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TITLE: Analysis of Alk-1 Signaling In Endothelial Cells and its Role in Breast Tumor Angiogenesis

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14. ABSTRACT
The process of tumor angiogenesis, i.e. the vascularization of the tumor mass by formation of new blood vessels, is important for tumor growth and metastasis. Inhibition of tumor angiogenesis is therefore an important goal for therapeutic intervention in breast cancer. Understanding the regulation of tumor angiogenesis at the molecular level is a prerequisite for successful manipulation of this process. Angiogenesis is regulated by the coordinated action of various growth factors and their receptors, among the Alk-1, a member of the TGF-β receptor family. While convincing genetic evidence has demonstrated an essential role of Alk-1 in developmental angiogenesis and the maintenance of the vascular system in the adult, the possible role of Alk-1 in tumor angiogenesis as well as its cellular and molecular functions in endothelial cells need to be investigated. To this end we have generated a variety of tools for the analysis of Alk-1 function and have made further progress in elucidating its role in the regulation of endothelial cell growth and survival. These studies have the potential of leading to the identification of novel targets in the TGF-β signaling system for anti-angiogenic intervention in breast cancer.

15. SUBJECT TERMS
Growth factors, receptors, signal transduction, transcription, endothelial cells, angiogenesis, hematopoietic stem cells

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Marcus Kretzschmar. Ph.D. the presented data are unpublished and should be protected

Introduction

Inhibition of tumor angiogcnesis is a promising avenue of therapeutic intervention in breast cancer as well as other forms of cancer. Identification and characterization of the molecules that control and regulate the process of tumor angiogcnesis is a prerequisite for successful manipulation of this process. One of the growth factor receptors that is critical for developmental aneiogenesis is the TGF-β family receptor Alk-1. Functional deletion of Alk-1 in mice causes embryonic lethality due to defects in angiogenesis. Furthermore, haploinsufficiency of Alk-1 is the cause of hereditary hemorrhagic telangiectasia (HHT) type II, a genetic disease in man that is characterized by defects in the adult vasculature. Based on this information the idea was proposed that Alk-1 signaling may also be important for tumor angiogenesis in breast cancer. Furthermore, it was hypothesized that Alk-1 serves as a functional receptor for TGF-β, a growth factor that is abundantly expressed in breast tumors, and thereby mediates some of the pro-angiogenic activities that have been ascribed to TGF-β. The objective of the project is to investigate the validity of these hypotheses. Specific goals are to determine the expression patterns of Alk-1 and Alk-5/TβR-I (the known TGF-β type I receptor) in the vasculature of normal breast tissue and tumor tissues from progressive stages of breast cancer, and to study the functions of these receptors in endothelial cells. These studies have the potential of leading to the identification of Alk-1 as a novel target for anti-angiogenic therapeutic approaches.

Progress

Aim 1: to determine the expression patterns of Alk-1 and Alk-5 in the vasculature of normal breast tissue, ductal carcinomas *in situ* (DCIS), and primary tumors derived from patients with premetastatic or metastatic invasive breast carcinomas.

We previously described the initial characterization of a monoclonal antibody against the extracellular domain of human Alk-1. This antibody displayed a high degree of specificity for Alk-l as compared to other members of the TGF-β type I receptor family in Western blots (Figure 1). Furthermore, the antibody recognized not only denatured Alk-1 but also surface-expressed, native Alk-1 in human dermal microvascular endothelial cells (HMEC-1) (Figure 2). These results suggested the usefulness of this antibody for the planned immunohistochemical analysis.

