Bleomycin: A Study of DNA Damage and the Cell Cycle

John Jeevarajan
Northwestern University
Dr. Honglu Wu
Dr. Ye Zhang
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

- Galactic Cosmic Radiation (GCR) (Protons to Iron Nuclei)
- South Atlantic Anomaly (Protons)
- Inner Radiation Belt (Protons)
- Outer Radiation Belt (Electrons)
- Outer Radiation Belt (Electrons)
- Solar Particle Event (Protons to Iron Nuclei)

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 $G_1$ phase
- Cyclin A: high expression $S$ phase, low expression in early $G_2$ phase
- Cyclin B1: $G_2$ 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: γ-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) γ-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

![Images of 53BP1, γ-H2AX, and 53BP1 and γ-H2AX with corresponding bar graphs showing cell distribution of foci counts for γ-H2AX and 53BP1 at different bleomycin concentrations (0, 0.1, 1, and 10 µg/mL).]
Results: γ-H2AX and 53BP1 Foci Counts

- Slight dose response for 53BP1, but not for γ-H2AX
- All significant changes except between 0.1 and 1 for γ-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.

γ-H2AX: % of Cells Per Type

53BP1: % of Cells Per Type
Results: Cell Cycle Study

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

Cyclin D1: Dose vs. Cell Type Distribution

Cyclin D1: Dose vs. Average Number of Foci

Cyclin D1 and γ-H2AX
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
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

10 µg/mL
Apoptosis and Cell Cycle Arrest
CDKN1A
IL18
IFNG
DDX11

1 µg/mL
Cellular Processes and Immune Responses
IL18
IFNG
DDX11

0.1 µg/mL
Cellular Processes and Antiproliferative Effects
CDKN1A
IL1B
PMAIP1
TNFRSF10D
TNFSF9
MDM2
PPM1D
PGF

DNA Damage Repair
DDB2
GADD45A
PCNA
POLH

National Aeronautics and Space Administration
Bleomycin: A Study of DNA Damage and the Cell Cycle
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

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<th>Absorption Ratio</th>
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### MDM2 Dose Response

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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.
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