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Tumors as Organs: Biologically augmenting radiation therapy by inhibiting TGF β actions in carcinomas

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Abstract

Transforming growth factor β (TGF β) plays critical roles in regulating a plethora of physiological processes in normal organs, including morphogenesis, embryonic development, stem cell differentiation, immune regulation, and wound healing. Though considered a tumor suppressor, TGF β is a critical mediator of tumor microenvironment, in which it likewise mediates tumor and stromal cell phenotype, recruitment, inflammation, immune function and angiogenesis. The fact that activation of TGF β is an early and persistent event in irradiated tissues and that TGF β signaling controls effective DNA damage response provides a new means to understand both tissue and tumor responses to radiation. Here we discuss pre-clinical studies unraveling TGF β effects in radiation responses and review TGF β biology in lung cancer as an example of the opportunities for TGF β pathway inhibition as a pharmaceutical approach to augment radiation therapy.

Keywords

TGF β ; lung cancer; tumor microenvironment

Genetic and cell biology studies indicate that cancer should be considered as an organ in which malignancy, like function, is not just determined by parenchymal cells themselves, but also by their stroma and microenvironment¹. Tumor companion cells, such as myeloid cells, phagocytes and immune cells, intermingled in the tumor-associated fibroblastic stroma comprises most of the tumor mass in carcinomas and provides the soil in which malignant cells will grow, invade and metastasize². In order to treat cancer, this growing body of evidence that solid tumors are organized in a manner that resembles an organ requires better understanding of the complex ways in which cancer cells interact with their surroundings, both locally and systemically.

Radiation therapy is prescribed to more than half of cancer patients³. Although mechanisms of radiation response and tumor cells are well understood on a molecular level, much remains uncertain concerning the interaction of cells within irradiated tumor microenvironment. In studies spanning two decades, we and others have identified transforming growth factor β (TGF β) as a key extracellular signal in irradiated tissues and tumors. Among all radiation-induced cytokines in the tumor microenvironment, TGF β

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arguably elicits the most complex and far-reaching effects in determining outcome. Evidence from pre-clinical models suggests that TGF β inhibition may provide an effective means to improve the therapeutic response to radiation. Here we consider cellular and multicellular processes mediated by TGF β in response to radiation, with particular reference to TGF β biology and the therapeutic opportunity in treating non-small cell lung cancers.

TGF β Signaling

The effects of TGF β are mediated by three TGF β ligands— TGF β 1, TGF β 2 and TGF β 3 — through TGF β type 1 (TGF β R1) and type 2 receptors(TGF β R2)⁴. All three mammalian TGF β genes share a high degree of sequence homology in the ligand region and functional overlap. TGF β 1 is the best studied protein of these three differentially expressed isoforms. During protein synthesis of the 390 amino acid polypeptide, intracellular proteolysis processes the precursor to yield a C-terminal peptide that dimerizes to form the mature 24 kDa cytokine (FIG 1). The remaining N-terminal peptide forms a 75 kDa glycosylated homodimer called the latency associated peptide (LAP)⁵. LAP acts as a chaperone to ensure proper folding of TGF β and contains the signal peptide for secretion. Non-covalent association of LAP with its respective TGF β cytokine forms the latent TGF β (LTGF β) complex, referred to as the small latent complex. The so-called large latent complex in which LAP is covalently bound via a disulfide linkage to a protein called latent TGF β binding protein facilitates LTGF β sequestration within the extracellular matrix. Thus, biological activity of TGF β is restricted by the fact that it is secreted and sequestered in this latent state.

Latent TGF β 1 is abundant and stored in extracellular reservoirs until activation by extracellular stimuli, such as proteases, reactive oxygen or radiation, that release TGF β to bind to cell surface receptors. TGF β signals via ubiquitously expressed cell surface type I and type II receptors that in turn phosphorylate Smad proteins that translocate to the nucleus to mediate transcription directing differentiation, function, proliferation and apoptosis depending on cell type and context (reviewed in⁶). Despite 25 years of study, TGF β actions are still being defined and new activities are being discovered.

Release of TGF β from its LAP, referred to as activation, is required before the cytokine can bind to cell surface receptors, which places the modes of activation as critical determinants of its biological activity^{5,7}. Although all three isoforms bind to the same cell surface receptors, gene knockout of the three genes results in distinct phenotypes⁸⁻¹⁰, suggesting that specificity of action may reside at the level of susceptibility to different modes of activation. LTGF β can be activated in solution using highly acidic or basic conditions or by heat treatment¹¹ but these activation mechanisms are not physiologically relevant. Most activation mechanisms require the participation of one or more additional proteins generally localized to the cell surface and these activation modes are relatively inefficient⁵.

