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ABSTRACT

Human mammary epithelial cells grown in a 3D matrix form polarized acini that closely resemble the cellular environment encountered in breast tissue. Consequently, acini are an excellent system for studying morphogenesis, and for modeling the role of cell-cell interactions in processes such as apoptosis, tumorigenesis and carcinogenesis. In this project, we studied the interaction of an aggressive mammary epithelial tumor cell line with the MCF-10A acini. We found that MCF-10A acini have the ability to induce apoptosis in tumor cells, whereas non-polarized MCF-10A cells (those in monolayers rather than acini) show no such mechanism. We also showed that tumor cell apoptosis induced by contact with MCF-10A acini is mediated by the extrinsic Fas death signaling pathway. We believe this is an example of tissue homeostasis, in which the normal mammary epithelial cells regulate the growth of aberrant epithelial cells. This model system can be used to study the ways in which tumor cells evade this early control mechanism enabling their uncontrolled growth and proliferation.

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INTRODUCTION

Human mammary epithelial cells form organized structures called acini when cultured in a 3-D matrix. The acini, which consist of a layer of polarized cells with a central lumen, resemble glandular tissue in morphology and function. These acini are, therefore, excellent models for the study of mammary gland morphogenesis, the role of extracellular matrix on cell function, and tumorigenesis. We used this model system to examine the direct interactions of human mammary epithelial tumor cells with polarized acini.

BODY

The specific goal of this project was to examine the mechanism by which the tumor cells were dying. Specific questions were:

1) Did cell death occur via apoptosis or necrosis?
2) Is cell death upon contact with acini specific to MDA-MB-231 cells or common to many cell types?
3) If it is a common mechanism, how do normal cells recognize the tumor cell as abnormal and how is the recognition signal transduced?

To address these questions, we used the 3-D model system previously described by Joan Brugge's laboratory (Debnath et al., 2003). We added human mammary epithelial tumor cells (MDA-MB-231) to normal MCF-10A acini. We then incubated the cells and monitored their interactions using live-cell confocal microscopy. We observed that the tumor cells migrated through the matrix toward the acini. MDA-MB-231 tumor cells demonstrated a directed migration toward the acini, extending long invadopodia (long, finger-like projections) and contacting the acini. Eventually the acini were surrounded by MDA-MB-231 tumor cells (Figure 1A-1C). We monitored the interactions of the tumor cells with the acinar cells for up to five days using live-cell imaging. We observed that those MDA-MB-231 tumor cells in contact with acini died (Figures 1D-1E), whereas those in the vicinity, but not in contact with the acini, did not die. We examined the tumor cells in contact with the acini by labeling for the presence of activated caspase 3, a protein activated during apoptosis, and determined that the tumor cells are dying via the process of apoptosis, rather than necrosis (Figure 1G-1I).

