Transforming Growth Factor-β and Lung Tumorigenesis

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Lung Cancer in 2014, USA

- Most common cause of cancer deaths in both men and women
- 224,210 diagnosed new cases
  - 116,000 men; 108,210 women
- 159,260 deaths due to lung cancer
  - 86,930 men; 72,330 women
- Most cases now occur in ex-smokers
- < 15% five year survival rate
Transforming Growth Factor-β (TGF-β)

- Multifunctional regulator of cellular growth
- Potent inhibitor of normal epithelial cell proliferation
- Widespread tissue expression
- Pivotal role in epithelial homeostasis
- Association with various types of cancers
- Context-dependent inhibition or stimulation of cell proliferation and neoplastic transformation

TGF-β is an attractive candidate for new therapeutic intervention approaches
Transforming Growth Factors: The Beginning

Sarcoma Growth Factor – Polypeptide secreted by Moloney murine sarcoma virus-transformed mouse fibroblasts that stimulated normal rat fibroblasts to form colonies in soft agar (transformation assay).
*De Larco & Todaro: PNAS 75:4001, 1978*

Two classes of TGFs isolated from MSV-transformed cells:
1. Competes with EGF for receptor binding (TGF-α)
2. Does not compete for EGF binding, but colony forming activity is enhanced by EGF (TGF-β)
Sarcoma growth factor = TGF-α + TGF-β
*Roberts, Anzano,…Sporn: Nature 295:417, 1982*

1983- Publication of the purification of TGF-β from:
Human platelets (*Rick Assoian*)
Human placenta (*Chuck Frolik*)
Bovine kidney (*Anita Roberts*)
Scale of TGF-β1 Purification from Bovine Kidney

- Extract with 8 liters of acid/ethanol
  - Centrifuge
  - Precipitate with 32 liters ether + 16 liters ethanol

- Redissolve in 2 liters 1M acetic acid
- Apply to 80 liter BioGel P-60 column
- Collect 1 liter fractions
- Lophilize and redissolve for further chromatography

Final Yield = 6 µg TGF-β1
purification fold = 230,000; recovery = 10%
The Columns for TGF-β1 Purification
The Assay: Growth of NRK Cells in Soft Agar

- Plate agar base
- Add mix of media, serum, NRK cells, EGF, sample
- 1 wk/37°C/5%CO₂
- Stain
- Count colonies >3100 µm² with Omnicon Image Analysis System

If no TGF-β is present

If TGF-β is present
The Final HPLC Purification

TGF-β
EUREKA!! TGF-β: Born at NCI

Michael Sporn & Anita Roberts
Sequence of mature TGF-β1 monomer

Pre-pro TGF-β 391 amino acids

Signal peptide (latency associated peptide, LAP) Mature TGF-β 112 amino acids
TGF-β: A Homodimer

Monomer chain

Hydrophobic pocket

Interchain disulfide bond

Daopin, S et al Science 257:369, 1992
The TGF-β Superfamily
Transforming Growth Factor-β

- 25,000 MW disulfide-bonded homodimer
- 3 highly homologous isoforms (TGF-β 1, 2 and 3)
- Principal sources - platelets, bone, spleen
- Most cells express TGF-β and its receptors
- Usually secreted in latent, inactive form
- Superfamily of TGF-βs, activins/inhibins, BMPs, GDFs
The TGF-β Superfamily: Central Control Modules for Many Biological Processes

- Development
- Immune System Function
- Reproduction
- Metabolic Regulation
- Tissue Repair and Response to Injury
- Aging
- Angiogenesis
- Proliferative Homeostasis
Major Biological Responses Regulated by TGF-β

- Inhibits proliferation
- Regulates apoptosis
- Regulates differentiation
- Regulates immune cell function
- Stimulates accumulation of extracellular matrix
- Promotes chemotaxis
Model for TGF-β Pathway

 activin
 TGF-β

 Receptors

 BMPs

 Smads
Clinical Observations

TGF-β is a tumor suppressor:

- Germline mutations in TGF-β pathway components cause familial predisposition to cancer
  \((Smad4 \text{ in juvenile polyposis syndrome})\)
- TGF-β pathway components are somatically mutated or deleted in some human cancers
  \((T\beta-RII \text{ in HNPCC}, Smad4 \text{ in pancreatic cancer})\)
- Reduced expression of TGF-β1 signaling pathway components or overexpression of endogenous pathway inhibitors are associated with disease progression
  \((T\beta-RII, T\beta-RI, Smad7, Ski)\)
Clinical Observations

**TGF-β is a tumor promoter:**

- TGF-β1 is elevated in many advanced human tumors and correlates with metastasis and/or poor prognosis

(breast, colon, stomach, liver, pancreas, prostate, lung, kidney, bladder, nasopharynx, melanoma, chondrosarcoma, osteosarcoma)

Prostatic adenocarcinoma stained for TGF-β1:

(Truong et al. Hum Pathol 1993)

TGF-β sits at the interface between tumor parenchyma and microenvironment
TGF-β in Carcinogenesis - Hero or villain?