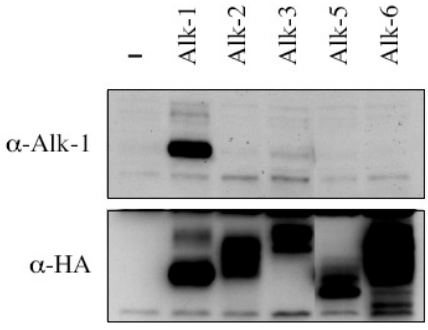

Figure 1. Specificity of the Alk-1 monoclonal antibody. COS cells were transiently transfected with expression constructs for HA-tagged members of the TGF-β type I receptor family. 48 hrs post-transfection whole cell lysates were prepared and analyzed by immunoblotting against the HA-tag and against the extracellular domain of Alk-1. The receptors tested included Alk-1, the activin receptor Alk-2, the bone morphogenic protein (BMP) receptors Alk-3 and Alk-6, and the TGF-β receptor Alk-5 (TβR-I).

Surprisingly, in immunohistochemistry experiments using paraffin-embedded sections of normal human breast and skin tissues the antibody stained not only the endothelial cell lining of blood vessels, as expected, but also the epitheiial cells of the mammary gland ductal system (see previous report).

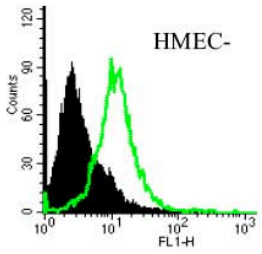

Figure 2. Cell surface recognition of Alk-1 by the monoclonal antibody. Human dermal microvascular endothelial cells (HMEC-1) were analyzed by FACS for cell surface expression of Alk-1. The closed curve represents the IgG control, the open curve represents the Alk-1 monoclonal antibody.

This result was unexpected since AIk-1 expression is thought to be restricted mostly to the vascular endothelium [1-3] and it therefore raised questions regarding the specificity of the antibody in immunohistochemical tissue staining. Since the antibody recognized a major band of about 9OkD molecular weight in Western blots of whole cell lysates of breast epithelial cells (data not shown) we determined by mass spectrometry whether this band represented Alk-1, a modified form of Alk-1, or an Alk-1 related protein. This analysis revealed that the antibody cross-reacted with an epithelial cell-specific cytoplasm protein that is unrelated to Alk-1 (data not shown). Since this molecule might also be recognized in immunohistochemical staining of breast epithelial tissues, this cross-reactivity casts doubt on the usefulness of this antibody for the planned analysis of tissue microarrays that contain cores of normal breast tissue and cancerous breast tissues derived from various stages of the disease. Thus, while the characterized antibody represents a valuable tool for Western blotting or surface analysis of Alk-1, the tissue microarray analysis will have to await the availability of a different antibody that has demonstrated specificity for Alk-l in immunohistochemical staining of epithelial tissues.

Aims 2 and 3: to study the functions of Alk-1 in specific cellular processes that is important for angiogenesis; to compare the activities of Alk-l in these processes with those of AIk-5, and to determine the respective contributions of these different receptors to the effects of TGF-β legends.

Using the tools described in the previous report we have continued to analyze the cellular functions of the Alk-1 receptor in endothelial cells and to compare its activities with those of Alk-5/TGFR-I, in particular in the response to TGF-β. Our previous experiments using RNA interference indicated that the Alk-1 and Alk-5 receptors have opposite roles in regulating endothelial cell death and survival, with Alk-1 acting as an inducer of apoptotic cell death and Alk-5 acting to suppress apoptotic cell death.

To confirm the differential roles of the two TGF-β receptors in inducing apoptotic cell death with a different method, we transfected primary endothelial cells with Alk-1 or Alk-5 receptors harboring a single amino acid substitution that confers constitutive kinase activity [4,5]. These constitutively active receptors (caAlk-1 and caAlk-5) were co-transfected with red fluorescent protein to mark the transfected cells and apoptotic cells were stained 48 hours post-transfection with FITC-VAD-FMK and analyzed by fluorescence microscopy. A significant number of apoptotic cells were observed in the caAlk-1 expressing cells, but not in control transfected cells

or cells expressing the constitutively active Alk-5 receptor (Figure 3). Furthermore, we transfected cells with a constitutive (i.e. CMV enhancer-driven) luciferase reporter to measure relative cell numbers 48 hrs post-transfection. Co-transfection of caAlk-1 caused a strong reduction of luciferase levels as compared to control-transfected cells, while co-transfection with caAlk-5 caused an increase in luciferase levels (Figure 4).

