Investigating a Role for DNA Mismatch Repair in Signaling a Benzo[a]pyrene Diol Epoxide-Induced DNA Replication Arrest

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Cancer affects everyone

Economic impact of cancer in 2007
Total : $226.8 billion
Direct medical costs : $103.8 billion
Indirect mortality costs : $123.0 billion
Source: NIH, American Cancer Society website (www.cancer.org)
Colon cancer is the second leading cause of cancer death

- In 2011, nearly 50,000 deaths in the U.S., many of which were preventable
- 5-year survival rates range from 6%-74%
- Early detection is vital
Factors that increase cancer risk

• Mutations in critical genes
  Mismatch repair deficiency
  (Lynch syndrome)

• Exposure to carcinogens
  Polycyclic aromatic hydrocarbon (PAH) exposure
Mismatch repair

• Highly conserved pathway focused on repair of DNA replication errors
• Conserved proteins include MLH1, PMS2, MSH2, MSH6
• MMR deficiency has significant impacts on human health (increased cancer risk)
MMR recognizes and repairs DNA replication errors

[Diagram showing DNA bases and proteins involved in MMR]
PAHs – they’re everywhere
Many studies link PAHs to cancer

- Scrotal tumors in chimney sweeps
- Lung cancers in smokers
- Colon cancer associated with grilled meat consumption
- Coal-tar induced skin tumors (mice)
- *In utero* PAH exposure linked to cancer as adult in mice
PAHs vary in structure and carcinogenicity

(From Neff, 1979; CCREM, 1987; NRCC, 1983; USPHS, 1990)
Benzo[a]pyrene – a model PAH

- Best known and most studied of PAHs
- Gold standard of PAH carcinogenicity
- Produced during combustion of organic compounds
- Recalcitrant pollutant - bioaccumulates
- Detected in air, water, food and soil
<table>
<thead>
<tr>
<th>Sample</th>
<th>PPB (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBQ steak, very well done</td>
<td>4.75</td>
</tr>
<tr>
<td>BBQ chicken (bone and skin), well done</td>
<td>4.57</td>
</tr>
<tr>
<td>BBQ hamburger, medium cooked</td>
<td>0.56</td>
</tr>
<tr>
<td>Pumpkin pie</td>
<td>0.47</td>
</tr>
<tr>
<td>Fast food french fry</td>
<td>0.22</td>
</tr>
<tr>
<td>Tomato (fresh)</td>
<td>0.19</td>
</tr>
</tbody>
</table>

BaP biotransformation into ultimate carcinogen

- Benzo[a]pyrene
- (+)Benzo[a]pyrene-7,8-epoxide
- (-)Benzo[a]pyrene-7,8-dihydrodiol
- (+)Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide
BPDE bonds to DNA and forms a bulky adduct

BPDE Lesion on DNA

Image courtesy of Zephyris

B[a]P-Adducted Guanine

Image courtesy of Peter Hoffman
Consequences of BaP-Derived Adducts
Two responses to BaP adduct

• Local (translesion polymerase recruitment)

• Global (reduction in DNA synthesis rate)
S-phase checkpoint helps ensure replication fidelity

- A normal response to DNA damage
- If damage cannot be resolved, cell may remain quiescent or signal for apoptosis
- Inability to activate checkpoint can compromise fidelity of DNA replication
Phases of the Cell Life Cycle

- **G1**: Organelle synthesis
- **S**: DNA Replication
- **G2**: Growth and protein synthesis
- **M**: Mitosis (Cell Division)
BPDE-induced S-phase checkpoint signaling

BaP-DNA adduct

 atolad replication fork

ATR

Chk1

MMR

Reported Involvement

Apoptosis

Inhibition of firing at origins of replication

DNA repair
Is S-phase checkpoint a mechanism by which MMR suppresses BPDE-induced mutations?

• Increased mutations seen in MMR-deficient cells exposed to BPDE
• BPDE induces S-phase checkpoint
• S-phase checkpoint suppresses mutations
• MMR is necessary for S-phase checkpoint induced by ionizing radiation and alkylating agents
Hypothesis

MMR is necessary for the activation of BPDE-induced S-phase checkpoint
Predictions

MMR-deficient cells will show reduced activation of S-phase checkpoint in response to BPDE exposure

- MMR-deficient cells will display lower levels of PChk1
- PChk1 can be measured using semi-quantitative immunoblotting
- Differences in S-phase checkpoint activation can be observed by flow cytometry
Model system: MMR deficient and proficient cell lines

- **HCT116 – 2 defective copies of MLH1 (Chr. 3)**
- **WT MLH1 Chr. 3 + neomycin resistance gene**
- **HCT116+3 – 2 defective copies of MLH1 (Chromosome 3) + 1 copy of WT MLH1 + neomycin resistance gene**
Experimental procedure

Cultured cells: HCT116, HCT116+3

BPDE treatment

Fixing and PI staining cells

Harvest of cells

Whole cell lysates

Flow cytometry

Protein immunoblot
Predicted results

Flow cytometry

HCT116+ch3
DMSO (control)

BPDE

S-phase arrest

BPDE

Protein Immunoblots

HCT116
DMSO  BPDE

PChk1

HCT116+ch3
DMSO  BPDE

PChk1
Results outline

• Confirmation of MLH1 expression in cell lines
• GAPDH loading control
• PChk1 accumulation
• Positive control for PChk1
• Flow cytometry
Confirmation of MLH1 expression in HCT116 vs. HCT116+ch3 cells

<table>
<thead>
<tr>
<th></th>
<th>MLH1</th>
<th>MSH6</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>Neg</td>
<td>Pos</td>
</tr>
<tr>
<td>HCT116+3</td>
<td>Pos</td>
<td>Pos</td>
</tr>
</tbody>
</table>

[Image: Western blot showing MLH1 and MSH6 expression levels in different conditions.]
What is the upper limit of the linear dynamic range of GAPDH signal?

