Non-targeted effects *in vivo*: from radiation to growing tumors, a unifying model?

*Alexandros Georgakilas, PhD*

*Department of Physics, School of Applied Physical Sciences, National Technical University of Athens*
Effects of track structure on the direct ionization of DNA (left panel) and on damage arising from the indirect action of reactive chemical species that diffuse and interact with the DNA (right panel).

\[ \cdot \text{OH} \text{ formed through the ionization of water diffuse on average about 4-6 nm as suggested in (100) in a cellular milieu. The random 'walk' of the \cdot \text{OH} may result in indirect damage to any one of at least 100 nucleotides.} \]
Types of DNA Damage

- Undamaged DNA segment
- Simple SSB (1 lesion)
- Simple DSB (2 lesions)
- Complex SSB (6 lesions)
- Complex DSB (11 lesions)
- Base damage (5 lesions)

Radiation

Abasic Clusters

- AP Endonucleases: human APE1 or APE2, E.coli Nfo protein.

Oxybase Clusters

- Glycosylases: Human OGG1/Nth1, or E.coli Fpg/Nth1
Detection of OCDLs in Human Cells
Detection of Bistranded Oxidative Clustered DNA Lesions (OCDLs) using:

Different Types of DNA Electrophoresis (SAGE and PFGE)

Neutral and Alkaline Comet Assay

Enzymatic Probes (Repair Enzymes)

Number Average Length Analysis (NALA)
Single Cell Gel Electrophoresis ‘Comet Assay’

Tail Moment = (TAIL LENGTH) x Tail % DNA / 100

Sensitivity 0.1-4 Gy for Alkaline Neutral 2-10 Gy
Detection of DSBs and OCDLS Acute Lymphoblastic Leukemia (NALM-6) Cells after exposure to 2-4 Gy of $\gamma$-Rays using modified Comet Assay:

Main mechanistic insights and conclusions from our studies on the repair of OCDLs:

<table>
<thead>
<tr>
<th>Lesions</th>
<th>SSBs</th>
<th>DSBs</th>
<th>OCDLs</th>
</tr>
</thead>
<tbody>
<tr>
<td>BER</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HR</td>
<td>+</td>
<td>+</td>
<td>+(BRCA1)</td>
</tr>
<tr>
<td>NHEJ</td>
<td>+</td>
<td>+</td>
<td>+(DNA-PK)</td>
</tr>
<tr>
<td>MMR</td>
<td></td>
<td></td>
<td>+(MSH2)</td>
</tr>
<tr>
<td>NER</td>
<td></td>
<td></td>
<td>?</td>
</tr>
</tbody>
</table>

Non-targeted effects

Main conclusion: DSB repair deficient cells exhibit a parallel OCDL repair deficiency, increased apoptosis and chromosomal instability (In collaboration with Dr. Pantelias).

Clinical studies perspective with Attikon Hospital on long term radiation effects

- 40 breast cancer patients undergoing radiation therapy
- Establish oxidative clustered DNA damage as reliable biomarker for long term effects of radiation in patients
- Complementary analysis:
  - Lipidomics (Dr. Chatgilialoglou group in Bologna)
  - Bystander –Non-targeted effects- Clastogenic factors
  - Use of antioxidants/Anti-inflammatory drugs
  - Inflammation ?
  - miRNA ?
Recent Results

Measuring DSBs in human breast cancer MCF-7 cells using the γ-H2AX assay.

Cells were exposed to 0.5-1 Gy of γ-rays and co-stained with APE1-antibody (abasic sites; green foci) and γ-H2AX for detection of DSBs (red foci). This approach can be used for the in situ detection of clustered DNA lesions. It is estimated that each DSB is accompanied by 5-10 abasic sites/DSB.
In collaboration with Dr. Pantelias group

γ-H2AX method on human lymphocytes

![Graph showing the number of foci per cell against dose (Gy) for donors A and B. The graph includes a linear regression line with the equation $y = 7.725x + 1.025$ and $R^2 = 0.994$. The number of foci per cell ranges from 0 to 20, and the dose (Gy) ranges from 0 to 2.5. The graph also indicates that the number of foci per cell is below 0.5 Gy for doses less than 0.5 Gy.]
Repair of foci - DSBs

Average from donors A, B

Number of foci per cell

Repair time (hrs)

1 Gy
0 Gy

Repair of foci - DSBs

"0" h 6 h 12 h 24 h 48 h

0 1 2 3 4 5 6 7 8 9 10
We investigated the effect of low dose X-ray radiation on 5 interventional cardiologists and 1 patient who underwent a percutaneous coronary intervention (PCI). Lymphocytes in blood samples were used for chromosomal aberrations detection.