• TGF-β, a proximal effector of the malignant phenotype

• TGF-β, potent growth inhibitor and tumor suppressor

• TGF-β, a pro-metastatic factor
Unifying Hypothesis: TGF-β Switches from Tumor Suppressor to Pro-oncogenic Factor During Cancer Progression

Changes in genetic and epigenetic context

NORMAL EPITHELIUM

INVASIVE METASTATIC CANCER

TGF-β responsiveness

TGF-β expression/activation

Suppressor activities dominate

Pro-oncogenic activities dominate
TGF-β Smad-independent Pathways
TGF-β Smad-independent Pathways
K-ras Protooncogene

- K-ras shows an activational mutation in ~25-50% of human lung adenocarcinomas
- Mutation of even one allele of K-ras increases appearance of lung lesions
- There is cross-talk between Smad-dependent pathway and the Ras/MEK signaling
- Activation of the Ras pathway can modulate TGF-β1 signaling through the Smads
- In-vitro studies show that TGF-β1 dominates over mitogenic effects of ras, but activated ras overrides antiproliferative effect of TGF-β1
TGF-β in Tumor Suppression/Promotion

• Activated Ras/MAPK = Tumor Promotion
• Determine the role of Transforming Growth Factor-β in the development and malignant transformation of lung epithelial cells

Epithelial Carcinogenesis Section
Cell and Cancer Biology Branch
Center for Cancer Research
NCI
Objectives

• Examine the effect of TGF-β1 deletion and K-ras mutation alone and in combination on lung tumor incidence and pathology

• Determine early events in the development of lung lesions and their progression

• Identify potential signal transduction pathway changes with tumorigenesis
Mouse Models

- A/J
- C57BL6 TGF-β1 HT
- AJBL6 TGF-β1 HT
- TGF-β1 HT/K-ras LA
Question

• Does lung tumorigenesis affect the TGF-β signaling pathway?

• Does the TGF-β signaling pathway affect lung tumorigenesis?
A/J Mouse Model

- Susceptible to chemically-induced lung tumors
- Tumors develop in a time-dependent manner
- Hyperplasia, adenoma and carcinoma
- Carcinomas are histologically similar to human lung adenocarcinomas
- Same molecular mutations in both human and mouse lung tumors (i.e., over-expression of ras, loss of p53)
Ethyl Carbamate Metabolism

**Detox Path**

Ethyl Carbamate → Ethanol + CO₂ + NH₃

**Bioactivation Path**

Vinyl Carbamate → Vinyl Carbamate Epoxide → Binding to Macromolecules + CO₂ + NH₃

CYP2E1

Esterase + H₂O
Production of Tumors in A/J Mice

- 2 Month Old Mice
- 1, 2, 3, 4, 6, 8, 10, 12 Months Sacrifice
- 20 Mice per Sacrifice

Inject Ethyl Carbamate
A/J Mouse Model
TGF-β1, RI and RII Proteins in Lung Tumors

<table>
<thead>
<tr>
<th></th>
<th>TGF-β1</th>
<th>TGF-β RI</th>
<th>TGF-β RII</th>
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<tbody>
<tr>
<td>2 Month</td>
<td>![Image A]</td>
<td>![Image B]</td>
<td>![Image C]</td>
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<tr>
<td>4 Month</td>
<td>![Image D]</td>
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<td>![Image F]</td>
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<td>8 Month</td>
<td>![Image G]</td>
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Decreased TGF-β RII protein in tumors
TGF-β in A/J Mouse Model

EC-induced Lung Tumors

TGF-βRI

TGF-βRII

A

C

B

D

IHC

IHC

Lung Tumor Derived Cell Lines

E10  E9  A5  LM1  PCC4*

28S

18S

TGF-βRI

TGF-βRII

Decreased TGF-β RII protein and mRNA

5.5 Kb

5.5 Kb
Expression of TGF-β1, RI and RII Proteins and mRNAs in BP-Induced A/J Mouse Lung Tumors

Decreased TGF-β RII mRNA and protein in tumors
TGF-β in Tumor Suppression/Promotion

- Reduced TGF-β RII = Lung Tumor Promotion
Question

- Does deletion of TGF-β1 affect lung tumorigenesis?

