Bleomycin: A Study of DNA Damage and the Cell Cycle

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Radiation
Objectives of Internship

• Enhance proficiency in cell culture (human fibroblast cells)
• Become familiar with assay and analysis techniques
  • Immunofluorescence assays, fluorescence microscopy, DNA microarray analysis
• Apply learned techniques to further bleomycin ground study in preparation for parallel in-flight study
• Learn about space travel and science in space from SLSSI lectures
Background

• Space is an environment filled with radiation
  ▪ Trapped protons, Galactic Cosmic Rays (GCRs), Solar Particle Events (SPEs)

• Assess astronaut’s radiation risk
  ▪ How cells respond to radiation
  ▪ More important for long-term missions and missions outside of Near Earth Orbit (NEO)

• Question: How is DNA repair altered in microgravity compared to on Earth?
  ▪ A controlled source of radiation is desired
  ▪ Funded by NASA Fundamental Space Biology Program
The Space Radiation Environment

- **SOLAR PARTICLE EVENT** (Protons to Iron Nuclei)
- **GALACTIC COSMIC RADIATION (GCR)** (Protons to Iron Nuclei)
- **OUTER RADIATION BELT** (Electrons)
- **INNER RADIATION BELT** (Protons)
- **SOUTH ATLANTIC ANOMALY** (Protons)

Space radiation: Energetic charged particles, high-LET (linear energy transfer)
Bleomycin

- Chemotherapy antibiotic
- Able to induce DNA double-strand breaks (DSBs) – radiomimetic
- Controlled source of DNA damage
γ-H2AX Assay and 53BP1 Assay

• Quantification of DSBs
  • Creation of foci as a result of DSBs from ionizing radiation
  • H2AX – histone component variant
    ▪ Phosphorylation of serine residue indicates DSB
    ▪ Previous study tested high concentrations of bleomycin (0-80 µg/mL) and found little dose response (Venkatachalam et. al, 2008)

• 53BP1(p53-binding protein 1): protein involved in DNA repair and tumor suppression
  ▪ Indirect quantification of DSBs
Cell Cycle Markers

- Determine if level of DNA damage correlates to stages of cell cycle
- Cyclin D1 and E: mid-to-late G\textsubscript{1} phase
- Cyclin A: high expression S phase, low expression in early G\textsubscript{2} phase
- Cyclin B1: G\textsubscript{2} phase. Expressed in nucleus in early M phase
- Phosphohistone H3: M phase
- Identify cell cycle stage that is best to study DNA damage and nuclear foci production
Bleomycin Treatment

• Growth of fibroblast cells in six 8-well tissue culture slides

• Once cells were 90% confluent, bleomycin treatment was performed
  • 0, 0.1, 1, and 10 µg/mL for 3 hours

• Cells fixed with 4% paraformaldehyde

• Immunofluorescence staining:
  1: γ-H2AX and 53BP1
  2: γ-H2AX and Cyclin A (S phase)
  3: γ-H2AX and Cyclin B1 (G2 phase)
  4: γ-H2AX and Phosphohistone H3 (M Phase)
  5: γ-H2AX and Cyclin D1 (G1 phase)
  6: 53BP1 and Cyclin D1 (G1 phase)
Results: $\gamma$-H2AX and 53BP1 Classification

Classification of cell types:

- Type I: Bright, pannuclear stain
- Type II: Uncountable bright foci or near pannuclear stain
- Type III: Countable foci
- Type IV: (only applies to 53BP1 analysis) $\gamma$-H2AX signal present, but observation of weak or no 53BP1 signal
Results: γ-H2AX and 53BP1 Foci Counts

- Distribution of foci counts: unimodal
- One type for all cell with countable foci

![53BP1](image1)
![γ-H2AX](image2)

**53BP1**: Cell Distribution of Foci Counts

**γ-H2AX**: Cell Distribution of Foci Counts

- 0 µg/mL
- 0.1 µg/mL
- 1 µg/mL
- 10 µg/mL
Results: $\gamma$-H2AX and 53BP1 Foci Counts

- Slight dose response for 53BP1, but not for $\gamma$-H2AX
- All significant changes except between 0.1 and 1 for $\gamma$-H2AX
Results: γ-H2AX and 53BP1 Foci Counts

As dose increases, Type I and II for γ-H2AX increase and Type I for 53BP1 increases.
Results: Cell Cycle Study

- Cyclin D1: G₁ phase
- Almost all cells are Type III
- Dose response observed for γ-H2AX foci
Results: Cell Cycle Study