<table>
<thead>
<tr>
<th></th>
<th>Aberrations/ cell</th>
<th>Estimated Dose (mGy)</th>
<th>95% CL</th>
<th>Hp 3 (mSv)</th>
<th>Hp 12 (mSv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doctor A</td>
<td>0</td>
<td>0</td>
<td>0 - 0.1227</td>
<td>0.62</td>
<td>2.99</td>
</tr>
<tr>
<td>Doctor B</td>
<td>0.0038</td>
<td>127</td>
<td>0.0192 – 0.2918</td>
<td>2.67</td>
<td>4.24</td>
</tr>
<tr>
<td>Doctor C</td>
<td>0.0020</td>
<td>68</td>
<td>0 – 0.2116</td>
<td>4.16</td>
<td>13.12</td>
</tr>
<tr>
<td>Doctor D</td>
<td>0.0010</td>
<td>28</td>
<td>0 – 0.1730</td>
<td>2.82</td>
<td>7.03</td>
</tr>
<tr>
<td>Doctor E</td>
<td>0.0020</td>
<td>68</td>
<td>0 – 0.2116</td>
<td>0.87</td>
<td>5.41</td>
</tr>
<tr>
<td>Patient</td>
<td>0.0070</td>
<td>207</td>
<td>0.0959 – 0.3463</td>
<td>96</td>
<td></td>
</tr>
</tbody>
</table>
Images with the results of Biodosimetry analysis for doctors

5 double minutes

Dicentric chromosome with its fragment
Detection of clusters \textit{in vivo}??

\textit{Bystander/distal effect}?

Joint project with Dr. Bonner’s group at NCI
(A) C57BL or BALB/C female age- and sex-matched control mice (a) were compared with mice subcutaneously injected with B16 melanoma, M5076 reticulum sarcoma, or COLON26 carcinoma cells (b). (B) Six-animal cohorts of mice injected with PBS, “NC” (negative control); mice injected with Freund’s adjuvant, “IC” (inflammation control); and tumor-bearing mice, “T”, were analyzed for each experiment.
γ-H2AX focal frequencies are elevated in the skin of COLON26 carcinoma-bearing mice.
OCDLs in mouse tissues

Oxidative clustered DNA lesions (OCDLs) in duodenum, colon, rectum, stomach and skin from NC, IC, and TST cohorts of COLON26 carcinoma-bearing mice. In TST mice, OCDLs were measured in the tumor, as well as in normal skin of three different locations: proximal to tumor, close to tumor, and far from tumor.

Redon et al. (2010) PNAS USA
OCDLs are increased in colon and duodenum of B6J WT but not in CCL2 KO mice.

TST1, M5076 sarcoma-bearing mice; TST2, B16 melanoma-bearing mice. Error bars are SDs (n=3-6).
Systemic DNA damage accumulation under *in vivo* tumor growth conditions can be inhibited by an antioxidant tempol.

The modified alkaline comet assay was performed in 4 cohorts of B6 mice, B16 melanoma (B16) - and Lewis lung carcinoma (LLC)-bearing mice without tempol treatment and with tempol treatment.
From the Cover

Cell Biology

*Tumors induce complex DNA damage in distant proliferative tissues in vivo*

Redon Et al.


Commentary "*Para-inflammation mediates systemic DNA damage in response to tumor growth*" *Commun Integr Biol*, volume 4 on page 78.
Non-targeted DNA Damage effects Unifying Model: Stress (Tumor growing in an organism or irradiated area)

Tumor-associated Inflammatory cells (macrophages etc.)

Release of reactive oxygen species (ROS)

Release of pro-inflammatory factors like chemokines, cytokines (CCL-2 etc).

Determining factors: Distance from the tumor or irradiated area, antioxidant and repair capacity of the tissue, background (endogenous) levels of DNA damage and immune status.