Rapid activation of LTGF β , as demonstrated by an increase in reactivity of TGF β epitopes that are masked by the latent complex, is observed in vivo after exposure to IR^{12, 13}. Because reactive oxygen species (ROS) are a product of the interaction of IR with water or with cell membranes, we postulated that the rapid activation of TGF β in vivo could be due to ROS generated by IR if the protein itself contained redox sensitive amino acids. Redox switches that alter conformation and activity are found in a variety of proteins. Examples include the clotting cascade mediator, thrombomodulin and transcription factors like Sox and p53. We showed that solution sources of ROS generated by Fenton chemistry efficiently release biologically active TGF β from recombinant LTGF β in the absence of cells or other proteins¹⁴. Indeed, solution sources of ROS generate more biologically active TGF β as measured by bioassay than does heat or acid, possibly because the latter can also denature

the ligand. This mode of activation has been confirmed in recent studies using asbestos generated ROS in solution¹⁵. Since ROS induces very efficient LTGFB activation that does not require participation of additional cellular machinery and since ROS are widely generated as by products of cellular metabolism, inflammation, ischemia/reperfusion injury, as well as exposure to exogenous agents such as IR, chemotherapeutic agents and asbestos, LTGFB could function as an extracellular sensor of oxidative stress¹⁴.

The specific mediators and requirements for the ROS mechanism of activation showed that redox sensitivity is restricted to LTGFβ1¹⁶. Although the three latent TGFβ proteins share 75% sequence identity, their respective LAP exhibit only 34-38% identity, which supports the idea that the isoforms differ in structural susceptibilities to modes of activation. Specific radical scavengers pinpointed hydroxyl radical as the critical ROS for activation in the Fenton chemistry cell-free model for generating ROS. It was postulated that oxidation of specific amino acids within LTGFβ1 causes a conformational change in the latent complex, allowing release of active TGFβ. The target of ROS modification is LAP.

Comparison of the primary amino acid sequences of the three LAP regions of the TGFβ isoforms. Each monomer of LAPβ1 contains five methionine residues, which are susceptible to oxidation. Of these five, two methionines (132 and 253) are unique to LAPβ1. Single point amino acid changes to alanine were made to methionines at positions 132 or 253 unique to LAPβ1. Like wild type, the expressed proteins containing site-directed mutations formed latent complexes that could be activated by heat treatment, however mutant M253A LAPβ1 was resistant to ROS mediated activation. The specificity and efficiency of the oxidative activation mechanism indicates that LTGFβ1 operates as a tissue sensor of oxidative damage and that TGFβ1 release is a signal transducer¹⁷. The redox switch provides a highly efficient mechanism by which radiation increases TGFβ activity.

Once released from the latent complex, TGFβ ligands bind to TGFβ receptor II (TBR2) with high affinity. Both type I and type II receptors contain serine/threonine kinase domains in their intracellular portions⁴. Binding of the ligand causes the formation of heterotetrameric active receptor complexes that result in the phosphorylation of the type I receptor by the type II receptor. TGF-βs elicit their effects by binding to cell surface receptors. The tumor suppressor activity of TGFβ is predominantly mediated through its effect on cell cycle progression and apoptosis. Inhibition of c-Myc, which is known to promote cell cycle entry into S phase by regulating various cell cycle related genes, by TGFβ signaling is very well established¹⁸. Repression complex of SMAD3, E2F4/5 and p107 which are induced upon TGFβ signaling repress the expression of c-Myc¹⁹. Stimulation of p15ink4b and p21cip1 expression by TGFβ leads to inhibition of cyclin D-CDK4/6 and Cyclin A/E-CDK2 activity²⁰.

Although TGFβ has an anti-proliferative effect on most epithelial cells and haematopoietic cells, it promotes proliferation of certain mesenchymal cells, including smooth muscle cells, through the induction of platelet-derived growth factor (PDGF)²¹. Similarly, TGFβ induces the proliferation of certain types of cancer cells, including glioma and osteosarcoma cells, through the induction of PDGFA or PDGFB^{22, 23}. Abnormal proliferation observed in cancer cells is caused by mutations that either increase positive growth signals or decrease negative growth control signals or a combination of both events. Tumors may use various mechanisms anywhere along alterations in TGFβ receptors, its primary cytoplasmic signal transducers, the Smad proteins, to circumvent the growth inhibitory effects of TGFβ²⁴. TGFβR2 mutations have been reported in several epithelial type human malignancies, such as pancreatic, and breast cancer, which lose their growth inhibitory response to TGFβ²⁵⁻²⁷.

Almost all cancer cells lose the proliferative arm of TGF β signaling by one means or another, but maintain some degree of response, and in general produce and activate significantly more TGF β than surrounding normal tissue; these events characterize the so-called TGF β switch from a tumor suppressor to a tumor promoter (reviewed in²⁸⁻³⁰)

TGF β is strongly implicated in the acquisition of invasive and metastatic disease, in large part via activation of the epithelial to mesenchymal transition (EMT) phenotypic program, usurped from development and wound healing where it provides a means for transient motility of epithelial cells.

TGF β 's Role in the Composition of the Tumor Microenvironment

The normal tissue stroma is essential for maintenance and integrity of epithelial tissues and contains a multitude of cells that collaborate to sustain normal tissue homeostasis. There is a continuous and bilateral molecular crosstalk between normal epithelial cells and cells of the stromal compartment, mediated through direct cell-cell contacts or by secreted molecules. There is a similarity between stroma from normal organs and tumors, because both entities have active angiogenesis and numerous proliferating fibroblasts secreting a complex extracellular matrix (ECM)³¹. Fibroblasts are mesenchymally derived cells present in the stroma of most tissues. During lung development they or their progenitors are involved in epithelial-mesenchymal crosstalk, helping to shape the organ.

