Fetal Arsenic Exposure and Adult Cancer

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Arsenic Exposure

• Millions of people worldwide
  – Are exposed to elevated levels of arsenic in the drinking water

• A human carcinogen:
  – At multiple sites
    • Clear: skin, urinary bladder (UB), lung
    – Potential: liver, kidney, and prostate

¹IARC (2004); IARC (2009)
Arsenic Background

- **Inorganic arsenic**
  - Is an effective cancer chemotherapeutic
  - Curing certain otherwise fatal leukemias
    - In part by apparently resetting leukemic stem cells (SCs) back to “normal”
    - We hypothesize this ability to alter SC phenotype
      - May indicate an affinity for SCs
      - And may not always occur in the “correct” fashion
Basic Qualities of SCs

• Example: Mouse skin SCs
  – “Immortal”
  – Quiescent until needed
  – Limitless self-renewal
    • Controlled here by Rac1
  – Pluri-potent with directed commitment
    • Controlled here by c-Myc
  – Occupy a niche
    • Follicular bulge with markers (CD34)

Transplacental (TPL) Model of Arsenic Carcinogenesis

• We have developed a TPL model of arsenic carcinogenesis
  – Fetal life stage is sensitive because of factors like:
    • Organogenesis, global proliferative growth, cell differentiation, etc., all of which involve SCs

• The emerging hypothesis: cancers arise in SC populations producing cancer SCs (CSCs)
  – Would apply in TPL carcinogenesis
Typical Transplacental Protocol for Inorganic Arsenic Carcinogenesis in Mice

<table>
<thead>
<tr>
<th></th>
<th>conception</th>
<th>birth</th>
<th>weaning</th>
<th>adulthood</th>
<th>pathology Up to 2 years</th>
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<tr>
<td>Control</td>
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<td>42.5 ppm</td>
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<td>85 ppm</td>
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Notes:
- Arsenic (as NaAsO₂) given in maternal drinking water (well tolerated)
- Done in several strains (C3H, CD, Tg.AC)
- Promoters included TPA, estrogens, etc.
TPL Arsenic Carcinogenesis: Female C3H Offspring

- Arsenic was a TPL carcinogen
  - Example: Lung carcinoma in adult female C3H offspring
  - Also caused or predisposed to tumors or preneoplasia of the liver, UB, adrenal, ovary, uterus, oviduct, vagina, etc.
    - In adulthood
    - In other strains
      - Long after arsenic exposure ends

![Graph showing lung carcinoma incidence vs maternal arsenic dose](image)

Lung Carcinoma Incidence (%)

Control 42.5 ppm 85 ppm

Trend $p = 0.009$
TPL Arsenic Carcinogenesis: Male Offspring

- Fetal arsenic exposure was also carcinogenic in males
  - In adulthood inducing:
    - Liver tumors including HCC
    - Also tumors or preneoplsia of adrenal, lung, kidney, UB, etc.
    - Alone or with postnatal treatments
    - In other strains

![Graph showing HCC Incidence (%) vs. Maternal Arsenic Dose](image)

HCC Incidence (%)

- Trend $p < 0.002$

Maternal Arsenic Dose

0 ppm 42.5 ppm 85 ppm

*
Additional Work with TPL Models

• Fetal arsenic exposure initiates tumors
  – Promotable later in life by:
    • Estrogens (CD1) causing urogenital system tumors
      » Including UB transitional cell carcinoma
    • Dermal TPA\textsuperscript{1} (C3H) causing liver and lung cancers
      » No skin tumors (C3H not sensitive) – important human site

• Fetal arsenic exposure alters carcinogenic response to later exposures
  – Points to “long-lived” cells (SCs)

\textsuperscript{1}12-O-tetradecanoyl phorbol-13-acetate; often used as a skin promoter but will promote internal cancers after dermal exposure
SCs and Skin Cancer in Mice

• To test this emerging SCs hypothesis we turned to a mouse skin cancer model
  – Keratinocyte SCs (KSCs) likely are key targets in mouse skin carcinogenesis
  – Tg.AC mice sensitive to skin cancer
    • $v$-$Ha$-$ras$ transgene in KSCs$^1$
      – Silent until activated by chemical like TPA

• If fetal arsenic attacks SCs, this sensitive strain should be responsive

$^1$Humble et al. (2005) Oncogene 24:8217]
Protocol: *In Utero* Arsenic and Postnatal TPA to Promote Skin Tumors in Tg.AC Mice

Notes:
- Arsenic from GD 8 to 18 in maternal drinking water (85 ppm)
- TPA given from week 4 to 40 at a standard dermal promoting dose
- Separate control, arsenic alone and TPA alone groups
TPL Arsenic Carcinogenesis in Tg.AC Mice

- Squamous cell carcinoma (SCC) multiplicity
  - Greatly ↑ by arsenic + TPA
    - Compared to TPA alone or arsenic alone (no effect)

- Fetal arsenic facilitates (SCC) formation in adults
  - Tumors arise from KSCs
  - Are much more aggressive after arsenic (invasive, etc.)

