[Frontiers in Bioscience 13, 7243-7249, May 1, 2008]

Influence of TBK-1 on tumor angiogenesis and microvascular inflammation

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1. ABSTRACT

Tank-Binding-Kinase 1 (TBK-1) has been proposed as a putative mediator in tumor angiogenesis. It was the aim of our study to gain insight into TBK-1s role in tumor angiogenesis and tumor-associated microvascular inflammation. TBK-1 overexpressing KB 3-1 cells were generated and their growth characteristics were analyzed. Expression of TBK-1, VEGF, RANTES and Il-8 were quantified using qPCR and western blot analysis. Intravital microscopy using the dorsal skinfold chamber model in nude mice addressed total (TIVD) and functional intratumoral vascular density (FIVD), perfusion index, vessel diameter and leukocyte sticking. Transfection of KB-3 cells resulted in significantly increased TBK-1, RANTES and IL-8 expression without affecting cellular growth. Supernatants from TBK-1 overexpressing clones induced HUVEC proliferation. Intravital microscopy identified an increase in leukocyte sticking paralleled by significantly increased TIVD and FIVD as a result of increased VEGF expression. Therefore, TBK-1 represents a novel mediator of tumor angiogenesis and exerts proinflammatory effects via upregulation of inflammatory cytokines. The TBK-1 pathway might be an important cross-link between angiogenesis and inflammation representing a possible target for anti-tumor therapy.

2. INTRODUCTION

New insights into the angiogenic process involved in tumor biology postulate an important interaction of the inflammatory system and angiogenesis (1). In this context a complex system of pro- and anti-angiogenic chemokines, primarily known for their inflammatory nature, are involved in modulating the formation process of new blood vessels and therefore represent a sensitively balanced regulation system for angiogenesis (2). Recently, TANK-binding-Kinase 1 (TBK-1) has been described as a novel kinase activating the inflammatory Nuclear Factor-kappaB pathway as well as the Interferon-regulatory-factor 3 (IRF-3) pathway, resulting in a proinflammatory immune response primarily directed against viral antigens (3, 4). However, using a novel functional genomics screening procedure for proangiogenic genes and pathways, the TBK-1-IRF-3 pathway was demonstrated to stimulate proliferation of human umbilical vein endothelial cells (HUVECS) *in vitro* (5). Furthermore, an increased expression of TBK-1 in solid tumors has been described, suggesting the TBK-1-IRF-3 pathway to be of importance for tumor angiogenesis (5). With regard of its putative tumorigenic and pro-angiogenic effects, it was the aim of our study to gain further insight into the role of the TBK-1-IRF3 pathway in

tumor biology and to address its role in mediating tumor angiogenesis and tumor-associated microvascular inflammation *in vivo*.

3. MATERIALS AND METHODS

3.1. Cell cultivation, tumor cell transfection and functional genomics screen

Tumor cells of the cell line KB 3-1 (human cervix carcinoma cells) were grown in DMEM/5% FCS. KB 3-1 cells were transfected by calcium phosphate coprecipitation to yield a TBK-overexpressing clone, designated KB 3-1_{TBK-1}. KB 3-1_{mock} cells, containing the empty vector, served as controls. In order to confirm that TBK-1 overexpression stimulates endothelial cell proliferation, we analyzed the effects of the cells supernatants on HUVEC proliferation following the screening procedure introduced by Korherr at al (5). To rule out adverse effects of TBK-1 transfection on proliferational behaviour of our tumor cell clones, growth curves of transfected clones were additionally assessed by Alamar Blue assay (Biosource, California USA). Fluorescence was measured as described above on day 1, 2, 3 and 4.

3.2. Analytical assay

To assess the expression of TBK-1, IL-8 and RANTES by tumor cell clones we used qPCR analysis utilizing the LightCycler (Roche): cDNA was synthesized from 1 µg of total RNA using random hexamers and avian myeloblastosis virus Reverse Transcriptase (Roche). The exact PCR conditions and further information on primer sequences have been described before (5). PCR reactions contained 0.5 µM each of sense (s) and antisense (as) primers, 3 mM $MgCl_2$, 1xSYBR green MasterMix and 2 µl of cDNA. References for quantification were G6PDH. Data were analyzed using LightCycler analysis software.