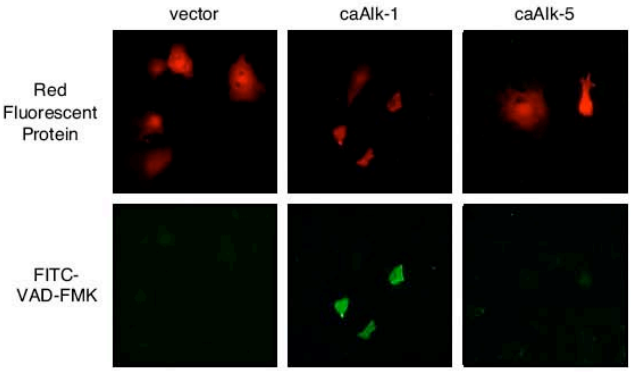

Figure 3: BAECs were co-transfected on slides with red fluorescent protein and empty expression vector (vector), constitutively-active Alk-1 (caAlk-1) or constitutively-active Alk-5 (caAlk-5), followed by staining with FITC-VAD-FMK and analysis by fluorescence microscopy. Representative sections are shown.

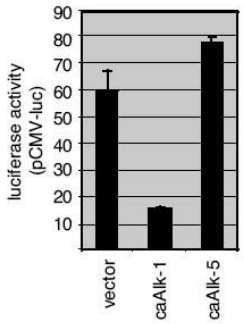

Figure 4: BAECs were co-transfected with a CMV promoter/enhancer-driven luciferase reporter (pCMV-luc) and empty vector, caAlk-1 or caAlk-5, followed by measurement of luciferase activity 48 hrs post-transfection.

In summary, these data support our conclusion derived from the RNA interference experiments that the ubiquitous TGF-β receptor Alk-5/TβR-I functions to suppress apoptotic cell death in endothelial cells, while the alternative receptor Alk-I induces apoptosis in response to TGF-β ligand.

These observations raise questions regarding the mechanisms underlying these opposite effects on cell death. We started to address these mechanisms by investigating the possible involvement of pro- or anti-apoptotic members of the Bcl-2 protein family. Members of this protein family regulate cell death and survival in many different cell types and the expression levels as well as the ratio of anti-apoptotic (e.g. BcI-2) to pro-apoptotic (e.g. flax) family members are important determinants of a cell's sensitivity to cytotoxic conditions and pro-apoptotic signals [6]. Up regulation of Bcl-2 protein levels is also an important mechanism employed by tumor cells to escape from cell death.

To test the possible involvement of this protein family in the regulation of cell death or survival by the two TGF-β receptors we analyzed the levels of various family members after TGF-β treatment of primary endothelial cells. We found that TGF-β signaling leads to an increase in Bcl-2 protein levels and a concomitant decrease in flax protein levels, thereby providing an anti-apoptotic regulation of the ratio of these two key family members (Figure 5,

lanes 1 and 2). Furthermore. RNA interference against Alk-1 and Alk-5 showed that this anti-apoptotic response is mediated by TGF-β signaling via the Alk-5 receptor, but not the Alk-1 receptor (Figure 5, lanes 4 to 6).

Future experiments will address the interesting possibility that Bcl-2 and Bax genes are directly regulated by Smad2 or Smad3 transcription factors in response to TGF-β /Alk-5 signaling, and thereby represent part of a direct, Smad-dependent survival signal.

