The Tumor Microenvironment and 3-D Tumor Models

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Outline

• The Tumor Microenvironment
  ➢ Chronic versus acute changes
  ➢ Consequences of tumor microenvironment
  ➢ Advances in measuring the tumor microenvironment
  ➢ Difficulties with *in vivo* models and clinical tumors

• 3-D Experimental Tumor Model Systems
  ➢ Types of model systems
  ➢ The multicellular spheroid tumor model
  ➢ Example of application of spheroids
  ➢ Recent developments and future work

• Mathematical Modeling in Tumor Biology
  ➢ Tumor microenvironment
  ➢ Genetic/proteomic/metabolic networks
  ➢ Tumor growth and development

• Questions?
Malignant Progression of Cancer

Important to realize: all of this happens in a 3-D context within a tissue!
Differences: Tumor and Normal Tissue Vasculature

Chronic Changes in Tumor Microenvironment

- Tumor cells grow faster than vasculature: cells located far from vessels
- Gradients in biochemistry of extracellular space
  - Nutrients (oxygen, glucose)
  - Metabolic wastes (pH, lactate)
  - Signaling molecules (promoters, inhibitors)
- Gradients in cell physiology
  - Proliferation
  - Metabolism
  - Viability
  - Motility, invasiveness
- Gradients in gene/protein expression
- Gradients in therapy response
- Generally occur over ~200 μm

Transient Changes in Tumor Microenvironment

- No organization to architecture of vasculature: driven by semi-random processes
  - Long, tortuous vessels
  - A-V shunts
  - Blockages

- Disorganized function
  - No smooth muscle or nerve cells
  - Varying pressure gradients
  - Trapping of white/red cells

- Transient microregional variations in flow
  - Slowed, stopped, reversed flow
  - ~10-20 minute period most frequent

- Time-varying nutrient supply and waste removal
- Superimposed on chronic gradients
- Altered by therapy

Kimura et al., Cancer Res. 56: 5522, 1996
Both Chronic and Transient Hypoxia

Microenvironment Involved in Tumor Progression

Bindra & Glazer., *Mutat. Res.* 569: 75, 2005
Microenvironment Involved in Metastasis

Therapeutic Impact of Tumor Microenvironment

• Hypoxia causes radiation resistance
  ➢ Major explanation for radiotherapy failure
  ➢ Major focus of drug development and imaging

• Cell cycle arrested cells more resistant
  ➢ Resistant to most common chemotherapies, radiation
  ➢ Able to repopulate tumor after treatment

• Limited drug delivery
  ➢ Poor penetration (chronic) & limited delivery (transient)
  ➢ Problem for new therapies (antibodies, nonparticles)

• Induction of drug resistance and genetic instability
  ➢ Gene expression and protein modifications
  ➢ Mutations: drug resistance, survival phenotypes

• Stimulation of angiogenesis and metastatic spread
  ➢ Induction of pro-angiogenic factors
  ➢ Increased local invasion and distant metastases
Effect of Hypoxia on Therapy


Cervical Cancer

H&N Cancer

\[ pO_2 > 10 \text{ mm Hg} \]

\[ pO_2 < 10 \text{ mm Hg} \]

\[ p=0.005 \]
Imaging in Window Chamber Tumors

Sorg et al., *J. Biomed. Optics* 10: 044004, 2005
Imaging in Human Tumor Sections

Metabolic Analysis of Tumor Microenvironment

Wallenta et al., *Biomol. Engineer*. 18: 249, 2002
Advanced MRI of Tumor Microenvironment


- Vascular volume
- Vascular permeability
- Histology
- V & P
- V & P & pH

Advanced MRI of Human H&N Tumor

Limitations to *in Vivo* Tumor Biology

- Enormous complexity and heterogeneity both within and between tumors
- Non-reproducibility of even the best rodent tumor model systems
- Poor understanding of extent and control of transient variations: basically chaos
- Inability to control experimental parameters
- Inability to perform mechanistic experiments on humans
- Therefore, advances in basic understanding of tumor biology (and progress in therapy?) require *in vitro* experimental models of tumor
**In Vitro** Experimental Tumor Models

- Most basic: monolayer or suspension cell cultures
  - Useful for very basic studies
  - A very poor model of a 3-D tissue
  - Do not mimic any aspect of the tumor microenvironment
- Several different 3-D *in vitro* models have been developed
  - Cells embedded in external matrix material
  - Bioreactors: cells within artificial capillary structure
  - ‘Sandwich’ culture: cells trapped between two plates
  - Multicell layers: 3-D layers of cells on a membrane
  - *Ex vivo* explants of tumor pieces
  - Multicellular aggregates: spherical 3-D cultures (‘spheroids’)