3.3. Immunohistochemistry

For histological visualization of TBK-1 overexpression, KB 3-1_{TBK-1} and KB 3-1_{mock} tumors were resected, frozen in liquid nitrogen and cryosectioned. Sections were incubated with the primary antibodies at 4 °C over night (anti-TBK-1: 1:200) after non-specific reactivity was blocked with goat serum (DAKO). After washing, biotinyilated goat anti rabbit serum (DAKO) was added for 60 minutes at room temperature. After being washed, the reagents of the ABC kit (Vectastain, Vector, Burlingame, CA) were added. Finally, sections were counterstained and mounted in glycerine gelatine.

3.4. Western blotting

In order to investigate the potential mechanism of TBK-1 induced angiogenesis we investigated VEGF expression in KB 3-1-10_{TBK-1} and KB 3-1-10_{mock} tumors using Western blotting techniques. Tumor lysates (50µg/ml) containing protease inhibitor cocktail (Roche, Mannheim, Germany) were run on 10% SDS-PAGE gels. Western Blots were probed with polyclonal antibodies against human VEGF (1µg/ml, A-20, Santa Cruz Biotec, Heidelberg, Germany), and visualized using an AP-conjugated anti rabbit antibody (Jackson Immuno/Dianova, Hamburg, Germany) by chemiluminescence (Lumi-Phos, Pierce, Bonn, Germany).

3.5. Dorsal skinfold chamber model

The experiments were performed in athymic nude mice (nu/nu; male, 28 to 32 g; n = 6 per group), bred and maintained within a specific pathogen-free environment. The microsurgical techniques for the implantation of the dorsal skinfold chambers have been previously described in detail (6). The observation window, used as implantation site for KB 3-1 cells, contains one layer of striated mucle, s.c. tissue and epidermis. It permits recurrent observations of tumor microcirculation. To eliminate the effects of surgical trauma, chambers were implanted 2 days before tumor cell implantation. The animals tolerated the skinfold chamber well and showed no signs of discomfort. This model is characterized by solid tumor growth patterns and represents a versatile technique for the noninvasive analysis of tumor microcirculation and hemodynamics.

3.6. Intravital microscopy

The tumor microvasculature was analyzed by intravital fluorescence videomicroscopy (epi-illumination) using a modified Axiotech Vario microscope, Zeiss, Oberkochen, Germany with UV (340 to 380 nm) and blue (450 to 490 nm) filter blocks (6). FITC-conjugated dextran was used to visualize tumor microvasculature by contrast enhancement (6). Leukocyte attachment to the microvasculature was visualized using leukocyte-specific Rhodamin staining. FITC-conjugated dextran and Rhodamin were injected into the tail vein of the experimental animal prior to microscopy. All microcirculatory measurements were made using a computer-assisted image analysis system (CAPIMAGE; Zeintl Software Engineering, Heidelberg, Germany). For microcirculatory analysis, the newly formed microvasculature of the tumor was divided into 3 radial zones (peri-tumoral, marginal and central). Each zone was assessed separately (2 to 3 observation fields/zone), resulting in a total of 6 to 9 measurements per animal. Quantitative analysis included total intratumoral vascular density (cm^{-1}), which was defined as the length of all newly formed microvessels in the tumor area, functional intratumoral vascular density, which was defined as the length of all newly formed perfused microvessels in the tumor area and vessel diameter. As a functional parameter the perfusion index PI was calculated as the percentage of functional intratumoral vascular density (FIVD) and total intratumoral vascular density (TIVD) based on following formula: PI = FIVD/TVD * 100. Sticking leukocytes were defined as adjacent leukocytes without further movement along the vessel wall for at least 30 seconds. The number of sticking leukocytes per mm² surface of the observed microvessel segment was calculated based upon the diameter and length of the microvessel segment and the corresponding number of sticking leukocytes. The following formula was used: $2 * \pi * r * h$ with r representing vessel segment diameter/2 and h representing the length of the observed microvessel segment.