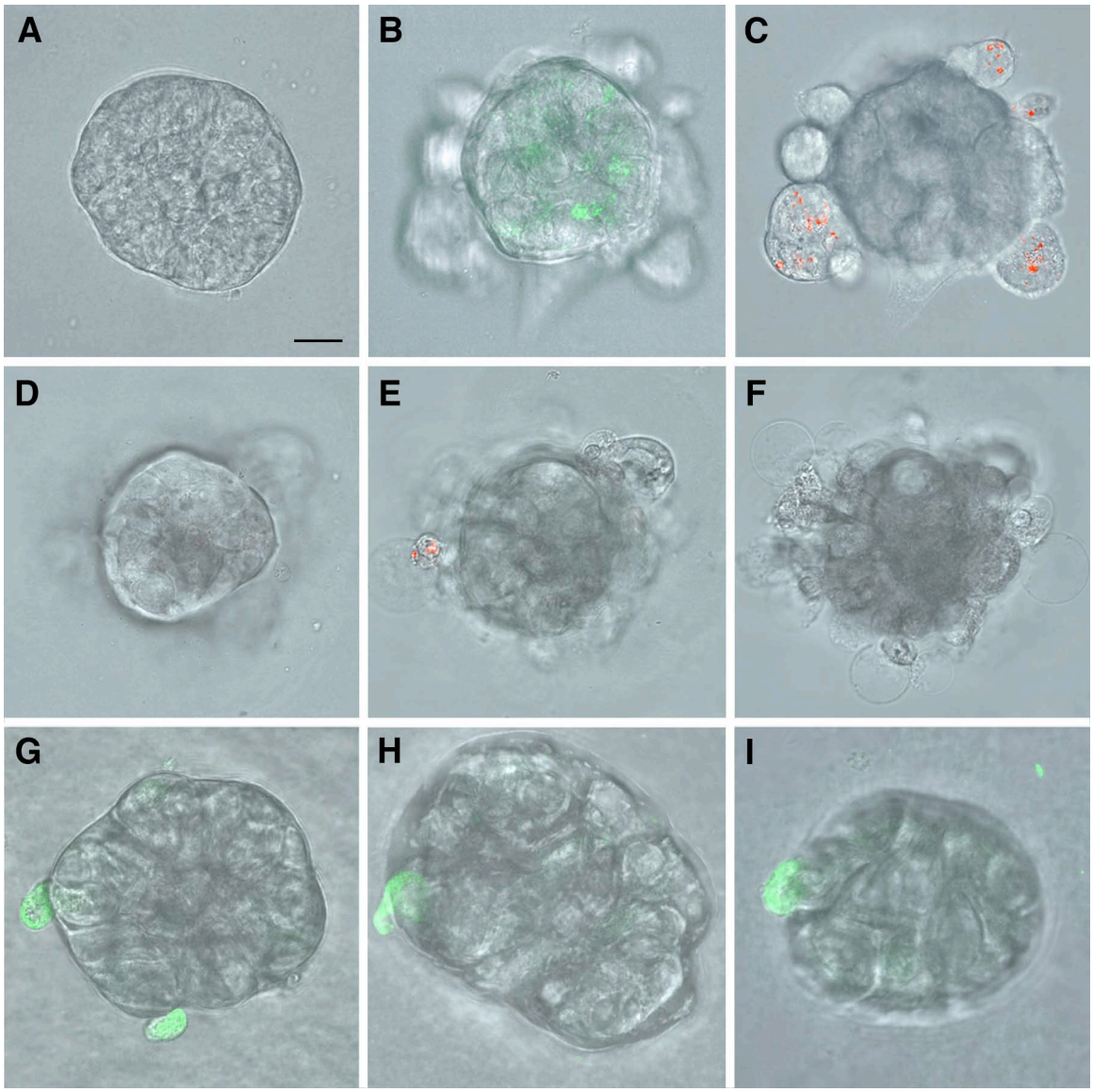

Fig. 1. (**A)** Human mammary epithelial cells (MCF-10A) form acini after growing in a 3-D matrix (Matrigel) for 10 days (Debnath et al., 2003); **(B-C)** Human mammary epithelial tumor cells (MDA-MB-231) migrate through basement membrane and contact MCF-10A acini. After 24 hr, an acinus formed from an MCF-10A cell pre-loaded with green-emitting QDs (**B**) is surrounded by MDA-MB-231 tumor cells containing red-emitting QDs (**C**); (**D-F**) MDA-MB-231 tumor cells contact MCF-10A acini and undergo apoptosis. Confocal microscope optical sections through co-culture show an acinus surrounded by numerous MDA-MB-231 cells (tagged with red-emitting QDs) and imaged two days after their addition to the acini. (**D**) Optical section at the deepest region of co-culture shows the acinus. Optical sections through the upper portion show numerous dying and dead tumor cells attached to the acinus at 90 µm (**E**) and 26 µm (**F**) from the top of the culture. Many of these cells have leaked organelles and QDs into the medium, but newly attached cells in the deepest region of the culture still retain red-emitting QDs. (**G-I**) Immunostaining with antisera to activated caspase-3, the death effector caspase, shows that the attached tumor cells seen12 hours after addition to culture are undergoing apoptosis. Bars = 20 µm.

