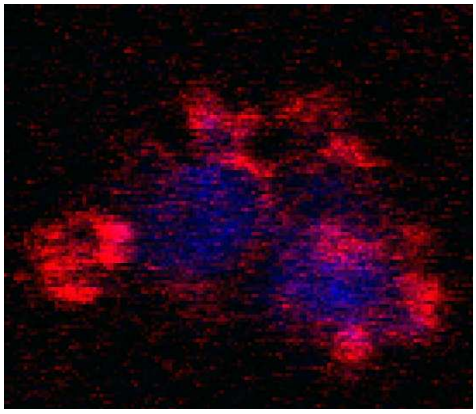

Figure 6. Alpha V integrin (red) was densest around U251 (blue) self pairs as opposed to U251-F3 pairs

Antibody Inhibition. Alpha 3 integrin primary antibody, which blocks ligand binding to the surface receptor, was added to HB1.F3 neuroprogenitor cells and U251 glioma cells before being mixed and placed in Puramatrix. Alpha 3 integrin inhibition caused F3 self pairing to decrease but F3-U251 pairing to increase (See Figure 7).

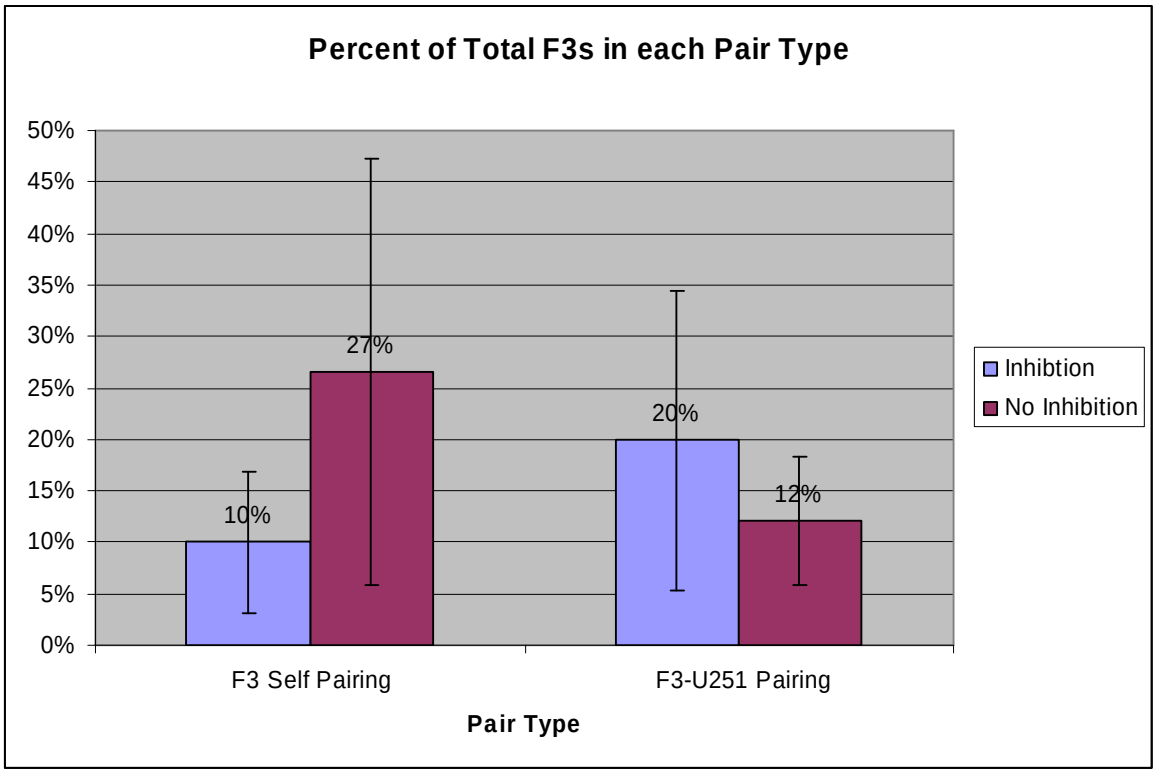

Figure 7. Percent of total F3s in each type of pair was measured. Each F3 self pair counted as two cells. The inhibition data was taken from 20 fields in two experiments. The no inhibition data was taken from 74 fields in 4 experiments. T-value for F3 self pairing was 1.07×10^{-4}. T-value for F3-U251 pairing was 2.2×10^{-4}.

