Bleomycin: A Study of DNA Damage and the Cell Cycle

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Bleomycin: A Study of DNA Damage and the Cell Cycle
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)
- **SOLAR PARTICLE EVENT** (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 $G_1$ 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

53BP1

γ-H2AX

53BP1 and γ-H2AX

γ-H2AX: Cell Distribution of Foci Counts

53BP1: Cell Distribution of Foci Counts
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**

**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

10 µg/mL
- Apoptosis and Cell Cycle Arrest
  - CDKN1A
  - IL1B
  - PMAIP1
  - TNFRSF10D
  - TNFSF9
  - MDM2
  - PPM1D
  - PGF
  - DDB2
  - GADD45A
  - PCNA
  - POLH

1 µg/mL
- DNA Damage Repair
  - BTG2
  - TOB1
  - HTRA3
  - ESR2
  - IL18
  - IFNG
  - DDX11

0.1 µg/mL
- Cellular Processes and Antiproliferative Effects
  - CDKN1A
  - IL1B
  - PMAIP1
  - TNFRSF10D
  - TNFSF9
  - MDM2
  - PPM1D
  - PGF
  - DDB2
  - GADD45A
  - PCNA
  - POLH

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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
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 G<sub>1</sub> 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 G<sub>1</sub> 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 $G_1$ 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