To demonstrate that apoptosis was occurring via an extrinsic, rather than intrinsic, pathway, we investigated the role of the Fas pathway. We showed that blocking the Fas pathway enabled growth of the MDA-MB-231 cells.

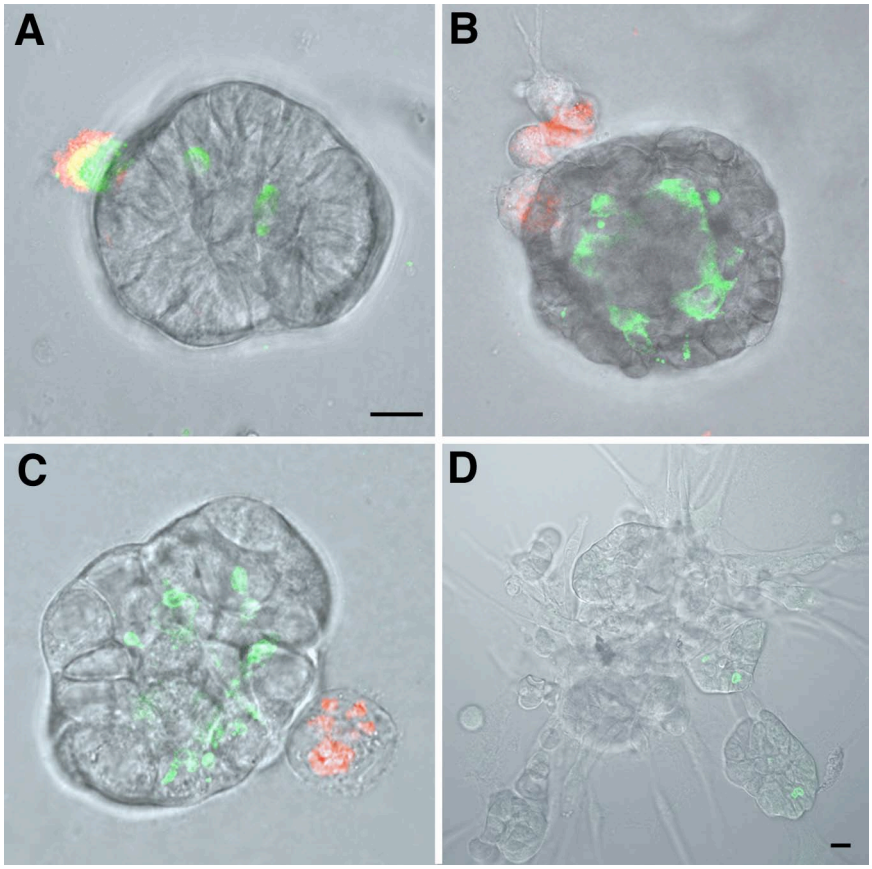

Fig 2. Blocking the Fas pathway prevents MDA-MB-231 cells apoptosis in the co-culture. In absence of Fas blocking antisera, MDA-MB-231 cells (labeled with Texas Red dextran) undergo apoptosis (detected with Annexin V in green) upon contacting MCF-10A acini (yellow = overlap of Texas Red dextran and Annexin V) (**A**). In the presence of antagonistic antibodies to either FasL (**B**) or Fas (**C**), MDA-MB-231 cells do not undergo apoptosis upon contact with acini. After extended culture in presence of antagonistic Fas antibodies, the tumor cells proliferate and engulf the MCF-10A acini (**D)**. Activated caspase-3 immunostaining was examined using confocal microscopy of optical sections through the center of the acini; the only cells undergoing apoptosis are those in the lumen-forming region of acini. Bars = 20 μm.