Modified from: Waalkes et al. (2008)
**v-Ha-ras in Skin Cancers**

- The *v-Ha-ras* gene drives Tg.AC skin cancer
  - Located in KSCs
    - Greatly increased by fetal arsenic + TPA in SCC
      - Compared to TPA alone
    - *v-Ha-ras* is up in fetal skin
  - Arsenic alters SC number or response

### Assessment of *v-Ha-ras* Expression

Assessed at 40 weeks

![Graph showing relative v-Ha-ras expression levels.](chart)
CSCs In Skin Tumors

- The SC/CSC marker in skin, CD34
  - Is greatly over-expressed in
    - In SCCs induced by fetal arsenic + TPA
      - Fetal arsenic greatly increases tumor CSCs
      - See increased CD34 expression in fetal see after arsenic too
Arsenic, Skin SCs and Skin Cancer

• Fetal arsenic facilitates skin cancer in adulthood:
  – Apparently by increasing SCs
    • More SCs for TPA to target produces more CSCs
      – Data *in vitro* indicates arsenic distorts skin SC differentiation and causes accumulation\(^1\)

Mouse TPL Model Issues

People are exposed during all periods of their lives. We only tested the fetal life stage in mice. Testing at any one stage is not “environmental”

In Utero

Tested here: sensitivity high in mice

Sensitivity unknown

Childhood

Sensitivity unknown

Adolescence

Negative in rodents: but not fully “environmental”

Adulthood
Whole Life (WL) Arsenic Carcinogenesis in CD1 Mice

Notes:
- Arsenic (as NaAsO₂) given in drinking water
- Mice observed for up to 2 years
WL Compared to TPL Response

• Doses approaching human
  – Increase response in many target tissues
    • E.g.: Carcinoma in female offspring
  – Target tissues the same in WL as TPL
    • Response higher and higher grade in WL at lower dose
    • Tumors at 6 ppm

Modified from Tokar et al., 2010 (in press)
CSCs in WL Tumors

- Lung adenocarcinoma
  - After WL arsenic
    - CSC overabundance
    - Based on ALDH and CD133
      - A widely accepted SC/CSC markers
      - Compared to spontaneous or transplacental ENU-induced
    - Same with liver tumors

Modified from Tokar et al., 2010 (in press)
Arsenic TPL Carcinogenesis in Mice

Maternal System 10 days Fetus

Oral Inorganic As^{3+} GD 8-18

Fetal exposure ends

Possible Mechanisms:
Aberrant DNA methylation, altered gene imprinting, oxidative DNA damage, Stem cell dysfunction

Birth Adulthood

Two Years or Less

UB Tumors
Skin Tumors
Lung Tumors
Liver Tumors
Kidney Hyperplasia

/+ Promoters Including additional arsenicals

Arsenic can be active alone;
Human targets of arsenic include UB, skin, lung, liver, kidney and prostate
**In Vitro Hypothesis Testing**

- **Hypothesis:**
  - Arsenic directly attacks SCs
    - Inducing malignant phenotype to form CSCs
      - In the process causes an overabundance of CSCs
    - As opposed to attacking mature epithelium to induced reacquisition of “stemness”
Key Issues

• Emergence of CSCs from normal SCs during chemical carcinogenesis is still debated
• Thus, we sought to determine if arsenic
  – Directly transforms SCs to produce CSCs
  – Increases SCs during transformation of heterogeneous epithelial populations
    • Similar to fetal induced tumors
Model of Isogenic Human UGS Cell Lines

RWPE-1
- non-tumorigenic
- heterogeneous

single cell dilution cloning

WPE-stem
SC characteristics:
- high ABCG2, Bmi-1, CK5, p63, etc.
- Prostasphere formation.
- Ductal structures in matrigel.