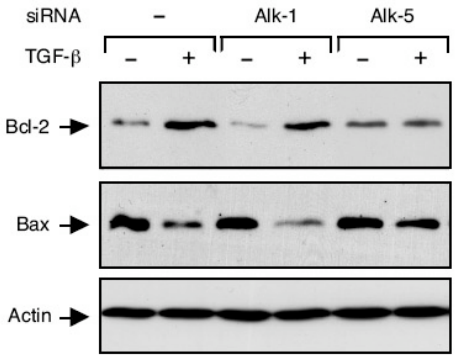

Figure 5: BAECs transfected with siRNAs as indicated were treated with TGF-β1 for 48 hrs. Cell lysates were analyzed by immunoblotting against Bcl-2 and Bax.

Aims 4 and 5: to determine whether TGF-β can activate Smad 1, 5, or 8 through the endogenous Alk-1 receptors in endothelial cells and thereby lead to transcriptional regulation of Smad1 responsive target genes; to identify genes that are regulated in response to Alk-1 signaling in microvascular endothelial cells.

We have previously reported the identification of the Tie1 and Tie2 genes as targets of Alk-1 signaling in endothelial cells. The Tie genes encode endothelial-specific tyrosine kinase receptors that are involved in vessel remodeling, organization, and integrity, and therefore play a role in similar processes than Alk-1 [7-9]. Furthermore, we previously demonstrated that in the human microvascular or bovine aortic endothelial cells TGF-β signals through both Alk-l and Alk-5 receptors, thereby activating two distinct branches of Smad signaling activity, the Smadl/5 branch and the Smad2/3 branch.

To investigate whether the Tie genes represent direct targets of the Alk-l activated Smad transcription factors we are now conducting an analysis of the Alk-I responsive region in the Tie2 gene. The promoter and intronic enhancer regions of the murine Tie2 gene provide most if not all of the regulatory elements that are required for the specific expression in the vascular endothelium [10]. In transgenic mice these two regions together recapitulate the expression of the endogenous Tie2 gene. The intronic enhancer comprises about 1.7 kilobases of sequence in which a number of binding sites for known transcription factors have been identified [11]. Mutational analysis demonstrated the importance of several of these sites for Tie2 expression in cultured endothelial cells as well as in transgenic mice [10]. Based on this information we created reporter constructs that express the firefly luciferase gene under the control of these in vivo characterized promoter and enhancer regions of Tie2. These constructs allowed us to test and characterize the Alk-1 responsiveness of these genomic regions in cell culture assays. The reporter constructs were transfected into primary endothelial cells together with empty vector or expression constructs for constitutively activate [A1(Q/D)] and kinase-dead [A1(K/R)] Alk-1 receptor. This analysis revealed that the enhancer is activated about 5-fold by the activated Alk-

1 receptor and that this activation depends on the kinase activity of Alk-1 (Figure 6). The Tie2 promoter region was not activated by Alk-l signaling (data not shown). Interestingly, the analysis of additional Tie-2 enhancer constructs demonstrated that the activity of the Tie-2 enhancer depended on its orientation with regards to the associated basal promoter (Figure 6). Together these results indicate that the regulation of the Tie-2 gene by Alk-1 signaling is mediated by responsive elements contained in the intronic enhancer region.

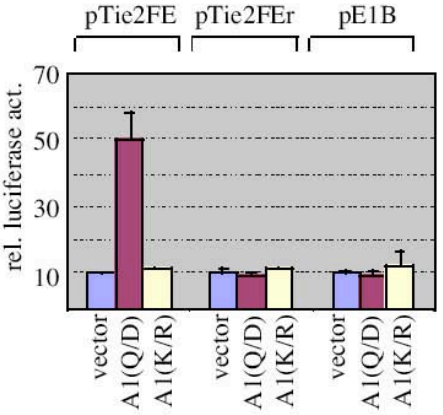

Figure 6. Various reporter constructs containing the full length Tie2 enhancer coupled to a heterologous basal promoter (E1B) were tested in transiently transfected BAECs for Alk-1 inducibility. pTie2FE contains the enhancer head-to-tail with the promoter, while pTie2FEr contains it in reverse orientation. The transfected cells were lysed 48 hrs post-transfection and the Alk-1 responsiveness of the constructs was determined by measurement of luciferase activity.