The initial signal that activates the Fas pathway, however, is not yet clear. Given the prevailing model for Fas activation, it would be expected that Fas on the surface of tumor cells is activated by interaction with FasL on the surface of acini. Immunolabeling experiments, however, demonstrate that although the acini contain cytoplasmic Fas (Figures 3A, A*), no FasL could be detected with immunolabeling using five different

antibodies (Figures 3D-F). The MDA-MB-231 tumor cells demonstrated Fas labeling, but only after contacting the acini (Figures 3B, B* and 3C, C*). Interestingly, they also demonstrate high levels of FasL, but only after contacting the MCF-10A acini (Figures 3D-F) These results suggest that contact of tumor cells with acini initiates activation of the Fas pathway in the tumor cells via an as yet unidentified initial signal. Once Fas is triggered in the tumor cells, there is an autocrine response causing a cascade of Fas and FasL activation. Identification of an initial signal remains to be determined.

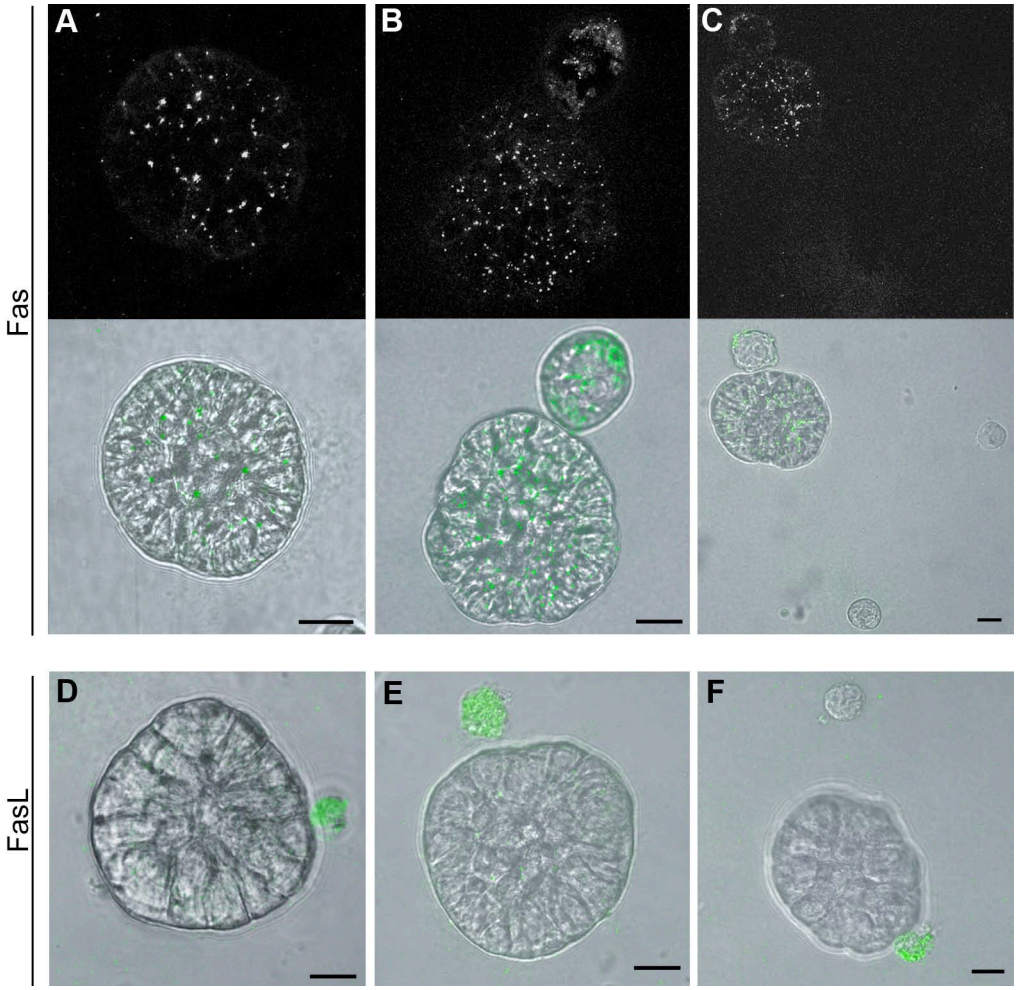

Fig. 3. Immunostaining shows increased levels of both Fas and FasL in MDA-MB-231 tumor cells contacting acini. **(A-C)** Multiple foci of Fas (clone CH11) are seen in the inner region, but not the basal portion, of the acinus; Fas is also seen in those MDA-MB- 231 cells in contact with acini (**B-C**), but could not be detected in those that had not contacted the acini (**C**). The first row shows fluorescent images that are projections of all optical sections through the culture; the second row is the superimposition of the projected fluorescence image with the transmitted light image. **(D-F)** FasL (clone G247-4) is seen in MDA-MB-231 tumor cells contacting MCF-10A acini, but not in those not 21 contacting acini (**E, F**). Images are superimpositions of all optical sections through the culture superimposed on transmitted light image. Bars = 20 µm.