Malignant Transformants

MNU transformed
Cadmium transformed
Arsenic transformed

B26
CTPE
CAseE-PE
Survival Selection

- Survival selection is common to SC and CSCs
  - Survive to perpetuate the tissue after injury SCs
  - Occurs with advanced tumor CSCs during chemotherapy
  - Association with carcinogen initiation incompletely defined
Acute Apoptotic Resistance

- Human SCs
  - Were innately resistant apoptosis induced by acute high level arsenic
    - Relative to total parental population

![Graph showing basal apoptotic rate](image)
Basis of Apoptotic Resistance to Arsenic in SCs

- Key factors in basal resistance:
  - ABCC1 – efflux transporter
  - GST-\(\pi\)
  - Glutathione (GSH)
- Combine to increase efflux and resistance in SCs
Cellular Resistance to Arsenic

• Cellular resistance to inorganic arsenic:
  – Involves enhanced efflux of an arsenic-GSH trimer
    • Assisted by GST and ABCC1
Hyper-adaptability of SCs to Arsenic

- Chronic pre-transformational exposure (6 weeks)
  - To low level arsenic (5 µM)
  - SCs more able to adapt to subsequent acute lethal arsenic exposure

- Involved:
  - Up-regulation of GST, ABCC1, etc.
  - Overproduction of GSH
    - Modified metabolism, biokinetics, etc.

![Graph showing LC₅₀ (µM) for Parental and WPE-Stem with comparisons indicated by † and * symbols. † signifies different from initial parental, *= different from initial stem.]
Arsenic Transformation of SCs

- Show survival selection but
  - Can arsenic induce a malignant phenotype
    - In these SCs?
- Continuous arsenic exposure
  - Environmentally relevant level
    - Periodically assess
      - Markers of malignant phenotype
        » MMP-9, etc.
      - Xenograft studies when transformation likely

MMP = Matrix Metalloproteinase, a common tumor cell marker
SCs Rapidly Transformed

- Rapid transformation
  - Arsenic greatly increased secreted SCs MMP-9 activity at only 18 weeks
    - Parental cells show only a 2-fold increase at 30 weeks
  - To xenograft study

![Graph showing arsenic treatment and MMP-9 activity](image)
CSC-like Phenotype Rapidly Acquired

• After injection into mice
  – Chronic arsenic exposed SCs form tumors in only 2 weeks
  – Highly malignant, aggressive, undifferentiated with immature epithelial- and mesenchymal-like cells

• Pluripotent cell of origin
  – Formation of CSC phenotype
Sphere Formation in Arsenic Transformants

- Spheres are free floating clusters of viable cells *in vitro*
  - Both SCs and CSCs form spheres *in vitro*
  - Spheres enriched in SCs or CSCs in cancer lones

- Arsenic transformation
  - Increased spheres by 230%
Formation of CSCs

- MMP-9 activity greatly increased in arsenic spheres
  - Thus, CSCs likely reside in arsenic spheres
Arsenic, Sphere Formation and CSCs

- SCs and CSCs both form spheres
  - Spheres highly enriched for SC/CSCs
  - Only the arsenic transformant formed highly abundant spheres
Second Xenograft Study

- Sphere cells separated from adherent cells and injected
  - Tumors formed only with arsenic sphere cells
  - Same histology, etc.
    - with mixed floater/adherent
- CSCs were clearly formed by arsenic
Gene Expression During CSC Formation

• Arsenic induces aberrant
  – SC differentiation
  – Then de-differentiation to CSCs
    • As assessed by SC/CSC markers
• De-differentiation occurs with loss of *PTEN* expression
  • Tumor suppressor gene
  • Often inactivated in cancers
  – Loss enhances SC self-renew, but depletes SCs and drives CSC formation
    • Likely causes CSC overproduction
Conclusions

• *In vivo* fetal SCs may be targets of arsenic carcinogenesis because fetal arsenic exposure:
  – Causes cancer much later in life
  – Predisposes to other carcinogens
  – Causes CSC overabundance
  – May dictate cancer site

• *In vitro* SCs exposed to arsenic:
  • Show survival selection, acquired CSC phenotype, and CSC overabundance
Final Thoughts

• In mice, early life is sensitivity to arsenic carcinogenesis
• Similar data have emerged in humans
  – Where arsenic acts as early life carcinogen in the lung, liver, and kidney¹
• Low arsenic water supplied during development could prevent human cancer

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