Non-Genotoxic Carcinogenesis: A Challenge for Scientists and Regulators

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The Multistep Process of Carcinogenesis

Initiation

Mutation

Promotion

Selection

Mutation

Progression

Selection

Further Mutations

Benign tumor

Mutation by chance - selection of the fittest“ Charles Darwin 1859
Genotoxicity & Carcinogenicity Testing

Why focus on Non-Genotoxic Carcinogenesis (NGC)?

- No sufficiently accurate or well-validated short-term assays to identify NGC
- Need early mechanism-based biomarkers for the design of more predictive tests & improved cancer risk assessment

Genotoxicity testing

- Required for IND
- Genetox battery
- Time: 1-3 month

Carcinogenicity testing

- Required for NDA
- 2-year bioassay
- Time: 3 years

DNA damage
- Base alteration
- Crosslink
- Abasic site
- Double strand break
- Pyrimidine dimer
- Single strand break

Chromosome damage
- Point mutations

Carcinogenesis Multistage process

Non-genotoxic mechanisms

Cancer

• Required for IND
• Genetox battery
• Time: 1-3 month

• Required for NDA
• 2-year bioassay
• Time: 3 years
The standard two-year-bioassay for carcinogenicity

- time consuming
- expensive
- the „Three Rs“
- impracticable to perform animal experiments to test all substances
## Frequency of Target Sites for Non-Genotoxic Carcinogens

<table>
<thead>
<tr>
<th></th>
<th>In Rats (n = 354)</th>
<th>In Mice (n = 299)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>143 (40%)</td>
<td>171 (57%)</td>
</tr>
<tr>
<td>Lung</td>
<td>31 (9%)</td>
<td>83 (28%)</td>
</tr>
<tr>
<td>Mammary</td>
<td>73 (21%)</td>
<td>14 (5%)</td>
</tr>
<tr>
<td>Stomache</td>
<td>60 (17%)</td>
<td>42 (14%)</td>
</tr>
<tr>
<td>Kidney</td>
<td>45 (13%)</td>
<td>12 (4%)</td>
</tr>
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</table>
Problem Statement

- Substantial resources are spent in unravelling irrelevant findings in rodent carcinogenicity assays.

- Greater understanding in this areas will provide considerable benefits for efficient drug development - An issue of current high priority is receptor-mediated carcinogenesis.

- A better understanding of the mechanisms of non-genotoxic carcinogenesis will contribute for the definition of the human risk associated to their use and give support to risk management.
2000 to the present: New priorities and approaches

**US EPA** carcinogen risk assessment guidance (published 2005): consideration of a chemical’s mode of action (MOA) in determining the relevance to humans

**NTP** have been conducting mechanistic studies → chemical compounds may be suspected of being potentially carcinogenic even though never been tested in a long-term animal bioassay.

**IARC** workshops on short- and medium-term tests for carcinogens: strong mechanistic data could be used to evaluate potential carcinogenicity

**REACH** carcinogenicity study may be considered required if substance is of widespread use / frequent or long-term human exposure / produced at >1,000 tons per year.
The Multistep Process of Carcinogenesis

Initiation

Promotion

Progression

Normal cell → Initiated cell → Clone of initiated cells → Benign tumor

Mutation → Selection → Mutation → Selection → Further Mutations

Polyclonal Malignant Tumor
Growth Stimuli: Preferential Growth of (Pre)Malignant Cells

NORMAL CELL

(PRE)NEOPLASTIC CELL

MITOSIS

CELL DEATH

"Overreactin" of (pre)malignant cells towards growth stimuli due to mutations in critical growth-regulatory genes
Categories of Growth Stimuli in Adult Tissues

**Regenerative growth**
Loss of cells via acute or chronic toxicity → replacement via increased cell proliferation

Original organ size

---

Damage

---

**Adaptive or hormonal induced growth**
Initially increased cell proliferation → chronic hyperplasia only as long as growth stimulus is present