We demonstrated that apoptosis of MDA-MB-231 cells occurred only upon contact with MCF-10A acini, not with non-polarized MCF-10A cells. When the two cell lines were simultaneously added to Matrigel and incubated for 24 hours, they formed large masses of co-mingled MDA-MB-231 and MCF-10A cells (Fig. 4A), with no evidence of apoptosis of either cell type. When MDA-MB-231 cells were added to MCF-10A cells that had been cultured in basement membrane for 24 hours (Fig. 4B) or 72 hours (Fig. 4C), tumor cells accumulated around the periphery of MCF-10A cell clusters and continued to grow with no evidence of apoptosis of either cell type. These experiments indicate that only polarized MCF-10A acini can trigger apoptosis of the tumor cells.

To determine the limits of the ability of acini to induce tumor cell apoptosis, we added increasing concentrations of tumor cells tagged with Texas Red dextran to acini and monitored the co-cultures for 72 hours. After this time we counted the percentage of surviving, unaffected acini that were not overwhelmed by tumor cells. When tumor cells were added at a ratio of three per acinus, the majority of MDA-MB-231 cells underwent apoptosis (Fig. 4D). At a ratio of nine tumor cells per acinus, only 37% of the acini were free of tumor cells (Fig. 4E); and at a ratio of 18 tumor cells per acinus, all but 18% of the acini were completely overwhelmed by the tumor cells after 72 hours (Fig. 4F).