GAPDH is not useful as a loading control when quantities greater than 30 μg are loaded.
BPDE-induced PChk1 accumulation

Immuno-blot probed with anti-PChk1 (S345) polyclonal antibody

- DMSO (control)
- 100 nM BPDE
- 200 nM BPDE

MW (kDa)
- 75
- 50
- 37

Time following exposure
MLH1 Status

<table>
<thead>
<tr>
<th>MW (kDa)</th>
<th>24</th>
<th>48</th>
<th>24</th>
<th>48</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>Def Pro</td>
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<td>Def Pro</td>
</tr>
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</table>

- PChk1 accumulation similar in MLH1-proficient and deficient cells
- HCT116 cells show sustained PChk1 accumulation relative to HCT116+3 cells
- .
BPDE-induced S-phase checkpoint activation in MLH1-proficient and deficient cells

<table>
<thead>
<tr>
<th>Time after Exposure (hours)</th>
<th>MLH1-Deficient</th>
<th>MLH1-Proficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BPDE 100 nM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BPDE 200 nM</td>
<td></td>
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<tr>
<td>24</td>
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<tr>
<td>48</td>
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</table>

DMSO: Dimethyl Sulfoxide
Selecting gel type

- Nonspecific signal (NS) in 4-12% Bis-Tris gels interfered with PChk1 signal
- Could this problem be resolved with a different gel?
### 7.5% Tris HCl gel used for identification of putative PChk1 signal

<table>
<thead>
<tr>
<th>MW (kDa)</th>
<th>DMSO (control)</th>
<th>200 nM BPDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>24 Def Pro Def Pro</td>
<td>24 Def Pro Def Pro</td>
</tr>
<tr>
<td>50</td>
<td></td>
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**Marker**

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
<td></td>
<td></td>
<td>PChk1</td>
</tr>
</tbody>
</table>

- Similar PChk1 accumulation observed in previous gel
- Excellent signal resolution and band separation
BPDE exposed MLH1-proficient and – deficient cells with earlier timepoints

- Signal fairly uniform within cell lines
- Is this signal really PChk1?
Positive control for PChk1

- HeLa cells treated with 25 μM etoposide or DMSO (solvent control)
- Repeated exposure four times
- Putative PChk1 signal weak at best
- Results were inconsistent

Etoposide (μM)

<table>
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<tr>
<th></th>
<th>0</th>
<th>25</th>
<th>0</th>
<th>25</th>
</tr>
</thead>
</table>

NS
PChk1
Protein immunoblots

- Preliminary results suggested MMR is not required for S-phase checkpoint activation
- This was not observed in subsequent experiments
- Many blots had technical problems, some of which remain unresolved
- Ultimately we were not sure if the antibody detected PChk1
Flow cytometry

- Cells are counted and their viability determined by lasers
- Cellular DNA content is measured by intensity of propidium iodide (PI) fluorescence
- Peak nearest origin is G1; peak twice as far from origin is G2
- Mathematical models used to fit curves of the histogram
- Area under curves used to estimate percentage of cells in each phase
Technical issues with flow cytometric analysis

HCT116+ch3 (MLH1-proficient) cells exposed to DMSO

- DMSO control populations showed abnormal cell cycle distribution
- Similar distribution in HCT116 (MLH1-deficient) samples
Comparison of flow cytometric profiles of MLH1-proficient cells treated with BPDE and DMSO

- No consistent differences between two treatment groups apparent
- MLH1-deficient cell populations gave similar results
Flow cytometry

- Difficulty in fitting cell cycle patterns to Dean-Jett-Fox model
- Abnormal profiles of control cells – likely technical issues
- No consistent differences found between cell lines
Conclusions

- Preliminary results suggest sustained S-phase arrest in MLH1-deficient cells
- Preliminary results suggest MMR is not necessary for S-phase checkpoint activation
- No pattern in S-phase checkpoint activation determined from flow cytometry
Technical changes for future experiments

- Subculture and synchronize cultures before exposure using non-chemical methods
- Use siRNA to create MMR-deficient cell lines
- Use PChk1 as positive control
Benefits of research

• Identify S-phase checkpoint as a mechanism by which MMR suppresses mutations
• Understand how MMR deficiency and PAH exposure interact to increase mutation risk
• Identify individuals most vulnerable to accumulation of mutations
• Help direct intervention efforts to vulnerable individuals
Future research

• Investigate other markers of S-phase checkpoint activation
• Analyzing downstream effects of prolonged checkpoint activation
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