To localize the Alk-1 responsive element within the 1.7 kb Tie2 enhancer we then carried out a deletional analysis by creating progressive deletions from the 5' and 3' ends of the enhancer within the context of the luciferase reporter construct. As shown in Figure 7 a region of about 400 base pairs was identified as the Alk-1 responsive region. Interestingly, AIk-1 responsiveness was lost when either half of this region was deleted, indicating the possibility that more than one transcription factor binding site contributes to Alk-l responsiveness (Figure 7).

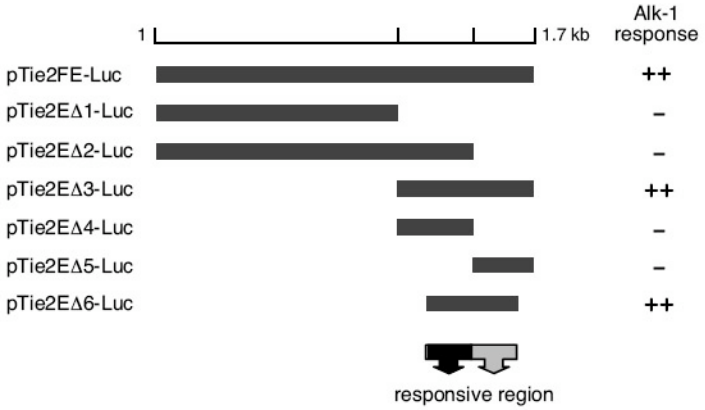

Figure 7: BAECs were transfected with the indicated *Tie2* enhancer reporter constructs and either empty vector or an expression construct for constitutively activated Alk-1 receptor. The transfected cells were lysed and the Alk-1 responsiveness of the constructs was determined by measurement of luciferase activity.

With the sequencing of various genomes, including the human and mouse genomes, comparative genomes has developed into a powerful tool for the identification of important regulatory DNA sequences [12,13]. We therefore carried out a genomic sequence comparison between the murine and human A/k-1 loci in order to determine whether the identified Alk-1 responsive region is conserved between the two species. Remarkably, disregarding a short insert found in the mouse sequence the 400 bp region containing the Alk-1 response elements is conserved between the human and mouse genomes with an identity of more than 90 percent (data not shown). This strongly supports the idea that the Alk-1 responsiveness of the Tie2 gene

in endothelial cells is mediated via a highly conserved, endothelial-specific intronic enhancer region. The identification of this 400 hp region should now facilitate our analysis of the involvement of Alk-l activated Smad transcription factors in the direct regulation of the Tie2 gene.

Additional aim introduced after change of PI from Dr. M Kretzschmar to Dr. H Snoeck

One of the interests of the Snoeck laboratory is TGF signaling in hematopoietic stem cells (HSC). We have shown that *Tgfb2*$^{+/-}$ mice have a defect in HSC function and renewal. As TGF-β is typically considered an inhibitor of stem cell function, this phenotype was rather unexpected and it signaling mechanism most likely involves an alternative pathway (14). We discovered that the *Tgfb2* locus also encodes a conserved alternative splice form of transforming growth factor-beta2 (TGF-β2), Δ6-TGF-β2, which is frameshifted in the TGF-receptor binding portion (Fig. 8a). Δ6-TGF-β2 is induced in stem and progenitor cells during recovery from hematopoietic damage and upon aging, conditions where HSC cycling is enhanced (Fig. 8b,c). Δ6-TGF-β2 is a secreted molecule (Fig. 8d,e).