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**NFCR**

National Flow Cytometry Resource

**Los Alamos**

National Laboratory

**EST. 1943**
Multicellular Tumor Spheroids

Proliferating cells

Quiescent cells

nutrients

wastes

Spheroid Volume

S-Phase Fraction (percent)

Viable Rim Thickness

Time of Growth (days)
Similarities: Spheroids and Tumors

• 3-D, tissue-like structure
  - Cell-cell contacts
  - Extracellular matrix
  - Microenvironment develops spontaneously

• Heterogeneous microenvironment
  - Gradients in extracellular biochemistry
  - Gradients in cellular physiology
  - Gradients in cellular metabolism
  - Gradients in gene/protein expression

• Therapy resistance
  - Radiation (ionizing, UV, microwave)
  - Many forms of chemotherapy
  - Hyperthermia
  - Photodynamic therapy
  - Biologicals (antibodies, liposomes, nanoparticles)
Advantages: Spheroids vs Tumors

• Highly reproducible
  ➢ Very small inter-spheroid variability
  ➢ Excellent long-term ‘stability’ (decades)

• Symmetrical
  ➢ Gradients are radially distributed
  ➢ Various gradients are tightly correlated
  ➢ Enables some unique experimental manipulations
  ➢ Ideal for mathematical modeling

• Experimental control
  ➢ External environment controlled
  ➢ Reproducible manipulation of experimental conditions
  ➢ Easy to manipulate individual spheroids
  ➢ High ‘data density’
Research applications of spheroids

• Therapy testing and mechanistic studies
• Basic tumor biology
  ➢ Cell cycle regulation
  ➢ Metabolic regulation
  ➢ Cellular physiology
  ➢ Cell-cell interactions
  ➢ Regulation of gene/protein expression
  ➢ Malignant progression
• Co-cultures
  ➢ Tumor-normal cell mixtures
  ➢ Angiogenesis models
• Non-cancer applications
  ➢ Artificial organ research
  ➢ Drug production
  ➢ Normal tissue models
Example: Cell Cycle Regulation

• Despite common (mis)conception that malignant cells have escaped growth control, majority of tumor cells in a solid tumor are not proliferating
• Common (mis)dogma is that cell cycle arrest in tumors is due to lack of nutrients, specifically oxygen
• Although recent imaging and molecular techniques have documented spatial distribution of proliferation in rodent and human tumors, controlled manipulation and mechanistic experiments are not possible
• Actual molecular mechanism of cell cycle arrest in tumors is currently unknown
• Spheroids are a good *in vitro* model to perform mechanistic studies on this question
Multicellular Tumor Spheroids

250,000 cells/spheroid

Fraction 1
Fraction 2
Fraction 3
Fraction 4
Necrosis

Distance from Surface
(μm)

Fraction of Cells Remaining in Spheroid

Time of Dissociation (minutes)
Cell Cycle Proteins in Spheroids

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<td>p21</td>
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<td>CDK4</td>
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<td>cyclins</td>
<td>cycA</td>
<td>cycE</td>
<td>cycD1</td>
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**Graphs:**
- Relative CKI Protein (fraction 1 = 1)
- Relative CDK Protein (fraction 1 = 1)
- Relative Cyclin Protein (fraction 1 = 1)

**Distance from Surface (μm):**
- 0
- 0.5
- 1
- 1.5

**Relative Protein Levels:**
- p18
- p21
- p27
- CDK2
- CDK4
- CDK6
- Cyclin A
- Cyclin D1
- Cyclin E
G1- Versus S-phase Arrest

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EMT6

Mel28

outer → inner

Distance from Surface (μm)

S-phase Fraction (percent)

DNA content

BrdU Uptake

DNA Content

BrdU Uptake

0 50 100 150 200

0 10 20 30 40 50 60

0 50 100 150 200

0 10 20 30 40 50 60

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Cell Cycle Arrest After Acute Oxygen Deprivation

- **O2** → **N2**

**Graphs:**
- **Relative Cell Number**
  - Time of Culture (hours): 0, 5, 10, 15, 20, 25
  - Relative Cell Number: 0.5, 1.5, 2.5, 3.5, 4.0
  - Oxygen: blue dots, Nitrogen: red dots

- **Fraction of Cells (percent)**
  - G1, S, G2
  - Time of Culture (hours): 0, 5, 10, 15, 20, 25
  - Fraction of Cells (percent): 0, 25, 50, 75, 100