C57BL/6 TGF-β1 Mouse
The C57BL/6 TGF-β1 Knockout Mouse

Increased tumor incidence in TGF-β1 HT mice
AJBL6 TGF-β1 HT Mouse Derivation

A/J TGF-β1 WT X C57BL/6 TGF-β1 HT

AJBL6 TGF-β1 HT + TGF-β1 WT (F1)

Carcinogen

Lung Tumors
AJBL6 TGF-β1 HT and WT Mouse

IHC Staining & In Situ Hybridization

Ab
WT

Ab
HT

Ab + Ag
HT

IHC

In Situ

Antisense

Antisense

Sense

Northern Blotting & Competitive RT-PCR

TGF-β1 WT

TGF-β1 HT

2.5 Kb

WT

28S rRNA

HT

Copies of Competitor Added

5x10^5

2.5x10^5

1.25x10^5

6.25x10^4

3x10^4

Reduced expression of TGF-β1 in HT compared to WT
Production of Tumors

2 Month Old Mice

Inject Ethyl Carbamamate

Months Sacrifice

Groups

TGF-β1 HT
TGF-β1 WT

20 Mice per Sacrifice
Carcinogen-Induced Lung Tumorigenesis in AJBL6 TGF-β1 HT & WT Mice

Increased tumor incidence and multiplicity and decreased tumor latency in TGF-β1 HT mouse.
TGF-β RII Protein in Lung Lesions from AJBL6 TGF-β1 WT and HT Mice

Hyperplasia

WT  WT  HT  HT

Adenoma

Carcinoma

Decreased TGF-β RII in tumors of TGF-β1 HT mice
Relative TGF-β RII mRNA Levels
Lesions from AJBL6 TGF-β1 HT Mouse Lungs Treated with Ethyl Carbamate

Decreasing TGF-β RII mRNA with increasing lung tumorigenesis
Question

- Does deletion of TGF-β1 and mutation of K-ras affect lung tumorigenesis?

TGF-β1 HT/K-ras LA Mouse
To Study the Interplay of TGF-β1 and K-ras:
Generation of TGF-β1/ K-ras LA Mice

**TGF-β1**
- HT
  - (C57Bl/6)

**K-ras**
- LA
  - (SV 129)

TGF-β1 HT/K-ras LA - HT/LA Double Mutant
TGF-β1 WT/K-ras LA - WT/LA Single Mutant
TGF-β1 HT/K-ras WT - HT/WT Single Mutant
TGF-β1 WT/K-ras WT - WT/WT Wild Type
TGF-β1 and K-ras Mouse Lungs

A: TGF-β1 HT, K-ras LA
   HT/LA

B: TGF-β1 WT, K-ras LA
   WT/LA

C: TGF-β1 HT, K-ras WT
   HT/WT

D: TGF-β1 WT, K-ras WT
   WT/WT
Effect of TGF-β1 Gene Deletion and K-ras Mutation on Mouse Survival
Effect of TGF-β1 Gene Deletion and K-ras Mutation on Mouse Survival

Decreased lifespans in HT/LA and WT/LA mice
Pathology of Lung Lesions

Increased hyperplasia & adenoma in WT/LA
Increased carcinoma in HT/LA
TGF-β1 and TGF-β RII in Lung Lesions

Reduced TGF-β1 & RII in HT/LA adenocarcinomas
TGFβ RII and Smad3 in Lung Tumorigenesis

HT/LA: Expedited TGF-β RII reduction & Smad3 production
## TGFβ Pathway in HT/LA Lung Tumorigenesis

### Western Blot:
- **TGF β RII**: Expedited TGF-β RII reduction
- **Smad3**: Expedited Smad3 production
- **Smad4**: Reduced Smad4 production
- **Smad7**: Reduced Smad7 production
- **K-ras**: Expedited K-ras production
- **Raf-1**: Expedited Raf-1 production

### Real Time RT-PCR:
- Reduced Smads 2, 3, 4 & 7 in adenomas
- Reduced TGF-β RII & Smads in carcinomas
Apoptotic Index in WT/LA & HT/LA Mouse Lung Adenomas

Reduced apoptosis in HT/LA adenomas
TGF-β in Tumor Suppression/Promotion

- Decreased TGF-β RII = Lung Tumor Promotion
- Activated Ras/MAPK = Lung Tumor Promotion
- Decreased Smad4 = Lung Tumor Promotion
- Compromised Apoptosis = Lung Tumor Promotion
Acknowledgements

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