3.7. Statistical analysis

Quantitative data are given as mean ± SD. Mean

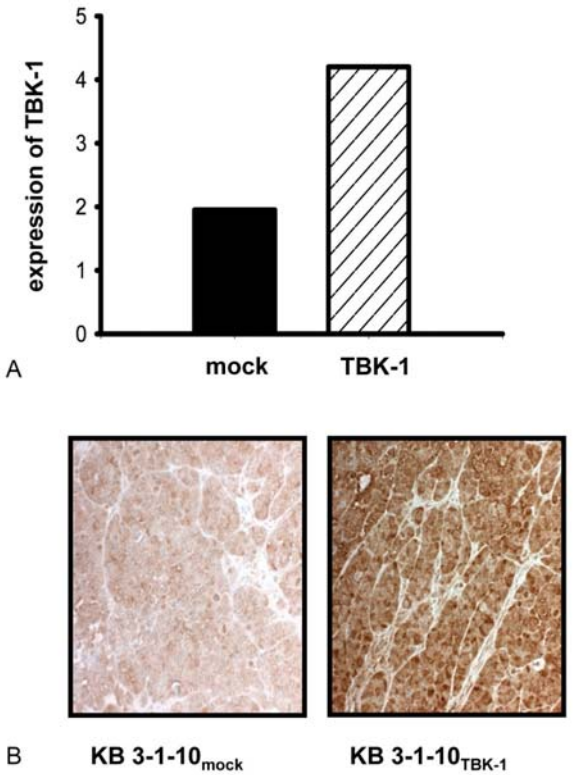

Figure 1. A : TBK-1 expression assessed by qPCR showing a more than 2 fold increase in TBK-1 expression; B: TBK-1 overexpression assessed by immunohistochemistry demonstrating markedly enhanced TBK-1 staining in TBK-1 overexpressing tumors

values of microcirculatory data were calculated from the average values in each animal. For analysis of differences between groups, 1-way ANOVA followed by unpaired Student's *t*-test was performed. Results with $p < 0.05$ were considered significant.

4. RESULTS

4.1. Generation of TBK-1 overexpressing tumor cell clones

Calcium phosphate coprecipitation generated successfully TBK-1 overexpressing KB 3-1 cells. Normalized ratio of TBK-1 expression was found to be doubled in TBK-1 transfected cells compared to mock cells, which were transfected with an empty vector (KB 3-1_{TBK-1}: 4.2 * 10^3, KB 3-1_{mock}: 1.95 * 10^3**;** see Figure 1A). Immunohistochemistry for TBK-1 demonstrated markedly enhanced staining in KB 3-1_{TBK-1} tumors as compared to KB 3-1_{mock} tumors (see Figure 1B). Furthermore, TBK-1 transfection exerted no influence on proliferating activity of KB 3-1_{TBK-1} cell clones. Over an observation period of 4 days we could find no differences in cellular growth rate between both cell lines using the Alamar blue assay (see Figure 2 A). Whereas TBK-1 expression obviously had no effect on tumor cell proliferation, the supernatant of KB 3-1_{TBK-1} cells showed a stimulatory effect on the proliferating activity of HUVECs as compared to supernatants of KB 3-1_{mock} cells (KB 3-1_{TBK-1}: 54.6 ± 1.7; KB 3-1_{mock}: 38.5 ± 2.3; see Figure 2B). These results confirmed TBK-1's role as an endothelial cell mitogen (5).

4.2. TBK-1-IRF3 pathway facilitates tumor angiogenesis

We next addressed the question whether this stimulatory effect on endothelial cells could translate into a relevant pro-angiogenic activity *in vivo*. Therefore, we implanted KB 3-1 cells in dorsal skinfold chambers in athymic nude mice followed by intravital microscopy. Intravital microscopic analysis represents the primary tool for the analysis of tumor microcirculation allowing direct visualization of tumor vascularization, blood-flow, vascular permeability and cell-endothelial interactions. Total intratumoral vascular density (i.e. the length of perfused and non-perfused blood vessels / cm²) in KB 3-1_{TBK-1} tumors reached a significant difference to KB 3-1_{mock} tumors after 21 days of observation (KB 3-1_{TBK-1}: 268 ± 30 cm/cm²; KB 3-1_{mock}: 183 ± 21 cm/cm²; $p < 0,05$; see Figure 3C). This difference was not observed 7 and 14 days after implantation. Moreover, functional intratumoral vascular density was significantly increased in response to TBK-1 transfection (KB 3-1_{TBK-1}: 204 ± 54 cm/cm²; KB 3-1_{mock}: 93 ± 40 cm/cm², $p < 0.05$; see Figure 3D). Representative intravital microscopic images are illustrated in Figure 3 A+B. Regarding the perfusion index (i.e. the ratio FIVD/TIVD), KB 3-1_{TBK-1} tumors demonstrated higher values than mock tumors without a statistically significant difference over the course of the experiment (PI after 21 days: KB 3-1_{TBK-1}: 74.9 % ± 12.7; KB 3-1_{mock}: 51.2 % ± 24.9). Also, no differences in vessel diameter were induced by TBK-1 during the entire experiment (after 21 days: TBK-1 group: 8.31 ± 2.82 µm; control group: 9.31 ± 4.70 µm).