- Cyclin A: S phase
- Most cells are Type III
- No dose response observed, due mainly to the high background in the control

**Cyclin A: Dose vs. Cell Type Distribution**

**Cyclin A: Dose vs. Average Number of Foci**

- Cyclin A
- γ-H2AX
- Cyclin A and γ-H2AX
DNA Microarray

- Analyzed microarray data from previous microarray samples
  - GenePix4000B for data acquisition
  - GenePix Pro 7 for data retrieval
  - Microsoft Excel and EASE for analysis
  - 0, 0.1, 1, and 10 (µg/mL)
  - Up-regulated or down-regulated

DNA microarray system:
  - Human GE 4x44K v2 Microarray
  - ~44,000 transcripts
  - Target 27,958 Entrez Gene RNAs
Significant Genes

- Major categories affected (all concentrations):
  - Apoptosis (cell death, programmed cell death, death)
  - Regulation of Cell Proliferation
  - DNA Repair or Response to DNA Damage/Endogenous Stimulus
Significant Genes

- **CDKN1A**
- **IL1B**
- **PMAIP1**
- **TNFRSF10D**
- **TNFSF9**
- **MDM2**
- **PPM1D**
- **PGF**
- **DDB2**
- **GADD45A**
- **PCNA**
- **POLH**
- **BTG2**
- **TOB1**
- **HTRA3**
- **ESR2**
- **IL18**
- **IFNG**
- **DDX11**

**10 µg/mL**

- Apoptosis and Cell Cycle Arrest
- Cellular Processes and Antiproliferative Effects

**1 µg/mL**

- DNA Damage Repair

**0.1 µg/mL**

- Cellular Processes and Immune Responses

**1 µg/mL**

- DNA Damage Repair

**0.1 µg/mL**

- Cellular Processes and Immune Responses

**10 µg/mL**

- Apoptosis and Cell Cycle Arrest
- Cellular Processes and Antiproliferative Effects
Significant Genes

- **CDKN1A**
  - Regulates G1 checkpoint by inhibiting activity of cyclin-CDK4 and cyclin-CDK2
  - Involved in p53 pathway in response to DNA damage
- **MDM2**
  - Part of autoregulatory negative feedback loop of the p53 pathway
  - Component in complex which links growth factor and DNA damage response pathways

***CDKN1A Dose Response***

- Absorption Ratio vs. Bleomycin Dose (µg/mL)

***MDM2 Dose Response***

- Absorption Ratio vs. Bleomycin Dose (µg/mL)
Significant Genes

- **GADD45A**
  - Increase in transcription usually follows environmental stresses and will lead to activation of the p38/JNK pathway
  - Activation of this gene due to DNA damage is mediated by p53 and other pathways

- **DDB2**
  - Required for DNA repair (especially UV-induced DNA repair)
  - Ubiquitination of histones H3 and H4 which aids cellular response to DNA damage
Conclusions

- 53BP1 and γ-H2AX are typically used markers for DNA DSBs caused by ionizing radiation, as indicated by colocalization of nuclear foci. However, similar to UV radiation, cells with bleomycin-induced DNA damage generally have more γ-H2AX foci than 53BP1 foci.

- 53BP1 and γ-H2AX have both shown dose dependent characteristics in identifying damage-induced foci. 53BP1 may be a better for DSBs because it showed a dose response more consistently.

- In terms of the cell cycle, cells in G₁ phase tend to have fewer damage-induced foci than cells in S phase.

- In general, an increase in number of genes involved in apoptosis, gene repair, and regulation of cellular processes is seen as the dose of bleomycin increases.

- Based on these data, we established that a concentration of 1 µg/mL should be used for the flight study. In addition, cells in the G₁ phase should be analyzed for comparison to the ground study results.
Future Directions

- Conduct further gene studies based on DNA microarray data including pathway analysis and cluster analysis.
- Test bleomycin treatment for different lengths of time and allow specific time intervals for DNA repair to occur following treatment.
- Complete and analyze remaining cell cycle stains in order to conclude that ground and flight analysis should be completed based on G₁ phase cells.
- Become a practicing physician and aid the space industry in finding countermeasures to mitigate the effects of spaceflight on humans.
Acknowledgements

• Dr. Honglu Wu
• Dr. Ye Zhang
• Radiation Lab

• Judith Hayes
• Dr. Lauren Merkle
• Blythe Starkey
• Jackie Reeves
• Jan Connolly

• Dr. Ron McNeel
• Dr. Amanda Hackler

This work is partially funded by National Space Biomedical Research Institute via NASA Cooperative Agreement NCC 9-58