Consistent with these findings, Δ6-TGF-β2 increased cycling of stem and progenitor cells and

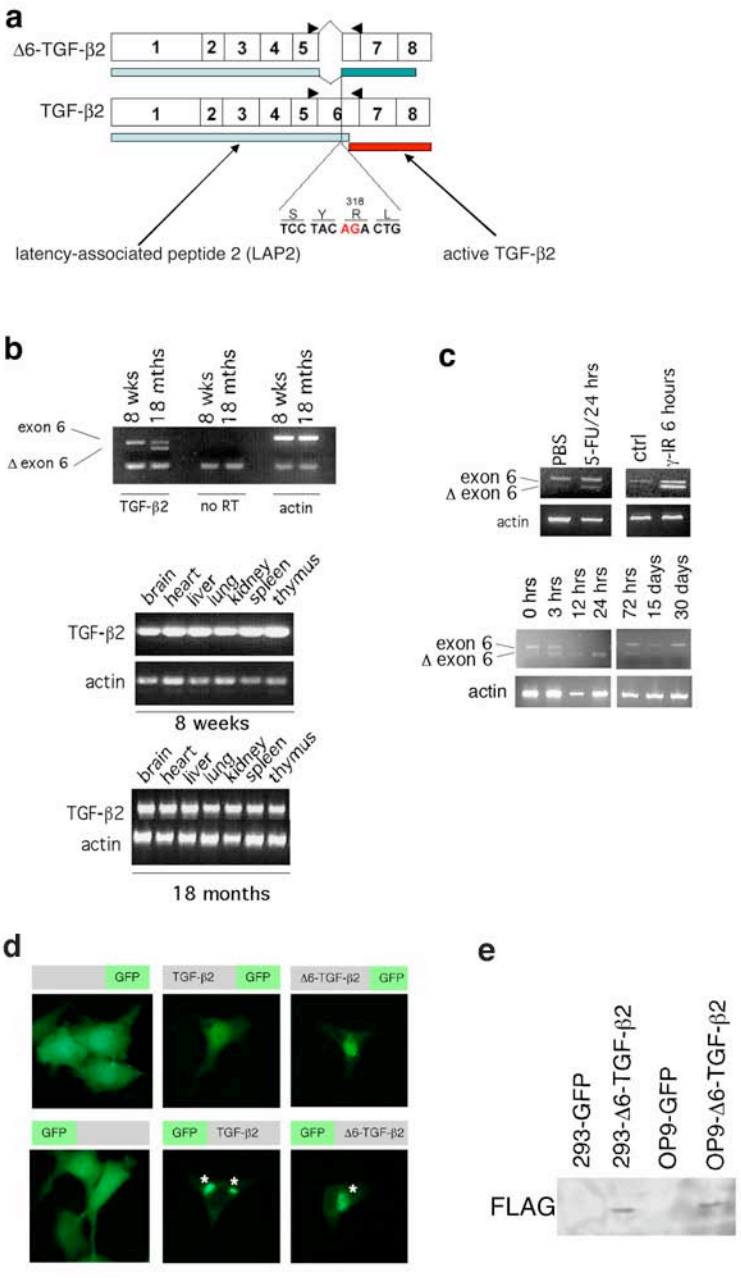

Figure 8: Expression of **D6-TGF-b2. (a)** Exon-intron structure of TGF-β2 and of Δ6-TGF-β2 mRNA. **(b)** Expression of Δ6-TGF-β2 mRNA in LSK cells and in various tissues from 8-week-old and 18-month-old C57BL/6 mice. **(c)** Expression of Δ6-TGF-β2 in LSK cells from C57BL/6 mice exposed to either 5-FU (150 mg/kg IP) or γ-irradiation (950 cG). The lower panel shows a time response after irradiation. **(d)** Distribution patterns of N-terminal and C-terminal fusions of Δ6-TGF-β2 with GFP in NIH-3T3 cells **(a,** white stars = Golgi**)**. **(e)** Detection of recombinant FLAG-tagged Δ6-TGF-β2 protein in the supernatant of Δ6-TGF-β2 transfected OP9 and 293 cells after immunoprecipitation.