TGF β also plays a central role in physiological fibrogenesis and pathological pulmonary fibrosis by promoting the activation, proliferation, and differentiation of epithelial cells and collagen-producing myofibroblasts³². After lung injury, TGF β from the recruited leukocytes trigger fibroblasts to proliferate and differentiate into myofibroblasts, which release ECM components. However, if this process becomes dysregulated it can lead to the development of a permanent lung pathogenesis such as pulmonary fibrosis and chronic obstructive pulmonary disease³³.

Most epithelial-derived tumors are characterized by the generation of mesenchymal-derived stromal cells, including intratumoral and peritumoral carcinoma-associated fibroblasts (CAF) associated with tumor growth, angiogenesis, invasion, and metastasis^{34, 35}. CAF are phenotypically distinguishable from normal fibroblasts. Although their heterogeneity is yet to be fully explored, at least a subset of CAF have been characterized as myofibroblasts based on expression of α -smooth muscle actin (α -SMA). The mechanism of fibroblast activation in cancer is not completely understood. Indeed, even the origins of activated fibroblasts in tumors are incompletely described. Currently it is believed that the majority of CAF arise from the activation of resident fibroblasts. However, activated fibroblasts can also originate from pericytes, vascular smooth muscle cells, bone marrow-derived mesenchymal cells, and from epithelial to mesenchymal cell transition³⁶. In adult tissues, differentiation from resident stromal fibroblasts into activated myofibroblasts occurs through paracrine signaling with TGF β generated by damaged or inflamed tissues³⁷⁻³⁹. Such TGF β -mediated activation of CAFs may occur in tumors. Both TGF β and interleukin-1 β induce differentiation of quiescent fibroblasts into activated myofibroblasts, but TGF β is considered the predominate inducer⁴⁰.

Importantly, the FAP promoter has an EGR-1 binding site, and EGR-1 binding is important for FAP expression⁴¹. It is rational to assume that FAP may be induced in resident tissue fibroblasts that are activated by TGF β released from stromal cells or epithelial cancer cells. The autocrine-signaling TGF β -SDF-1 loops initiate and maintain the differentiation of fibroblasts into myofibroblasts which gives rise to tumor-promoting CAF myofibroblasts during tumor progression⁴². CAFs also promote tumor progression through communications with pericytes and endothelial cells that promote tumor growth and angiogenesis through

elevated SDF-1/CXCL12 secretion⁴³. Communication between fibroblasts and cancer cells often involves other cell types: cancer cell-secreted platelet-derived growth factor (PDGF) can recruit macrophages, which then produce TGF β that, in turn, induces development of reactive fibroblasts⁴⁴. These examples underscore how TGF β activity is often amplified in the tumor microenvironment. TGF β produced by resistant cancer cells induces an activated phenotype, e.g. CAF, that then contributes more TGF β to the microenvironment, which promotes more recruitment, e.g. BMDC, and remodeling, the dysfunctional cancer dynamic leading to "the wound that does not heal"³¹.

TGF β pathway as an emerging target for anti-angiogenic therapy

Angiogenesis is the formation of new capillaries from pre-existing vessels which is of fundamental importance in development, normal organ maintenance and survival. This is a complex process that requires interaction between different cell types, the extracellular matrix and several cytokines and growth factors. Tumor angiogenesis relies on many of the same processes as those involved in physiological angiogenesis. Ischemia and hypoxic conditions, which initiate a cascade of highly coordinated cellular functions resulting in the establishment of new blood vessels and oxygen and nutrient supply, are major drivers of both physiological and tumor angiogenesis. Under physiological conditions, the chain of events and change in cellular function and composition recede following vascular perfusion.

By contrast, during tumor angiogenesis, the angiogenic cascade is persistent and unresolved, fuelled in part by tumor-secreted factors and tumor hypoxia. Angiogenesis contributes to the progression of cancer from a dormant *in situ* lesion to a life-threatening metastatic disease. The key role of vascular endothelial growth factor A (VEGFA) and its receptor VEGF receptor 2 (VEGFR2) in tumor angiogenesis is firmly established. VEGFA expression has been correlated with both vessel count and poor prognosis in patients with NSCLC, and anti-VEGF therapy has been approved by the FDA in this tumor type.

The ability of tumor cells to induce new blood vessel formation is essential for progressive tumor growth and blood-bone metastasis. Genetic studies in mouse and human have provided much evidence of the strong link between the TGF β signaling pathway and vascular morphogenesis and dysfunction. Deletion of the *Tgfb1* gene in the mouse results in embryonic lethality because of defective yolk sac vasculogenesis. Targeted deletions of *Acvr11* (ALK 1), *Tgfr1* (ALK 5), *Tgfr2* and *Eng* endoglin in mice result in vascular abnormalities⁴⁵. Studies in animal models and in humans suggest that the pro-angiogenic effects of TGF β 1 are dependent on the activation of its downstream signaling molecules: ALK1, which functions as a positive regulator of endothelial cell migration and proliferation, and ALK5, which promotes vessel maturation and is anti-angiogenic^{46, 47}. Therefore, the ratio of TGF β signaling through ALK1 and ALK5 probably determines its effect on angiogenesis. Furthermore, TGF β signaling might be modulated by the TGF β 1 type 3 receptor (endoglin) that is expressed on activated endothelial cells, which enhances TGF β 1-ALK1 signaling while exerting inhibitory effects on TGF β 1-ALK5 signaling⁴⁸. TGF β pathway acts as a potent inducer of tumor angiogenesis in several assays.