Original organ size

---

Growth stimulus

---

Stop of stimulus
Chronic Inflammation Promotes Tumor Development

Insulted cells recruit activated inflammatory cells

Activated granulocytes and activated monocytes/macrophages

Release of **pro-inflammatory cytokines**

**Superoxide** production may cause mutations?

Inflammatory cells express **growth factors** that stimulate cell growth and progression

Chronic activation promotes **continued inflammation**, **angiogenesis**, and **ECM remodeling**

Mutation

Normal cell  Initiated cell  Promotion  Benign tumor  Malignant conversion

Cancer
Selection of Non-Genotoxic Carcinogens (with Significant Cytotoxicity)

<table>
<thead>
<tr>
<th>Target Organ</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>Asbestos and Related Fibers</td>
</tr>
<tr>
<td>Nasal Mucosa</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Urinary Bladder</td>
<td>Saccharin, Limonene</td>
</tr>
<tr>
<td>Liver</td>
<td>Ethanol, Iron Overload, Chloroform, CC14</td>
</tr>
<tr>
<td>Esophagus</td>
<td>HCl (Reflux)</td>
</tr>
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Relevant to humans
Categories of Growth Stimuli in Adult Tissues

**Regenerative growth**
Loss of cells via acute or chronic toxicity → replacement via increased cell proliferation

**Adaptive or hormonal induced growth**
Initially increased cell proliferation → chronic hyperplasia only as long as growth stimulus is present
Hormonal Balance

Negative feedback

Hypothalamus

Pituitary

Stimulating hormones

Endocrine gland

Hormone

Target
Hormonal Dysbalance Induced by Chemicals

Negative feedback

Hypothalamus
Pituitary

Stimulating hormones

Endocrine gland

Size increases
Growth pressure on mutated cells

Hormone

Target
### Selection of Non-Genotoxic Carcinogens
**Hormonal Dysbalance and without Significant Cytotoxicity**

<table>
<thead>
<tr>
<th>Target Organ</th>
<th>Hormone Dysbalance in</th>
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<tbody>
<tr>
<td>Ovary</td>
<td>LH, FSH</td>
</tr>
<tr>
<td>Testis</td>
<td>LH</td>
</tr>
<tr>
<td>Thyroidea</td>
<td>TSH</td>
</tr>
<tr>
<td>Stomache</td>
<td>Gastrin</td>
</tr>
<tr>
<td>Mammary Gland</td>
<td>Prolactin</td>
</tr>
<tr>
<td>Whole Body</td>
<td>STH (human relevance ?)</td>
</tr>
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Relevant to humans
Induction of Growth via Receptor-Mediated Signalling

**Hormone Receptors:**
- Estrogens, Gestagens, Androgens
- Thyroid Hormones

**PPAR-Receptors:**
- Hypolipidemics, Phthales

**Dioxin-Receptor:**
- Dioxin, PAH

**Constitutive Androstan Rec./Pregnana-X-Receptor:**
- many chemical compounds

**Induction of gene patterns and of growth**
Adaptive Responses Include Liver Growth

Control rat liver  Rat liver treated with phenobarbital for 4 weeks
### Selection of Non-Genotoxic Carcinogens
(Receptor-Mediated and without Significant Cytotoxicity)

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<td>Skin</td>
<td>TPA, TCDD</td>
</tr>
<tr>
<td>Liver</td>
<td>Phenobarbital, TCDD and related agents, DDT, α-, γ-Hexachlorocyclohexane, Ethinylestradiol, Peroxisome Proliferators, Cyproterone acetate</td>
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Relevant to humans
The standard two-year-bioassay for carcinogenicity

- time consuming
- expensive
- the „Three Rs“
- impracticable to perform animal experiments to test all substances

Continuous treatment with test compound for "lifetime" ≥ 2 years (rats, mice, and/or hamster)
Liver Foci Test

Test for promoting potential

den

weeks
Liver Foci Test

**Parameters:**
- Number of foci per histological section area
- Total foci area per cm²

**Pros and cons:**
- Less time required (3 – 6 months), less animals, higher statistical reliability
- Specific for liver
Medium Term Tests  
(duration 3-6 months)