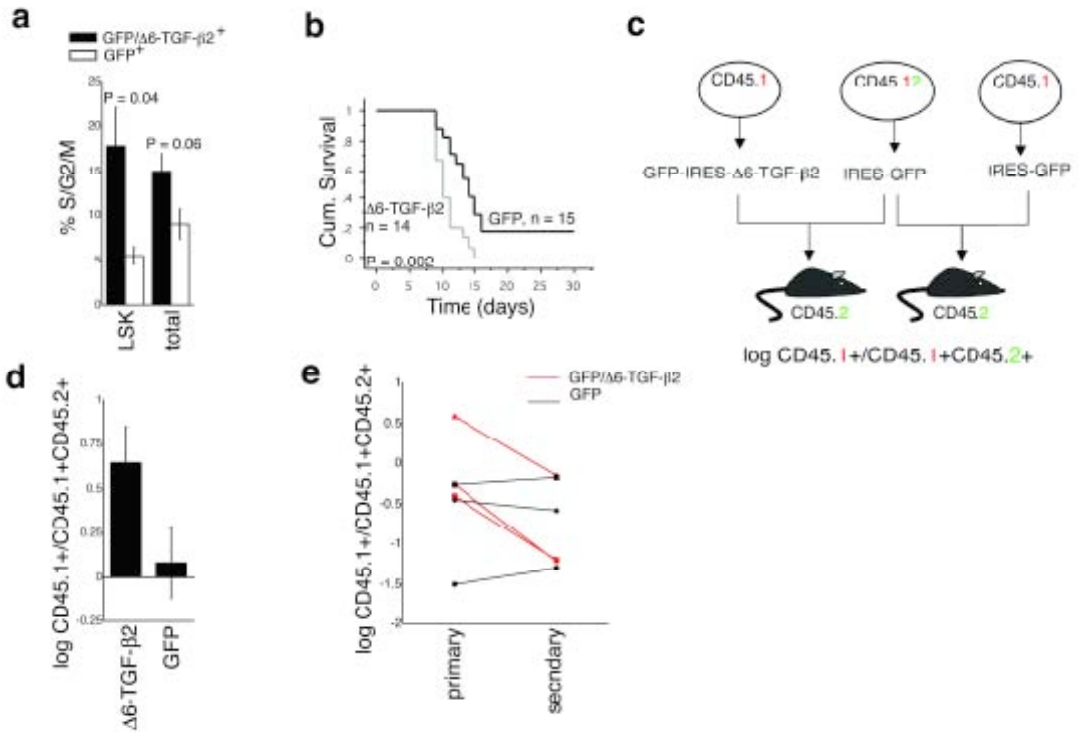

Figure 9: Δ6-TGF-β2 overexpression *in vivo*. (a) Fraction donor-derived LSK cells in cell cycle, as measured by staining of fixed bone marrow with Hoechst 33342 in mice that were reconstituted with CD45.1$^+$ GFP$^+$ or GFP/Δ6-TGF-β2$^+$ retrovirally transduced bone marrow cells. **(b)** Kaplan-Meyer survival curves of the survival of GFP/Δ6-TGF-β2 or GFP reconstituted mice after injection of 500 mg/kg 5-fluorouracil IV. **(c)** Experimental strategy for competitive repopulation studies using GFP and of GFP/Δ6-TGF-β2 transduced bone marrow cells. **(d)** Log ratios in primary transplantations with GFP and of GFP/Δ6-TGF-β2 transduced bone marrow cells. **(e)** Log ratio in secondary transplantations. Each data point in the secondary recipients represents the average of three mice (see text).

lethality from the cytotoxic agent 5-fluorouracil (Fig. 9a,b).
Furthermore, in competitive repopulation experiments, where bone marrow cells transduced with a Δ6-TGF-β2 expressing retroviral vectors were competed with control GFP vectors (see Fig. 9c for the experimental design), Δ6-TGF-β2 enhanced repopulation in the first round of transplantation (Fig. 9d). However, upon transfer to secondary recipients, Δ6-TGF-β2 expressing stem cells actually lost repopulation capacity (Fig. 9e), suggesting premature exhaustion, likely caused by enhanced cycling. Together, these finding indicate that Δ6-TGF-β2 is novel regulator of HSC cycling that is induced in conditions of stress. Knockout mice with a specific deletion of Δ6-TGF-β2 are currently being produced to determine the in vivo relevance of this protein.