Tumor angiogenesis is crucial for tumor growth and invasion, as blood vessels deliver nutrients and oxygen to the tumor cells and allow them to intravasate the blood system. Several models indicate the role of tumor cell-secreted TGF- β 1 in tumor angiogenesis. Increased expression of TGF- β 1 in transfected prostate carcinoma or hepatocellular carcinoma enhanced tumor angiogenesis, and local administration of neutralizing antibodies to TGF- β 1 strongly reduced tumor angiogenesis^{49, 50}. Intraperitoneal injection of TGF β antibodies reduced angiogenesis and tumorigenicity of a renal carcinoma cell line that lacks T β RII, and is therefore not responsive to TGF- β ⁵¹. TGF β blockade significantly inhibited the

expression of VEGF preventing abnormalization of diaphragm lymphatic vessels and completely abolished ascites formation⁵².

The mechanism of angiogenesis stimulation by TGF β signaling also includes the induction of key angiogenic factors such as connective tissue growth factor (CTGF) and VEGFA which directly act on endothelial cells to stimulate cell proliferation and migration^{53, 54}. In addition, TGF β can induce the expression, secretion and activation of matrix metalloproteinase 2 (MMP2) and MMP9, and downregulate the expression of tissue inhibitor of metalloproteinase (TIMP) in tumor and endothelial cells²⁸. These metalloproteinase activities result in the enhancement of migratory and invasive properties of endothelial cells, which are required for tumour angiogenesis. Indirect stimulation of angiogenesis by TGF β may occur through the potent chemoattractant activity of TGF β for monocytes, which release angiogenic cytokines⁵⁵. Thus, TGF β -induced changes in the microenvironment provide favorable conditions for endothelial cell migration and capillary formation.

TGF β a master regulator of inflammation in the tumor microenvironment

Inflammatory cells are components of the microenvironment of normal tissues and organs, regulating various processes during development, including epithelial growth and branching and clearance of apoptotic cells⁵⁶. Inflammation and the significant effect of the tumor microenvironment on tumor progression and aggressiveness are identified as the hallmarks of cancer⁵⁷. There is a progressive change in the composition of the immune cell infiltrate with tumor stage to one that is more conducive to tumor progression⁵⁸. Interactions between cancer cells and cells of the innate and adaptive immune system occur in the tumor organ and illustrate the complexity and dynamics of the tumor tissue. The tumor microenvironment comprises inflammatory cells, including Tie2+, VEGFR1+, CD11b+, and F4/80+ populations; innate and adaptive immune cells, including natural killer (T) cells, T cells, and B cells; as well as cancer-associated fibroblasts we discussed above⁵⁹. These stromal cells collectively create an environment that promotes tumor progression by providing growth factors, pro-angiogenic factors, proteases, and adhesion molecules that facilitate tumor cell proliferation, angiogenesis, invasion, and metastasis^{60, 61}. This dynamic tumor microenvironment provides a selective pressure for tumor cell variants that give rise to genomic instability, genomic heterogeneity, and epigenetic alteration⁶².

TGF β controls immune responses and maintains immune homeostasis through its impact on proliferation, differentiation and survival of multiple immune cell lineages. Overproduction of TGF β by tumor cells and tumor infiltrating leukocytes has an adverse effect on anti-tumor immunity and inhibits significantly host tumor immune surveillance⁶³. TGF β markedly and directly suppresses the cytotoxic program of CTLs and regulates both the clonal expansion of CD8+ T cells and CD8+ T-cell cytotoxicity *in vivo*⁶⁴. TGF β also has a significant impact on CD4+ T-cell differentiation and function. TGF β induces Foxp3 and generates induced regulatory T cells (Tregs)⁶⁵. Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature dendritic cells (DCs), macrophages, granulocytes and other myeloid cells in early stages of their differentiation and have properties similar to those that have been described for M2 macrophages. Increased levels of MDSC, marked by CD11b+/CD14+/CD15+/CD33+, were observed in the peripheral blood of advanced stage NSCLC patients (n =87) compared with healthy controls⁶⁶. In late stages of tumor development, TAMs and MDSCs can produce TGF β and are classically involved in cancer progression and metastasis⁶⁷. It is not clear whether TGF β is directly involved in converting TAM from an M1 to M2 phenotype or whether, TAM may contribute to the general immunosuppressive tumor microenvironment by producing large amounts of TGF β .

Similar to TAM, tumor-associated neutrophils (TAN) also have polarized functions. Although no published studies have investigated the role of TAN in human NSCLC, recent preclinical work by Fridlender and colleagues showed that in mouse models of lung cancer, TGF β induces a population of TAN with protumor function (N2), whereas TGF β blockade results in antitumor neutrophils⁶⁸. Depletion of these N1 neutrophils resulted in increased tumor growth⁶⁸. TGF β also regulates production of IL-1 to control chronic inflammation, and the production of chemokines and chemokine receptors that recruit inflammatory cells⁶⁹. These chemokines include CXCL12, a key regulator of hematopoietic stem and progenitor cell trafficking between the peripheral circulation and targeted tumor tissues⁶⁹. SDF-1 mediates its effects on chemotaxis through its receptor, CXCR4, which is highly expressed on putative stem and progenitor cells⁶⁰. CXCR4 is the receptor for CXCL12. In lung adenocarcinoma, tumor expression of CXCL12 has been shown to correlate with accumulation of CXCR4-expressing immune cells, 30% of which were protumor Treg⁷⁰.