Final effect: Induction of oxidatively-induced DNA damage in adjacent and/or distant sites and organs ('bystander' effect)
ACKNOWLEDGEMENTS

◆ The Georgakilas Group at NTUA
  ◆ Zacharenia Nikitaki
  ◆ Anastasios Mangelis
  ◆ Danae Laskaratou
  ◆ Ifigenia Mavragani
  ◆ Martha Xabibi

◆ Thanks to my Collaborators
  C. Chatgilialoglou, CNR, Bologna, Italy
  V. Gorgoulis, University of Athens, Greece
  O. Martin, Peter MacCallum Cancer Center, Australia
  G. Pantelias (G. Terzoudi and V. Hatzi), NCSR ‘Demokritos’, Greece
  R. Stewart, University of Washington, USA
  W. Bonner, NCI, USA

This work partially funded International Union Against Cancer (UICC), EU Marie Curie IRG Grant, Thalis’ Grant GSRT Greece,
Thank you for your attention!
Questions??
Supplementary material on cluster detection:
Why non-DSB Oxidative Clustered DNA Lesions (OCDLs) ?

- Signature of ionizing radiation and some radiomimetic drugs like bleomycin and neocarzinostatin.

Fact: Prompt DSBs only ~20-30% of radiation-induced clustered DNA damage!

- Oxidative metabolism/Endogenous
Repair Resistant

Produce additional DSBs (?)

Detected in Human cells of different origin

Potential Role in Carcinogenesis/Transformation?
Bistranded DNA Damage Clusters

*Two or more lesions on opposing strands within 1 or 2 helical turns*

- AP site
- Poor cleavage by AP endonucleases
- Complete cleavage by AP endonucleases
- SSB
- DSB
- No strand break
- Radiation:
  - Nfo protein:
  - Clustered DNA damage

- Possible incomplete cleavage by AP endonucleases.

Georgakilas *et al.* NAR 2002
Using PUTR method for detection of AP clusters in human monocytes

- γ-rays
- Prepare cells for Pulsed Field Gel Electrophoresis (CHEF)
- Lysis of cells embedded in agarose DNA plugs
- Treatment of cell plugs with enzyme or PUTR (+).
- Treatment of cell plugs with buffer control: (-).
Measurement of OCDL in Human DNA

Pulsed Field Gel Electrophoresis (PFGE) and Number Average Length Analysis (NALA)

J. Sutherland et al. 2001.

NALA

1. Calculate number average length (Ln) for each lane: Ln(−) and Ln(+)

2. DSBs = 1/Ln^(R) (+) - 1/Ln^(R) (−)

3. OCDL = 1/Ln^(E) (+) - 1/Ln^(E) (−)

Radiation (measure DSBs)

Enzyme (OCDL)
Pulsed Field Gel Electrophoresis
Modified Comet Assay using human repair enzymes as damage probes

Cells and agarose mixture

Microscope slide

cell lysis

Electrophoresis (Alkaline or neutral conditions)

DNA migration

Cells:

Control

+ Radiation

Radiation or enzyme treatment

+ Radiation + enzymatic probe

Fluorescence labeling of DNA prior to analysis

Detection

SSBs, DSBs and OCDL

New approaches on the detection of clustered DNA lesions. Using immunofluorescence (IF):

Measurement of Clustered DNA damage induced low LET radiations

Analysis of 53BP1, EGFP-XRCC1, and hOGG1 foci in individual cells after γ-rays or H2O2 treatment indicates that the co-localization of different surrogate markers was not due to IR-induced chromatin dynamics.

Asaithamby A et al. PNAS 2011;108:8293-8298
Conclusions-Future directions

- **Detection** of OCDLs in mammalian tissues.
- **Oxidative stress via inflammatory pathways** can lead to regeneration-induction of clusters in tissues and accumulation especially in the case of a tumor!
- **Bystander effects.** ‘The controversial abscopal effect’ (Kaminski et al. Cancer Treat Rev. 2005).

- Possible implications in **radiotherapy** or **chemotherapy** or tissues subjected to high oxidative stress like cancer tissues.

- **Biomarkers of oxidative stress in cancer**
Clusters and Hypoxia

Effects of oxygen on the induction of DSB and non-DSB (Fpg and Endo III) clusters in HeLa cells exposed to 5 Gy of \( \gamma \)-rays.

**Symbols:** measured data obtained with PFGE assay. Standard deviation for the Fpg and Endo III clusters approximately same as standard deviation for DSB (*not shown*).

**Solid line:** Predicted DSB yield normalized to 12.5 DSB Gbp\(^{-1}\) Gy\(^{-1}\) at 21% \( O_2 \) (same as the measured DSB yield). For visualization purposes, measured data for 0% \( O_2 \) are shown in the Figure at the 0.1% \( O_2 \) level.