4.3. Proinflammatory activation of tumor microcirculation

Besides its role as a potential pro-angiogenic molecule, the TBK-1 pathway is known to be a potent activator of NF-kappaB. In order to gain further insight into the proinflammatory role of TBK-1, we analyzed microvascular leukocyte adherence. KB 3-1_{TBK-1} tumors displayed significantly increased leukocyte sticking as compared to KB 3-1_{mock} tumors (Figure 3E). Leukocyte sticking increased over the course of the experiment in the TBK-1 group, whereas leukocyte sticking in the control group declined after day 7. Consequently, significant differences between both groups developed over the course of the experiment (day 21: KB 3-1_{TBK-1}: 562 ± 332 n/mm²; KB 3-1_{mock}: 100 ± 73 n/mm²; $p < 0.005$; see Figure 3E).

4.4. Expression of pro-angiogenic and pro-inflammatory cytokines

In order to better understand the mechanisms underlying the pro-angiogenic and pro-inflammatory role of TBK-1, we assessed the expression of angiogenic and inflammatory cytokines. KB 3-1_{TBK-1} tumors displayed increased expression of VEGF as compared to KB 3-1_{mock} tumors. Therefore, VEGF is a potential mediator responsible for TBK-1 induced angiogenic effects (see Figure 2C). Additionally, we observed a more than 12 fold increase in RANTES expression and a 3 fold increase in Il-8 expression in KB 3-1_{TBK-1} cells (RANTES expression:

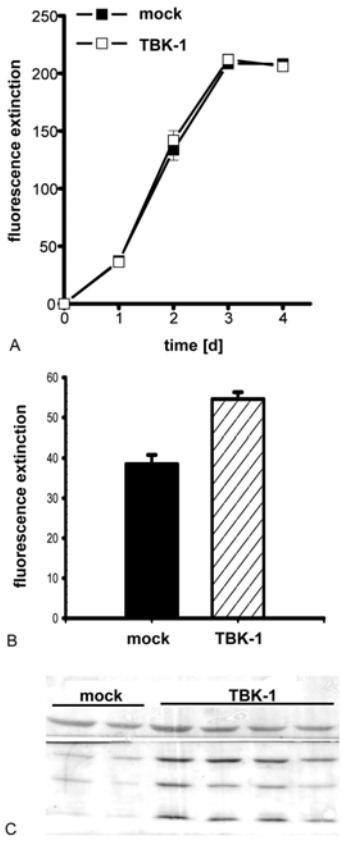

Figure 2. A: growth rate of transfected clones show the same behavior over the observation period of 4 days; B: effects of TBK-1 overexpression on proliferational activity of HUVECs assessed by Alamar Blue assay; C: western blot analysis demonstrating VEGF expression in KB 3-1_{TBK-1} and KB 3-1_{mock} tumors with markedly increased VEGF levels in TBK-1 transfected tumors.

KB 3-1_{TBK-1}: 6.6 * 10^3 , KB 3-1_{mock}: 0.46 * 10^3 , Figure 4A; IL-8 expression: KB 3-1_{TBK-1}: 0.1750, KB 3-1_{mock}: 0375; see Figure 4B). Based in this data, inflammatory chemokines RANTES and Il-8 are induced by TBK-1 and play an important role for its pro-inflammatory effects.