Inflammatory cells are another component of the tumor microenvironment. Brown and colleagues provided compelling evidence that ionizing radiation induced recruitment of tumor-protective BMDC, that promote blood vessel formation sufficient to support the growth of recurring tumors post-irradiation⁷¹. Suppression of BMDCs after irradiation with antibodies against CD11b greatly⁷¹ enhance the tumor radiation response in preclinical models. Conversely, inhibition of tissue resident macrophage before radiotherapy significantly protects against radiation-induced normal tissue damage⁷². Moreover, CAF promote further cell recruitment through secretion of chemokines such as SDF-1, which in turn enhance the recruitment of BMDCs involved in tumor vasculogenesis⁴³. As discussed above, TGF β activation is of great relevance, which includes the recruitment BMDCs to the tumor, aiding in the recovery of tumor growth by enhancing angiogenesis, supplying growth factors and creating a local immunosuppressive environment.

Alteration TGF β transduction pathways augment radiation response, from “Molecular” to “Microenvironment”

The sensitivity of LTGF β activation in vivo¹³ and demonstration of a redox switch^{14, 16}, suggested that it could have a role in orchestrating cell responses proximal to DNA damage. Consistent with this idea, epithelial cells of *Tgfb1* knockout embryos did not undergo radiation-induced apoptosis or cell cycle arrest after dams were irradiated with 5 Gy⁷³. TGF β depletion, by either gene knockout or by using TGFB neutralizing antibodies, also decreased p53 ser-18 phosphorylation in irradiated tissue. These data indicate that TGF β is essential for p53-mediated cellular responses that mediates cell fate decisions in vivo⁷³.

Studies in primary *Tgfb1* null and wildtype mammary epithelial cultures established that the effects of TGFB on radiation-induced molecular events and cell fate decisions are cell intrinsic, and are compromised as a function of TGFB abundance (i.e. both heterozygote and null cells were compromised when compared to wildtype cells, and when compared to each other). Treatment with TGF β restored the molecular and cell fate response. Moreover the mouse phenotype was replicated in human cells using a small molecule inhibitor the TGFB type I receptor (TBRIKI). In both models, TGF β treatment prior to irradiation restored radiation-induced signaling events. Thus, although radiation elicits TGF β activation through its redox sensor function, epithelial cells have severely compromised DNA damage response if TGF β is not present before they are exposed. Inhibition of TGF β signaling in normal epithelial cells, either by chemical or by genetic means, leads to increased radiosensitivity as measured by clonogenic survival⁷⁴.

It is interesting to ask why extracellular TGF β controls the intracellular DNA damage response, but it is also interesting to ask how. ATM is a nuclear sensor of DNA damage that

initiates, recruits and activates a complex program of checkpoints for cell cycle, apoptosis and genomic integrity^{75, 76}. TGF β 1 depletion compromises radiation-induced ATM kinase activity and autophosphorylation, leading to reduced phosphorylation of critical DNA damage transducers γ H2AX, Chk2, p53 and Rad17⁷⁴. Likewise, TGF β 1 antisense was also demonstrated to block ATM kinase and DNA damage response in A549 lung cancer cells⁷⁷. Recent studies further support the generality that TGF β inhibition prior to radiation attenuates DNA damage responses and increases clonogenic cell death in human and murine breast cancer and glioma cells^{78, 79}.

Recent studies shed light on the importance of the tumor microenvironment in modulating the tumor response to radiotherapy and open new opportunities for the development of novel therapeutic strategies to synergize radiotherapy. Increasingly evidence shows that ionizing radiation induces modifications of the tumor microenvironment, which profoundly impacts tumor biology which is particularly relevant to radiation response^{71, 80}. Strategies to improve radiotherapy are changing from “molecular” regulatory cancer cell intrinsic sensitivity to targeting of the “microenvironment” for better irradiation responses. As in normal organs, tumor vasculature is an important component in tumor stroma to ensure growth and development. Endothelial cell apoptosis precedes tumor cell apoptosis by 3 to 5 days, suggesting that tumor cells are dependent on endothelial cells for survival⁸¹. Tumor vascular endothelium plays a crucial role in tumor radiation response. Paris et al. also suggest that early phase microvascular endothelial apoptosis is mandatory for tumor cure⁸². Recent data indicate that radiation-induced endothelial cell apoptosis⁸³ can lead to vascular destruction and secondary tumor cell death⁸⁴.

TGF β Biology in Non-Small Cell Lung Cancer

Human lung cancer can be divided into two main histopathological groups: non-small cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). About 80% of lung cancers are NSCLC, which can be further subdivided into adenocarcinomas, squamous cell, bronchioalveolar, and large cell carcinomas⁸⁵. Radiotherapy is a well-established therapeutic modality in NSCLC. However cancer recurrences after radiotherapy often develop into more aggressive disease that is difficult to treat and associated with poor prognosis.