Key Research Accomplishments

- Identification of TGF-β1 as an agonist of the Alk-1 receptor in endothelial cells
- Characterization of an Alk-1 specific monoclonal antibody with respect to its usefulness for

Western blotting, FAC 5, and immunohistochemistry

- Establishment of adenoviral vectors expressing constitutively active or kinase-inactive mutant forms of Alk-1 as tools for the analysis of Alk-1 mediated cellular and gene responses

- Establishment of stable endothelial cell lines expressing a constitutively-active or a dominant-negative form of the Alk-1 receptor

- Establishment of RNA interference as a tool to study TGF-β regulation of endothelial cells by selective knock-down of the expression of endogenous Alk-1 or Alk-5 receptors

- Identification of cell cycle stimulation as a cellular response to TGF-β signaling via the Alk-1 receptor in endothelial cells

- Identification of induction of apoptosis as a cellular response to TGF-β signaling via the Alk1 receptor in endothelial cells

- Identification of enhanced survival as a cellular response to TGF-β signaling via the Alk-5 receptor in endothelial cells

- Identification of anti-apoptotic regulation of Bcl-2 and Bax as part of the Alk-5 mediated survival response

- Identification of Tie1 and Tie2 as target genes of Alk-1 receptor signaling in endothelial cells

- Identification of a 400bp intronic enhancer sequence as the Alk-1 responsive region

- Identification of an alternative splice form of TGF-b2, Δ6-TGF-β2, as a novel positive regulator of the cycling activity of hematopoietic stem cells.

Reportable Outcomes

- Poster presentation at the 'Era of Hope" meeting

- Establishment of stable endothelial cell lines expressing a constitutively-active or a dominant-negative form of the Alk-1 receptor

- Cloning and protein expression of Δ6-TGF-β2

Conclusions

Our results have provided evidence that TGF-β functions as an agonist of the Alk-1 receptor in endothelial cells, in addition to the known TGF-β receptor Alk-5/TβR-1. Our data therefore demonstrated that TGF-β can signal via two distinct type I receptors and consequently via distinct Smad signaling pathways in these cells. We have furthermore developed tools to dissect the roles of the individual receptors in mediating angiogenic functions of TGF-β in endothelial cells. Our results show that TGF-β can control cell cycle progression and cell death/survival in endothelial cells in opposite ways by signaling predominantly via one or the other receptor. Alk-l signaling induces apoptotic cell death, while Alk-5 signaling provides a survival signal that

involves the anti-apoptotic regulation of the Bcl-2 and Bax proteins. Furthermore, we identified Tie1 and Tie2 as target genes of Alk-1 signaling, suggesting a direct functional interplay between the TGF-β/Alk-1 and Angiopoietin/Tie signaling systems in the regulation of endothelial cells. Our analysis of Tie2 gene regulation by Alk-1 signaling revealed an Alk-1 responsive region within a highly' conserved intronic enhancer region.

These studies have the potential of leading to the identification of novel targets in the TGF-β signaling system for anti-angiogenic intervention in breast cancer. Understanding the specific cellular functions of the two distinct TGF-β type I receptors may allow to manipulate selectively pro-angiogenic functions of TGF-β while leaving unaffected its important roles in tumor suppression and in controlling normal tissue homeostasis. Such selective inhibitors of TGF-β function could provide a significant new tool for the control of breast cancer progression.

The work on hematopoietic stem cells has identified a novel splice form of TGF-β2, Δ6-TGF-β2 as novel regulator of HSC cycling that is induced in conditions of stress.

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Appendices

None