5. DISCUSSION

As the principal novel finding of our experiments, we have demonstrated pro-angiogenic as well as pro-inflammatory effects *in vivo* for the TBK-1-IRF3-pathway. Furthermore, we have additionally provided evidence for increased VEGF levels in tumor xenografts as a result of TBK-1 overexpression. We have also shown increased expression of Il-8 and RANTES *in vitro* suggesting these mediators to be involved in the pro-inflammatory mechanism responsible for our results. Therefore, the TBK-1 pathway represents an important cross-link between tumor angiogenesis and tumor-associated inflammation. Angiogenesis is controlled by a sensitive balance between angiogenic and angiostatic mediators (7). Interestingly, among those mediators are chemokines that are primarily known as proinflammatory mediators like RANTES and Il-8 (8-10). TANK-Binding-Kinase-1 (TBK-1) plays an important role in anti-viral responses by leading to increased expression of inflammatory mediators. Recently, the TBK-1-IRF3 pathway has been described as a potential new angiogenic pathway besides its known inflammatory activity (5). Korherr *et al.* further provided evidence for TBK-1 overexpression in solid tumors raising questions about the function of the TBK-1-IRF3-pathway in tumor biology (5).

5.1. TBK-1-IRF3-pathway induces tumor angiogenesis *in vivo*

TBK-1 has been identified to play an important role in the activation process of two different intracellular signalling pathways: the Nuclear Factor-kappa B (NF-kappaB) and the Interferon-regulatory-factor 3 (IRF3) pathway (3, 11).

The transcription factor IRF3 is required for the expression of proinflammatory chemokines in response to viral infections (12-14). NF-kappaB is also closely intertwined with inflammation mediating the effects of most inflammatory agents (15). Moreover, NF-kappaB has also been described to play an important role in tumor biology. Yu *et al.* demonstrated that increased expression of NF-kappaB contributes to angiogenesis in colorectal cancer (16). Therefore, the argument seems plausible that TBK-1-induced NF-kappaB activation contributes to the observed pro-angiogenic effects in our experiments.

However, previous experiments clearly show that other mechanisms explain our results. The experiments of Korherr at al. demonstrate the TBK-1-IRF3 pathway to induce HUVEC proliferation (5). In their functional genomics screen for proangiogenic pathways, TBK-1 and IRF3 were identified as independent functional "angiogenic" hits, whereas NF-kappaB activation did not alter HUVEC proliferation and consequently was not identified as a "proangiogenic" molecule (5). Consequently, the TBK-1-IRF3 pathway has to be the responsible molecular mechanism for the observed proangiogenic activity in our experiments.

5.2. Interleukin-8, RANTES and VEGF represent important mediators of TBK-1

The TBK-1-IRF3 pathway leads ultimately to the release of proinflammatory chemokines (13). In our *in vitro* experiments we could demonstrate increased expression of RANTES and Il-8 by our KB 3-1_{TBK-1} cells, identifying these chemokines as mediators responsible for the pro-inflammatory effects. Furthermore, both chemokines hold the potential to contribute to tumor-angiogenesis. RANTES was originally described as a leukocyte chemoattractant protein (17). It binds to different cellular receptors like CCR1, CCR3, CCR4 and CCR5, which can be found on various cell types including T cells, monocytes, dendritic cells and mast cells (17). RANTES is dramatically increased in inflammatory sites as well as in tumors, whereas in healthy adult cells RANTES is weakly expressed (18). Accordingly, increased RANTES levels are directly correlated with a more advanced disease and enhanced tumor progression in breast cancer (19). Moreover, exposure to RANTES has been described to