Premalignant lesions as endpoints:

1. Liver foci test with rats
2. Skin papilloma test with mice
3. Aberrant crypt foci test (mice, rats)
## Some Indicators for Prediction of Non-Genotoxic Carcinogens

### Non-Cytotoxic Compounds

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### Cytotoxic Compounds

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<td>Cytotoxicity</td>
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<td>Inflammation</td>
<td>Determination of immunological parameters</td>
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Conclusion I

- Lack of short-term or medium assay to predict non-genotoxic carcinogens due to complex mode of action

- With regard to the demands, posed by the REACH programme and other regulatory requirements, the development of such assays is urgently required
Innovative Medicine Initiative (IMI): MARCAR-Consortium

- **EFPIA**
  - Novartis
  - UCB Pharma SA
  - Bayer Pharma AG
  - Lundbeck
  - Boehringer Ingelheim

- **SME**
  - CXR Biosciences Ltd., UK

- **EMEA & FDA scientists**
  - via ad-hoc advisory board

- **Academic**
  - University of Dundee, UK
  - Medizinische Universitat, Vienna, Austria
  - Medical Research Council, Edinburgh, UK
  - Eberhard Karls Universitat, Tubingen, Germany
  - Natural & Medical Sciences Institute, Reutlingen, Germany
  - Institut National de la Sante et de la Recherche, Montpellier, France
MARCAR consortium focus on novel mechanisms & biomarkers for non-genotoxic carcinogenesis

• EPIGENETICS; non-coding RNAs; BIOINFORMATICS
• MOUSE MODELS CONTAINING HUMAN GENES IMPORTANT FOR NGC
  • TRANSLATIONAL IN VITRO CELL-BASED MODELS
  • NON-INVASIVE IMAGING & REPORTER MODELS
  • ROLE OF HEPATIC MESENCHYME

Goal:
Provide industry and regulatory scientists with new tools for earlier decision-making, mitigation of positive carcinogenicity findings and enhanced cancer risk assessment
Unaltered (GSTp-) and Initiated (GSTp+)
Hepatocytes In-Vivo and Ex-Vitro

N-Nitrosomorpholine

Liver cell separation

Initiated/preneoplastic

GSTp+

normal

GSTp-

Elevated DNA-synthesis of initiated hepatocytes in-vitro, as observed in-vivo
Two Protoypical Non-Genotoxic Hepatocarcinogens

Phenobarbital (PB):

- used as a third-line anti-epileptic drug.
- strong liver tumor promoter in rodents and exerts no genotoxicity.

Cyproterone Acetate (CPA):

- a gestagen with anti-androgen activity, used in contraceptive pills.
- produces liver tumors in rodents mostly by a non-genotoxic mode of action. Some genotoxicity which is considered to be irrelevant.
PB fails to exert direct effects in HC

Unaltered (GSTp-) and initiated (GSTp+) hepatocytes
Chemical Hepatocarcinogenesis in Rodents: Profound Impact of Activated Liver Mesenchyme

Hepatocarcinogenesis by geno-/cytotoxic compounds depends highly on:

- NFκB activity in Kupffer cells
  (for rev.: M. Karin and co-workers, Nature Rev. Immunol 2011,...)

- secretion of IL6 and TNFα
  (Park & Karin, Cell 2010, (Naugler et al., Science 2007)

- pro-oxidative and pro-inflammatory state of the liver
  (Slocum et al., Arch Toxicol 2011; Alwahaibi et al., J Gastro Hepatol 2010, Bishhayee et al., Pharm Res 2010)
Working Hypothesis: Non-Genotoxic Hepatocarcinogens Induce Profound Alterations in the Mesenchyme

Do non-genotoxic carcinogens act only directly on hepatocytes?

or

Do non-genotoxic carcinogens act also via the hepatic mesenchyme?