- **Relative Protein Level**
  - p18, p21, p27
  - Time of Culture (hours): 0, 5, 10, 15, 20, 25
  - Relative Protein Level (0 hr = 1.0): 1.0, 2.0, 3.0, 4.0
Regulation of Proliferation in Spheroids

• **Initial arrest is an *active* process regulated by a cyclin/CDK mechanism**
  - Little change in CDKs, loss of cyclin D1
  - Upregulation of p18 and p27, loss of p21
  - CKI binding to and inhibition of CDK activity
  - Bypassing initial G1-arrest allows S-phase arrest

• **Interior arrested cells continue to undergo alterations in cell cycle regulatory machinery**
  - Loss of all regulatory molecules: CDKs, cyclins, CKIs
  - May explain prolonged recovery lag time: unable to resume without rebuilding?

• **Inducers of initial arrest currently unknown**
  - Several CKIs, up- and down-regulated: multiple signals?
  - Initiated relatively close to surface (~50 μm)
  - Unlikely to be related to oxygen deprivation
  - Growth factor or inhibitor? Pressure sensing?
Limitations to Current Spheroid Model Systems

- Only mimics chronic nutrient deprivation
- Difficult for *in situ* assay of microenvironmental gradients (microelectrodes, histology)
- Separation of cells from different locations involves relatively long enzymatic treatment (complicates gene and protein expression data)
- Only applicable to adherent cells and those that proliferate in aggregate culture
- Difficult to use for controlled, reproducible experiments with co-cultures
Transient Deprivation System for Spheroids

The diagram illustrates a system for transient deprivation of oxygen. The system involves two chambers, one with 0% oxygen and the other with 20% oxygen. The oxygen partial pressure is plotted against time after switching from O2 to N2, showing a decrease in oxygen partial pressure over time.

The graphs on the right depict the oxygen partial pressure over time, with one graph showing the transition from O2 to N2 and the other showing periodic fluctuations in oxygen partial pressure over time.

The text also mentions the time of culture and oxygen partial pressure in mm Hg.
Effects of Transient Oxygen Deprivation

- 30 minutes oxygen
- 30 minutes nitrogen
- 30 minutes oxygen

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<th>Fraction of cells (percent)</th>
<th>G1</th>
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<td>Relative CKI Protein (fraction 1 = 1)</td>
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<tr>
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<th>Total Volume</th>
<th>Cell Number</th>
<th>Viable Rim</th>
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<td>12 hr cycle</td>
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Transient Nutrient Deprivation in Spheroids

• New culture system developed and validated for transient deprivation experiments
  ➢ Compact, portable culture chamber
  ➢ Ability to rapidly alter nutrient conditions
  ➢ Imposes external transient supply on pre-existing chronic gradients: more like tumor in vivo

• Preliminary experiments show essentially no effect of cyclic oxygen supply for up to 12 hours
  ➢ No change in spheroid growth rate or cell number
  ➢ No increase in central necrosis
  ➢ No alteration in cell cycle or CKI induction

• Preliminary experiments show remarkable resistance to nutrient deprivation
  ➢ Complete nutrient deprivation causes total loss of ATP and extremely acidic intracellular pH
  ➢ Complete recovery of normal cellular energetics after nutrient restoration
New *In Vitro* Model of Tumor Microenvironment
Preliminary Data with 1st Generation System

- **Cell Concentration**
  - $\times 10^{-7}$ cells per cm$^3$

- **Distance from Membrane** (mm)

- **S-phase Fraction** (percent)

- **Clonogenic Efficiency** (percent)

- **Relative p27 Protein** ($4° @ 0.4 \text{ mm} = 1.0$)

- **Distance from Membrane** (mm)
Current State of New Model System

• Demonstration of feasibility of design
  ➢ Spatial correlation of microenvironment and biology
  ➢ Potential for real-time, in situ measurement by NMR
  ➢ Allows rapid isolation of cells from different regions
  ➢ Experimental control over many parameters

• Produces physiological gradients similar to those seen in spheroids and tumors
  ➢ Cell proliferation and cell cycle distribution
  ➢ Cell death
  ➢ Induction of CKIs

• 1st generation system has problems
  ➢ Difficult and non-reproducible separation of cells from different regions, still requires matrix digestion
  ➢ No control over internal supply conditions
  ➢ Relatively low cell number to get extended gradients
Theoretical Modeling of Tumors