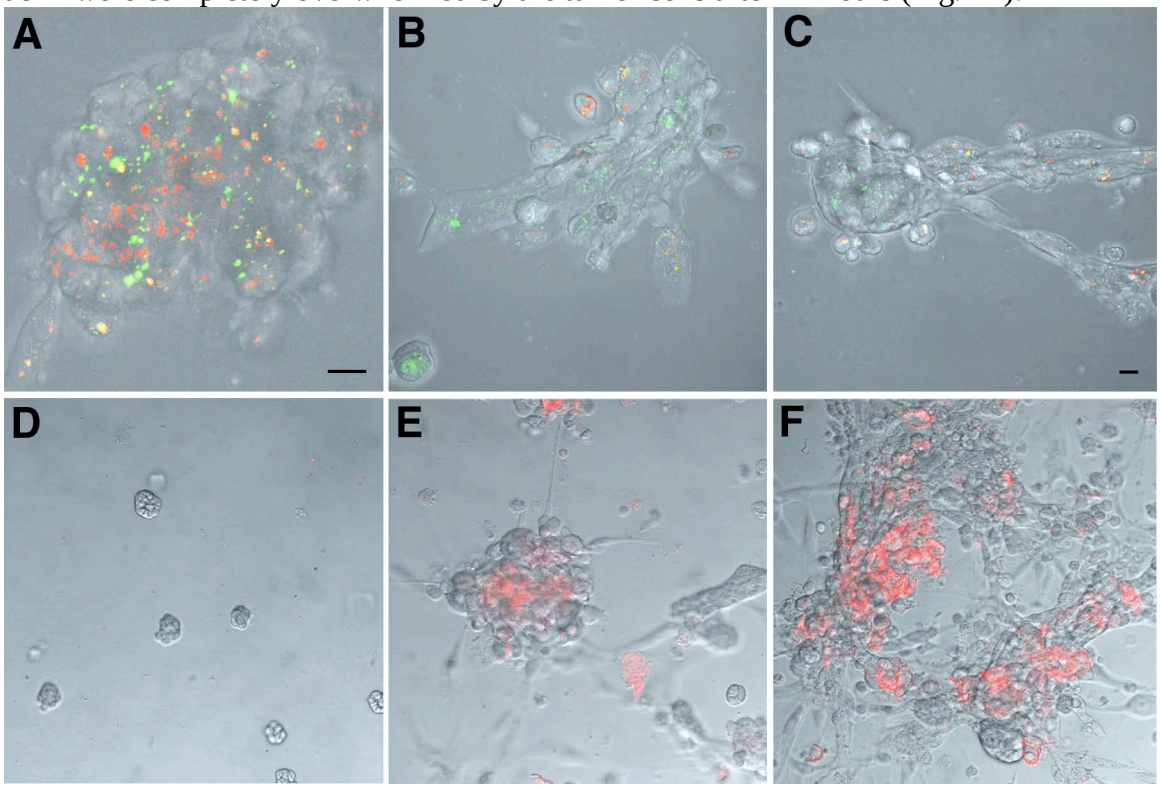

Fig. 5. Induction of apoptosis in MDA-MB-231 tumor cells depends on polarity of MCF- 10A cells and concentration of tumor cells. **(A-C)** Large masses of mixed cells are formed when MDA-MB-231 cells (tagged with red-emitting QDs) are added to the reconstituted basement membrane with non-polarized MCF-10A cells (tagged with green-emitting QDs) either simultaneously **(A)** 24 hr (**B**) or 72 hr after the MCF-10A cells (**C**). **(D-F)** MDA-MB-231 tumor cells (labeled with Texas Red dextran) escape apoptosis when added to acini at high concentrations. Virtually all tumor cells die when added to acini at a ratio of 3 tumor cells per acinus (**D**); increasing numbers of tumor cells grow and proliferate when added at a ratio of 9 cells per acini (**E**) and 18 cells per acini (**F**). Bars = 20 µm.

We are still evaluating other cell lines to determine whether the contact-dependent apoptosis seen in MDA-MB-231 cells is a common mechanism. We found that some cell lines did undergo apoptosis, but others were able to grow and proliferate, even when in direct contact with the acini. Our preliminary findings suggest that those cell lines possessing an intact Fas pathway do undergo apoptosis, whereas those cell lines with an aberrant pathway can survive and actually invade the acini. These investigations are still in progress.

KEY RESEARCH ACCOMPLISHMENTS

- We demonstrated that MDA-MB-231 human mammary epithelial tumor cells die upon contact with MCF-10A acini.
- We demonstrated that death of the MDA-MB-231 tumor cells requires direct contact with MCF-10A acini.
- We demonstrated that death of the MDA-MB-231 tumor cells occurs via apoptosis.
- We demonstrated that apoptosis of the MDA-MB-231 cells occurs via an extrinsic pathway, involving activation of the Fas death signaling pathway.
- We demonstrated that apoptosis of MDA-MB-231 tumor cells occurs only upon contact with polarized MCF-10A acini, not with non-polarized MCF-10A human mammary epithelial cells.

REPORTABLE OUTCOMES

- Rosanne M. Boudreau, Benjamin D. Engel, Weiwei Gu, A. Paul Alivisatos, Mark A. Le Gros, and Carolyn A. Larabell (2006). Breast Tumor Cells Undergo Apoptosis upon Contact with Human Mammary Epithelial Acini. *Cancer Research.* Submitted.

CONCLUSIONS

The results of this research project demonstrate that using the 3D model system to examine interactions of human mammary epithelial tumor cells with polarized human mammary epithelial acini can reveal processes that cannot be detected using the more traditional 2D culture systems. Using this model system, we observed that in culture, the normal cells can kill aberrant cells in their immediate micro environment. Tumor cells divide rapidly, however, and can overwhelm these early attempts to control their growth

by evading contact with the acini. It now remains to be determined whether comparable events occur in vivo.

REFERENCES

Debnath, J., S.K. Muthuswamy, and J.S. Brugge. 2003. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. *Methods (Duluth).* 30:256-268.