Collaboration with Dr. R.D. Stewart (Purdue University) and Dr. Koumenis (University of Pennsylvania). *Stewart et al. (2011) Radiat. Res.*
Table: Effects of radiation quality and oxygen concentration on the number of clusters Gy\(^{-1}\) Gbp\(^{-1}\) derived from Monte Carlo simulations in a representative mammalian cell.

<table>
<thead>
<tr>
<th>Radiation Type</th>
<th>LET(_{\infty}) (keV/um)</th>
<th>SSB</th>
<th>DSB</th>
<th>All</th>
<th>SSB</th>
<th>DSB</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anoxic (0% O(_2))</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(^{60})Co (\gamma)-rays</td>
<td>0.24</td>
<td>115.6</td>
<td>2.84</td>
<td>413.0</td>
<td>188.9</td>
<td>8.28</td>
<td>624.0</td>
</tr>
<tr>
<td>1 MeV electron</td>
<td>0.18</td>
<td>115.6</td>
<td>2.83</td>
<td>413.1</td>
<td>189.0</td>
<td>8.27</td>
<td>624.3</td>
</tr>
<tr>
<td>250 MeV proton</td>
<td>0.39</td>
<td>115.6</td>
<td>2.85</td>
<td>412.8</td>
<td>188.8</td>
<td>8.31</td>
<td>623.4</td>
</tr>
<tr>
<td>100 keV electron</td>
<td>0.42</td>
<td>115.5</td>
<td>2.86</td>
<td>412.7</td>
<td>188.7</td>
<td>8.33</td>
<td>623.0</td>
</tr>
<tr>
<td>10 keV electron</td>
<td>2.28</td>
<td>111.8</td>
<td>4.79</td>
<td>370.6</td>
<td>170.1</td>
<td>13.01</td>
<td>501.8</td>
</tr>
<tr>
<td>1 MeV proton</td>
<td>26.94</td>
<td>112.6</td>
<td>6.91</td>
<td>348.9</td>
<td>155.3</td>
<td>16.23</td>
<td>423.9</td>
</tr>
<tr>
<td>0.5 MeV proton</td>
<td>42.36</td>
<td>116.2</td>
<td>13.43</td>
<td>308.4</td>
<td>129.8</td>
<td>21.03</td>
<td>315.8</td>
</tr>
<tr>
<td>6.29 MeV (\alpha) particle</td>
<td>76.41</td>
<td>116.0</td>
<td>13.89</td>
<td>305.1</td>
<td>128.4</td>
<td>21.26</td>
<td>310.3</td>
</tr>
<tr>
<td>5.49 MeV (\alpha) particle</td>
<td>84.30</td>
<td>115.1</td>
<td>15.50</td>
<td>293.7</td>
<td>123.6</td>
<td>22.02</td>
<td>293.6</td>
</tr>
<tr>
<td>3.5 MeV (\alpha) particle</td>
<td>114.77</td>
<td>107.1</td>
<td>20.60</td>
<td>250.0</td>
<td>108.0</td>
<td>24.27</td>
<td>243.9</td>
</tr>
<tr>
<td>2 MeV (\alpha) particle</td>
<td>160.38</td>
<td>91.5</td>
<td>24.97</td>
<td>199.7</td>
<td>90.6</td>
<td>26.27</td>
<td>196.2</td>
</tr>
<tr>
<td>100 MeV (^{12})C ion</td>
<td>185.34</td>
<td>108.7</td>
<td>19.92</td>
<td>256.4</td>
<td>110.2</td>
<td>23.94</td>
<td>250.4</td>
</tr>
<tr>
<td>50 MeV (^{12})C ion</td>
<td>308.56</td>
<td>91.1</td>
<td>25.02</td>
<td>198.7</td>
<td>90.3</td>
<td>26.28</td>
<td>195.2</td>
</tr>
</tbody>
</table>

*Georgakilas et al., Radiation Research: July 2013, Vol. 180, No. 1, pp. 100-109*
Primary lymphocytes of 3 patients irradiated ex vivo with 2 Gy IR. Cells were incubated at either 37 or 13°C. Error bars are the average of 3 patient samples and astrices represent a significant difference with p<0.05. 
Detection of DSBs and OCDL in Human MCF-7 Cells using modified PFGE (5 Gy of γ-rays):

\[ \text{SIZE in Kb} \]

<table>
<thead>
<tr>
<th></th>
<th>MARKERS</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>0 (-)</th>
<th>0 (+)</th>
<th>5 (-)</th>
<th>5 (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5700</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3500</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2700</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>225</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.770</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.173</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textit{E. coli} Fpg enzyme has been used for the detection of oxypurine clusters.