Calcein Blue in HB1.F3 cells. HB1.F3 neuroprogenitor cells were loaded with Calcein Blue and then imaged in live culture. No transfer was recorded between the F3 and the U251 glioma cells. Most interactions looked like Figure 8 below.

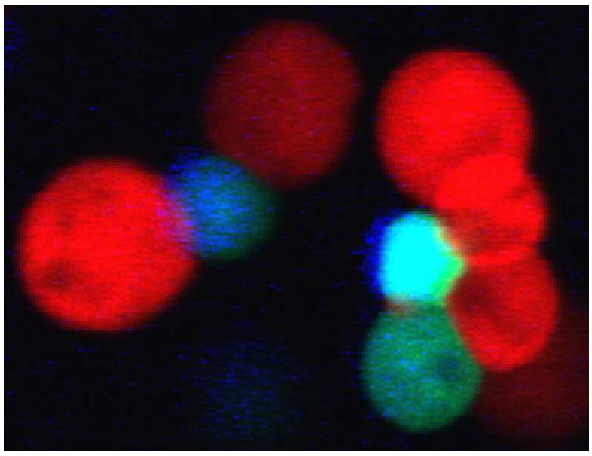

Figure 8. HB1.F3 (green) loaded with Calcein Blue (blue) did not display any transfer to U251(red) after 15 minutes in culture.

Discussion and Future Experiments

Varying Tumor Cell Lines. The invasiveness and origin of the cancer cell lines both affect the affinity of the HB1.F3 neuroprogenitor cells for the tumor cells as well as the morphology of their interaction. It seems that the more invasive the line, the more the stem cell attraction to it. This is evidenced especially in the glioma lines. There are probably other factors playing a role in these interactions as indicated by the presence (or lack) of the burst effect which does not correlate directly to tumor cell line invasiveness. The fact that the burst effect was only seen in those lines labeled with DiI gives rise to some questions as to the nature of the effect. It could be that the effect is due to the DiI leaking or some other experimental epiphenomenon. In the future, U251 dsRed 2 glioma cells should be labeled with DiI and then mixed with F3s in Puramatrix. If the burst effect appears, then it is probably not a physiologically meaningful interaction.

Immunocytochemistry. Each protein imaged had a particular trend in the confocal images. Connexin 43 was much more prevalent in U251 cells than HB1.F3 neuroprogenitor cells, both when the cells were interacting and when they were solitary in the Puramatrix. The low amount of gap junction protein in the neuroprogenitor cells and low amount expressed near F3-U251 interaction probably means that transfer is low between the cells.

Alpha 3 integrin was very prevalent on the U251s as well. Alpha 3 integrin is specific to neurons and integral in neuronal recognition. Even though we have not seen U251 cells move toward F3 cells, this high expression could mean that the U251 cell is engaging the F3 cell more than was previously thought.

The surface protein Alpha V integrin which aids in adhesion and is specific to radial glia was more prevalent on the U251 cell also. This could be because the U251 glioma cells share properties with radial glia stem cells and strengthens the hypothesis that this interaction is a recapitulation of inside-out development of the brain with the U251 cells acting as radial glia and F3 neuroprogenitor cells acting as the immature neuron.

Actin was local to U251-F3 interaction site, indicating that the HB1.F3 cells are actually moving to seemingly engulf the U251 cells in some cases. At times, the neuroprogenitor cells seemed to be forming podia to grab onto the tumor cell.

Antibody Inhibition. Alpha 3 integrin primary antibody blocks the ligand from binding to the surface receptor. This reaction caused a decrease in F3 self pairing and an increase in F3-U251 pairing. Therefore, the F3-F3 and F3-U251 binding morphology are likely to be of very different mechanisms. F3 self-recognition seems to be impaired since Alpha 3 integrin is integral in neural recognition. In the future, Alpha V Integrin primary antibody should be applied to block ligand in order to observe its effect on the interactions among cells.

Calcein Blue in HB1.F3 cells. HB1.F3 neuroprogenitors were loaded with Calcein Blue but no transfer was seen. Although we expect gap junctions to open immediately, it is possible that the short amount of time in culture could have produced a false negative. Calcein Blue is not fixable and is pumped out of the cells quickly due to being loaded by passive transport; therefore imaging must take place soon after incubation with the dye, leaving less time for the cells to interact. In the future, Cascade Blue (Molecular Probes) may be used as an alternative because it is fixable and less likely to leak out since it uses osmotic pressure and hypertonicity and hypotonicity as a mechanism to load.

References

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