- Overwhelming majority of literature based on mathematical models of tumor growth and development (~1200 papers since 1970)
- Interestingly, spheroid growth data very often used to ‘test’ models
- Limited development in other areas
  - Interactions with immune system
  - Regulation of cellular metabolism
  - Extracellular biochemical environment
  - Cellular invasion
  - Therapy response (radiation, chemo)
  - Protein regulatory networks
- Recent focus on developing biologically-based models of tumor growth and malignant progression
Modeling Hypoxia in Tumors

Modeling Hypoxia in Tumors

Modeling Angiogenesis in Tumors

Penetration of Chemotherapy Agent

Protein Network Model of Tumor Cell Invasion

Nested Deterministic Models of Tumor Growth

Two-parameter Models

- Generalized two-parameter
  \[ V' = aV^\alpha - bV^\beta \]
  
  \[ a = b \]
  \[ \beta = 1 \] (\(a = 1\))

- Generalized Gompertz
  \[ V' = aV^\alpha - bV^\beta \ln V \]
  
  \[ a = 1 \]

- Generalized Bertalanffy logistic
  \[ V' = \frac{aV^\alpha - bV^\beta}{V} \]
  
  \[ a < 1 \]
  \[ a = 1 \]
  \[ a > 1 \]

- Gompertz
  \[ V' = aV^\alpha - bV^\beta \]

- Logistic
  \[ V' = aV^\alpha - bV^\beta \]

- Bertalanffy
  \[ V' = aV^\alpha - bV^\beta \]

Functional Models

- Plantedi
  \[ \frac{V}{V} = \frac{1}{\left(1 + bV^\gamma\right)} \]
  
  \[ a = 0 \]
  \[ \gamma = 1 \]

- Autostimulation
  \[ \frac{V}{V} = \frac{1 + S}{1 + bV} - w \]
  
  \[ S' = aV^\alpha - bS^\beta \]

- Inhibition
  \[ \frac{V}{V} = \frac{1}{1 + bV} - w \]

Generic Models

- Hyper-Gompertz
  \[ V' = aV^\alpha \left(\frac{V}{V_0}\right)^\beta \]
  
  \[ r = 0 \]

- Hyper-logistic
  \[ V' = \frac{aV^\alpha}{1 - bV} \]
  
  \[ r = 0 \]

- Bertalanffy-Richards
  \[ V' = aV^\alpha \left(V_0 - V\right) \]
  
  \[ r = 0 \]

Marusic et al., *Cell Prolif.* 27: 73, 1994
Fits of 15 Models to 15 Independent Data Sets

Marusic et al., *Cell Prolif.* 27: 73, 1994
**Fits of 15 Models to 15 Independent Data Sets**

### Doubling Time

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### Thickness of Viable Cell Rim

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Marusic et al., *Cell Prolif.* 27: 73, 1994
Deterministic Tumor Models

• Wide variety available and more being developed
• Most can do a good job of fitting basic tumor (spheroid) growth data
• Useful for graphing, comparing and extrapolating data
• Most do a poor job of predicting any biological parameters
• Not really useful for advancing our understanding of tumor biology
  ➢ Generally not predictive
  ➢ Many not directly connected to biology
  ➢ Those that are have a very large number of parameters
  ➢ Difficult to distinguish one from the other
• The future of this field is in biologically-based models
Conceptual Model of Spheroid Growth Regulation

Freyer & Sutherland, *Cancer Res.* 46: 3504, 1986
Multi-Scale Mathematical Tumor Model

- Starts with single cell on 3-D lattice
  - ‘Programmed’ with metabolic, gene regulation, cell cycle, volume growth rate, adhesion and cell death parameters
  - Assumes limited inward growth factor penetration and internal growth inhibitor production
  - Simulation runs until lattice is filled or spheroid saturates: nothing ‘fit’ or constrained

- Three scales considered
  - Cellular (lattice Monte Carlo)
  - Gene regulation (Boolean network)
  - Extracellular (reaction-diffusion equations)
Final Conclusions

• Solid tumors are perhaps the most unique, complex, dynamic and chaotic biological system
• The tumor microenvironment is extremely heterogeneous, both spatially and temporally
• This microenvironmental complexity explains most therapy failures, as well as promotes the progression of malignancy itself
• Actual tumors in vivo are poorly suited to mechanistic experimentation
• Many 3-D in vitro experimental tumor models are available and important for advancing tumor biology
• Spheroids are an excellent tumor model system, but have limitations
• Theoretical modeling of tumors is in its infancy, but can contribute significantly in cancer research
Acknowledgements

• Spheroid projects
  ➢ Dr. Karen LaRue
  ➢ Antoinette Trujillo
  ➢ Anabel Guerra
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