NSCLC contain multiple cell types and extracellular matrix components and develop through complex interactions between these different components of the tissues using processes that often resemble those used by the lung organ. Distortion of TGF β signaling is perhaps the most important prerequisite in tumor progression in NSCLC^{86, 87}. Reduced TGF β RII expression was reported in 40-80% of NSCLC at the protein and/or mRNA level and decreased sensitivity to the growth inhibitory effects of TGF- β ^{88, 89}. It has been demonstrated that both microsatellite instability and promoter methylation are associated with TGF β R2 mutations in NSCLC^{89, 90}. Moreover, TGF β RII deletion increases proliferation, local inflammation, and TGF β ligand elaboration; TGF β RII knockdown in airway epithelial cells increases migration and invasion in both lung adenocarcinoma and squamous cell carcinoma indicating that TGF β RII loss plays a causal role in lung carcinogenesis⁸⁷.

Reduced TGF β RII expression in human NSCLC is associated with specific histologic subtypes, more aggressive tumor behavior, or reduced patient survival. Genetic variations in the TGF β pathway were also predictors of survival in advanced NSCLC⁸⁶. Interestingly, TGF β RII down-expression is more common in lung squamous carcinoma patients of males and smokers, and both are at higher risk of NSCLC⁸⁷. Long-term cigarette smoke reduced treatment reduced apoptosis, increased cell viability, decreased TGF β -mediated growth

suppression through down-regulation of Smad3 and enhanced tumorigenicity in human lung adenocarcinoma. These data provide evidence that cigarette smoking promotes NSCLCS tumorigenicity partly by abrogating TGF- β -mediated growth inhibition and apoptosis by reducing expression of Smad3²⁴.

One of the most consistent histological features of NSCLC invasion is the appearance of desmoplasia: stromal changes characterized by the activation of stromal fibroblasts into CAF, increased matrix protein disposition, new blood vessel formation, and immune cell infiltration. The cell surface serine protease fibroblast activation protein-a (FAP-a) is selectively expressed on CAF in lung tumors as well as granulation tissue and in fibrotic lesions⁹¹. TGF β is a powerful factor inducing FAP expression in NIH3T3 fibroblasts⁴⁰. The induction of fibroblasts and FAP by TGF β is thought to convert fibroblasts to a proinvasive state⁹². Targeting FAP- α inhibits tumor cell proliferation indirectly, increases accumulation of collagen, decreases myofibroblast content, and decreases blood vessel density in NSCLC⁹¹. Moreover, FAP-expressing cells are an immune-suppressive component of the tumor microenvironment in preclinical NSCLCs model. Depletion of FAP-expressing cells in established Lewis lung carcinomas, caused rapid hypoxic necrosis of both cancer and stromal cells in immunogenic tumors by a process involving interferon- γ and tumor necrosis factor- α ⁹³.

A recent study showed CAF significantly increases the invasiveness of co-cultured NSCLC cells compared with normal fibroblasts while enhancing tumorigenicity of A549 NSCLC cells in vivo⁹⁴. Forty-six differentially expressed genes were identified between CAFs and NFs from 15 patients. These genes are involved in signal transduction (14/46), response to stress (11/46), cell adhesion (7/46), and angiogenesis (3/46). Fourteen genes differentially expressed between CAFs and NFs were also commonly differentially expressed in NSCLC tumor stroma compared with normal lung parenchyma. Importantly, 7 of the 14 overlap genes were reported as transcriptional targets of the TGF β signaling pathway. There was prognostic significance of the CAF-associated gene-expression signature in multiple independent cohorts of NSCLC patients⁹⁴.

Increased TGF β expression correlates with poor prognosis, increased tumor growth and angiogenesis in NSCLCs⁹⁵. TGF β level was found significantly higher in patients with lymph node metastasis and advanced stage NSCLC⁹⁵. It has been confirmed that anti-VEGF therapy inhibits tumor angiogenesis and subsequent tumor growth and metastases in NSCLCs⁹⁶. A randomized trial demonstrated that adding bevacizumab to carboplatin and paclitaxel improved survival in advanced NSCLCs⁹⁷.

Inflammation and tumor microenvironment are critical players in NSCLC cancer progression. Cytotoxic T lymphocytes (CTLs), mostly CD8+ were associated with prolonged survival in squamous cell carcinomas in a investigation of 1,290 NSCLC tumors⁹⁸. Stromal CD4+ T-cell and co-localization of stromal CD8+ T cells and CD4+ T-cell have all shown association with improved NSCLC survival^{99, 100}. The protumor association of Treg tumor infiltration was examined in a study of 100 stage I to III NSCLC tumors showing that tumor-infiltrating Foxp3+ Tregs increased tumor recurrence¹⁰¹. Antitumor tumor-associated macrophages (TAM) in NSCLC are of the M1 phenotype and accumulate intratumorally, whereas protumor TAMs of the M2 phenotype accumulate in the stroma and express interleukin-8 (IL-8), IL-10, and trigger receptor expressed on myeloid cells (TREM-1).