Hepatic mesenchyme

Normal hepatocyte

Preneoplastic clone

Cancer

autocrine growth loops

paracrine growth induction
liver cell-type specific effects of NGCs:
proinflammatory pathways & secreted biomarkers

Collagenase perfusion

Separation of cell types by density gradient centrifugation steps

Treatment with PB, CPA, or solvent

Transcriptomics, proteomics & secretomics of hepatic mesenchymal & parenchymal cells
NGC Induce Multiple Alterations in the Transcriptome Profiles of the Hepatic Mesenchyme

PB treatment in-vivo (14d)  CPA treatment in-vivo (6d)

Pro-inflammatory signature  Induction of Growth Factors
The Secretome of PB-Treated Mesenchyme Supports Survival of Hepatocytes via TNFα

PB or solvent-Co

The secretome of BP-treated MC:
contains elevated TNFα and IL1β levels

sterile filtration of secretome and transfer to hepatocyte cultures

The secretome of PB-treated MC induces in HC:
- a pro-inflammatory reaction
- nuclear translocation and activation of NFκB
- enhanced survival of HC when treated with pro-apoptotic stimuli
- Secretome effects are abrogated by neutralizing TNFα via antibodies

solvent

mesenchymal cells

hepatocytes

Teresa Riegler et al., Carcinogenesis 2015
PB-Treated Mesenchyme Supports Survival of Hepatocytes via TNFα Secretion and NFκB Activation


Similar alterations observed in hepatocytes exposed to secretome from PB-treated mesenchyme

Teresa Riegler et al., Carcinogenesis 2015
**Immunological Effects of PB in Rat and Human Liver In-Vivo**

**Rat liver damaged by cytotoxic compound**

Riegler T, et al. *PB delays recruitment of cd68+ and cd163+ monocytes/macrophages to liver* Carcinogenesis 2015

**Humans Exposed to PB**


Mikawa K, et al. *Inhibitory Effects of Pentobarbital and Phenobarbital on Human Neutrophil Functions.* J Intens Care Medicine, 2011

Clarens et al., 2010. *Phenobarbital and Drug Induced Liver Injury*


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**Control rat liver**

**PB-treated rat liver**
NGC Induce Multiple Alterations in the Transcriptome Profiles of the Hepatic Mesenchyme

Induction of Growth Factors

Nejtab et al., manuscript in preparation
Investigations on the Secretome of CPA-Treated Mesenchyme

Treatment with CPA or Solvent

Treatment with N-Nitroso-morpholine

sterile filtration of secretome and transfer to hepatocyte cultures

mesenchymal cells

hepatocytes

Nejabat et al., manuscript in preparation
The CPA-Treated Mesenchyme Releases Growth Factors for Initiated Hepatocytes

Treatment with CPA or Solvent

Treatment with N-Nitroso-morpholine

sterile filtration of secretome and transfer to hepatocyte cultures

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Unaltered (GSTp-), Initiated (GSTp+) hepatocytes

Nejabat et al., manuscript in preparation
The CPA-Treated Mesenchyme Releases Growth Factors for Initiated Hepatocytes

The CPA-Treated Mesenchyme Releases Growth Factors for Initiated Hepatocytes

Treatment with CPA or Solvent

Treatment with N-Nitroso-morpholine

The secretome of CPA-treated MC: contains elevated levels of HGF

The secretome of CPA-treated MC induces in HC:
- DNA replication
- Secretome effects are abrogated by neutralizing HGF via antibodies

Nejtab et al., manuscript in preparation
Neutralizing anti-HGF Abrogates Largely Effects of CPA-treated Mesenchyme

Estrogen and progesterone receptor expression in macrophages and regulation of hepatocyte growth factor by ovarian steroids in women. Khan et al., 2005

Hepatocyte growth factor system in the mouse uterus: variation across the estrous cycle and regulation by 17-beta-estradiol and progesterone. Zhang et al., 2010
Conclusion II
Thank you for your attention and....

Teresa Riegler

Marzieh Nejabat

Wolfgang Huber
Birgit Mir-Karner
Helga Koudelka
Marzieh Nejabat
Tabea Reitinger
Rolf Schulte-Hermann