IL-8 is an angiogenic factor, and the angiogenic role of TAM in NSCLC has been shown by correlating macrophage density with intratumor microvessel counts and poor patient outcomes¹⁰². IL-10 is an immunosuppressive cytokine, and its expression by TAM has been

observed more commonly in stages II, III, and IV NSCLCs, thus correlating with decreased overall survival (26). TAM expression of TREM-1, which can initiate and amplify an inflammatory response, is increased in malignant pleural effusions of NSCLC patients¹⁰³. Furthermore, in 68 stage I to III NSCLC patients, an increased level of TREM-1 high TAM in resected specimens was an independent predictor of shorter overall survival¹⁰³.

In patients with early stage NSCLC who are unable to tolerate surgical resection because of medical co-morbidities, conventional radiotherapy remains the standard therapy. However local recurrence is still the main cause of NSCLC radiotherapy failure, with local failure of up to 50% and poor long-term survival of 15–30%^{41, 104}. There are essentially two approaches to improve local control with radiation therapy - either use a higher radiation dose or use more effective radiation sensitization.

Hypofractionated stereotactic body radiation therapy (SBRT) allows escalation of the fractional dose, which is important to improve the local control rate of tumors and overall survival for medically inoperable patients with early-stage NSCLC¹⁰⁵. However, there are still many issues to be elucidated. Firstly, radiation dose and fractionation vary because of a lack of biologically effective dose (BED) guidelines. Importantly, radiation induced normal tissue damage is still the biggest obstacle to limit the dose escalation of SBRT in NSCLC¹⁰⁶. Another approach that has enormous appeal involves the development of nontoxic, yet effective molecularly targeted radiosensitizers. If a drug selectively sensitizes the tumor response to radiation, one could use selective escalation of the effective biologic dose to the tumor, and therefore improve local control without increasing morbidity.

We hypothesize that the microenvironment regulates many tumor responses to radiation, thus providing novel routes for manipulating the response to ionizing radiation. Although little is known how TGF β modulates the irradiated tumor microenvironment, given its pleiotropic roles in NSCLC it seems reasonable that TGF β inhibition could both increase tumor cell radiosensitivity and shift microenvironment to augment NSCLC response to radiotherapy.

Preclinical studies support this conjecture that tumor growth, host cells and the composition of the microenvironment are highly interdependent (Du and Barcellos-Hoff, unpublished data). As found for brain and breast tumors^{78, 79}, most murine and human lung cancer cells were sensitized by TGF β inhibition prior to radiation as measured by in vitro clonogenic assays. Using the Lewis lung cancer syngeneic subcutaneous tumors, tumor growth control was significantly improved by use of TGF β neutralizing antibodies concurrent with single or fractionated radiation treatment. Notably, even though irradiated tumors treated with TGF β inhibition were significantly smaller at experiment termination, hypoxia was higher and vessel density was also significantly decreased, compared with non-irradiated, bigger tumors. Martin Brown has shown that hypoxia promotes mobilization of CD11b+ monocytes producing the pro-angiogenic factor MMP9 into the tumor microenvironment, which in preclinical GBM is necessary for tumor regrowth – and that blockade of this crucial event prevents tumor recurrence⁷¹. The combined treatment of radiation and TGF β inhibition decreased CD11b+/MMP9 monocytes, consistent with Brown and colleagues but suggestive that TGF β is necessary for the recruitment of the CD11b+/MMP9 cells and therefore, tumor regrowth.

Given that radiation induced immunity is critical for long term benefit¹⁰⁷, we also studied the response to the combined treatment of fractionated radiation and TGF β inhibition on the peripheral anti-tumor immune response. Analysis of monocyte maturation and activation markers CD11b and F4/80 in tumors suggest that distinct BMDC are recruited as a function of treatment: the F4/80+ macrophage population is more differentiated, while CD11b is

more immature. TGF β inhibition concurrent with radiation treatment also affects systemic maturation as evidenced by analysis of cells from spleens of treated mice. These preliminary data taken together suggest that TGF β inhibition concurrent with fractionated radiation treatment may cooperate in directing both the microenvironment and the immune system towards an anti-tumor response, which could lead not only to better control of the primary tumor growth but also to abrogation of relapse.

Summary

Although the radiation response of tumor cells has been far better characterized than that of the microenvironment, there is growing appreciation that the two are inextricably intertwined. The therapeutic effects of radiotherapy are traditionally considered as due to the induction of double strand DNA breaks in cancer cells causing cell cycle arrest, senescence or apoptosis¹⁰⁸. Accordingly, efforts aimed at understanding and improving the therapeutic efficacy of radiotherapy largely concentrated on the molecular mechanisms of DNA damage and repair. When cancer is treated with radiation, death of tumor cells coupled with changes in tumor microenvironment leads to tumor regression. It is likely that additional avenues of benefit may accrue by augmenting radiation effects on host cells, including innate and adaptive immune cells, CAF and endothelial cells.

As normal organ wound healing, the remaining tumor organ sends tissue damage signals to initiate remodeling and repair. TGF β is a key factor in normal organ wound healing following damage. Among all radiation-induced cytokines in tumor microenvironment, TGF β activation could potentially mediate enhancement of DNA damage repair, recruitment of BMDCs to the tumor, aiding in the recovery of tumor growth by enhancing angiogenesis, supplying growth factors and creating a local immunosuppressive environment which all contribute to tumor relapse following treatment.

There are several TGF β inhibitors in clinical trials, as recently comprehensively reviewed by Akhurst and Hata¹⁰⁹. We believe the therapeutic benefit from TGF β inhibitors will be best exploited in the context of radiation therapy; first because of its control of the DNA damage response leads to greater tumor cell kill and second, as occurs during normal tissue wound recovery, because it mediates many of the microenvironmental responses that promote tumor regrowth. The excellent safety profiles demonstrated in these clinical trials, as well as the possibility of protection from late complications in irradiated normal tissues¹¹⁰, provide further motivation for assessing TGF β inhibitors as an adjunct to radiation therapy of carcinomas.

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Abbreviations

TGFβ	transforming growth factor β
ECM	extracellular matrix
NSCLC	non-small cell lung cancer
EMT	epithelial to mesenchymal transition

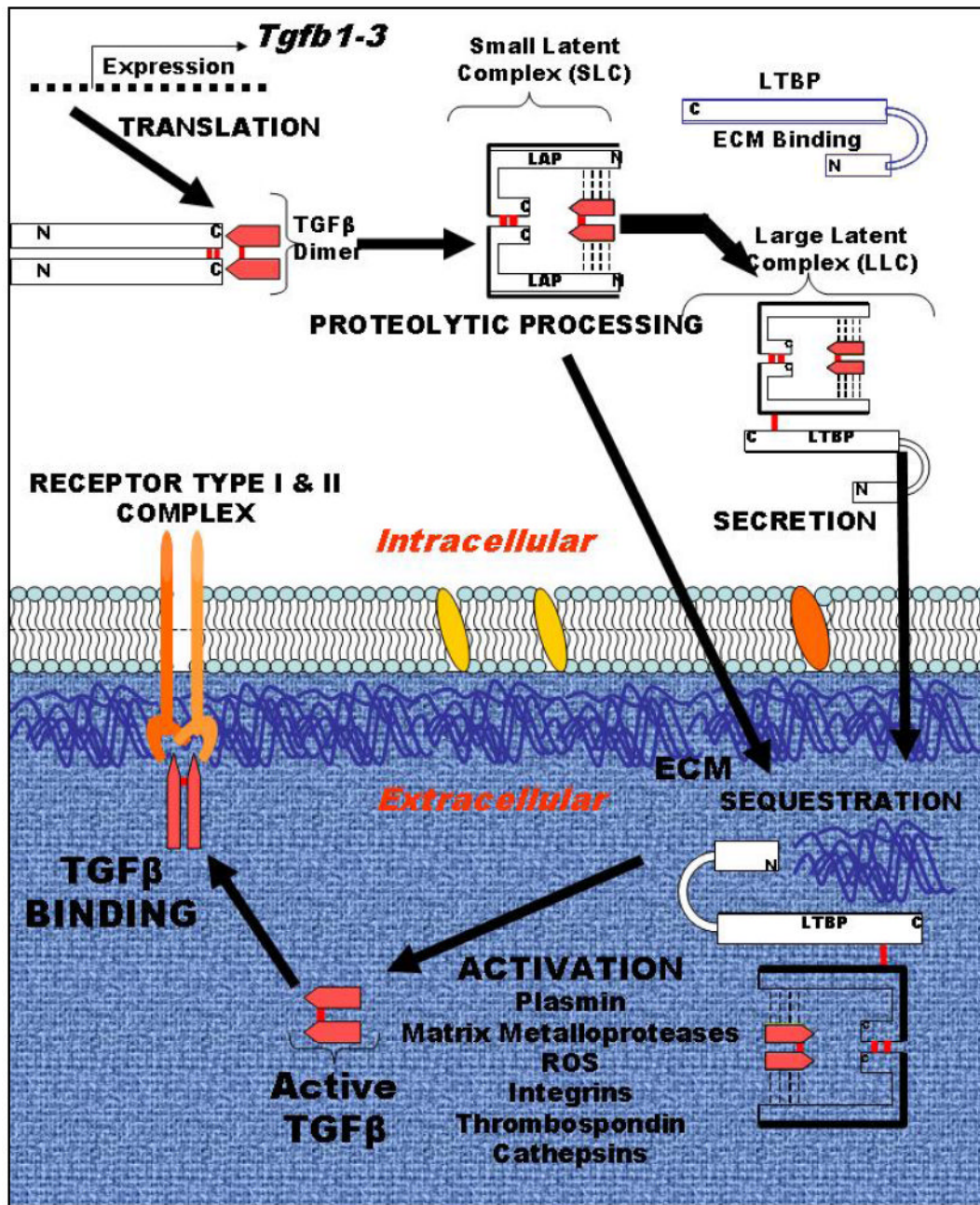


Figure 1. Schematic of TGFβ production as a latent complex

TGFβ 1-3 isoform genes encode a single polypeptide that is processed, cleaved to make a homodimer of the pre-pro peptide, which is designated as latency associated protein (LAP), that is non-covalently bound to TGFβ proper, which is a 24 kD homodimer, which associate in a non-covalent bound complex. LAP acts as a chaperone for proper folding, contains the signal sequence for secretion and sequesters TGFβ until activation. This so-called small latent complex can also form the large latent complex upon covalently binding the latent TGFβ binding proteins (LTBP), which sequester latent TGFβ in the extracellular matrix (ECM). Activation occurs by multiple relatively understudied mechanisms, as listed, to release TGFβ for binding to its receptors, type I and II, which then initiates the signaling cascade.