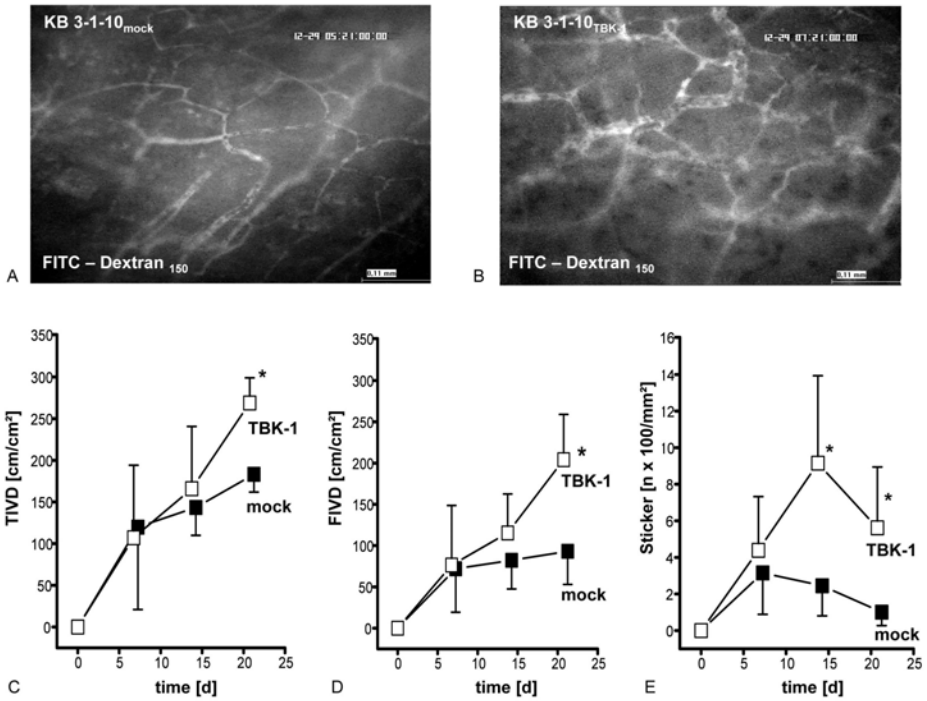

Figure 3. A + B: tumor microvasculature in KB 3-1$_{TBK-1}$ and KB 3-1$_{mock}$ tumors 21 days after tumor cell implantation; intravital fluorescence videomicroscopy, contrast enhancement with 2% FITC-Dextran$_{150}$ i.v.; TBK-1 overexpressing tumors (right image) demonstrating significantly increased microvascular density as compared to mock tumors (left image); C + D: total (TIVD) and functional intratumoral vessel density (FIVD) assessed by intravital microscopy; microcirculatory parameters were analysed offline using a computer-assisted image analysis system; the mean ± SD values are represented; statistical analysis was performed by using ANOVA followed by unpaired Student's t-test; * $p < 0.05$ vs. mock; E: leukocyte adhesion assessed by intravital microscopy; leukocyte stickers were analysed off-line and the number of sticking leukocytes per mm² vessel surface was calculated; the mean ± SD values are represented; statistical analysis was performed by using ANOVA followed by unpaired Student's t-test; * $p < 0.05$ vs. mock.

result in blood vessel thickening and an increased number of blood vessels implicating RANTES to induce angiogenesis (20). In our study we have observed increased RANTES expression by TBK-1 transfected clones. TBK-1 transfected tumors display increased angiogenic activity, therefore we conclude that RANTES contributes to tumor angiogenesis in our experiments. l-8 belongs to the CXC chemokine family and it has been shown to regulate pathological angiogenesis and tumor growth in various cases (21-23). The Il-8 receptors CXCR1 and CXCR2 are widely expressed on tumor cells and bind Il-8 with high affinity (24, 25). Murdoch *et al.* verified an increased expression of these mediators on HUVECs focusing on a possible direct interaction between Il-8 and endothelial cells (26). Further experiments by Li *et al.* demonstrated that Il-8 stimulates endothelial cell proliferation and capillary tube formation in a concentration dependent manner by direct interaction with endothelial cells (27). Regarding these data, TBK-1-IRF3 induced expression of Il-8 also contributes to our pro-angiogenic results. However, neither RANTES by itself nor Il-8 by itself were identified as proangiogenic "hits" in the functional genomics screen performed by Korherr *et al.* indicating that other, more potent angiogenic mediators must be involved in TBK-1 induced angiogenesis. Addressing this issue, we performed western blot analysis for VEGF expression and we identified increased VEGF levels in xenotransplanted tumors. Interestingly, Korherr at al were able to demonstrate a positive correlation between TBK-1 and VEGF expression in a cell culture model for hypoxia (5). These data clearly demonstrate evidence for the induction of potent pro-angiogenic factors, like VEGF, in response to TBK-1 overexpression.

5.3. TBK-1 induces a pro-inflammatory response of tumor microcirculation

Besides the pro-angiogenic response in our experiments, we demonstrate significantly increased leukocyte sticking in the KB 3-1$_{TBK-1}$ tumors. We conclude that proinflammatory mechanisms, leading to increased leukocyte activation and hence increased leukocyte sticking, are triggered via the TBK-1 pathway. Increased RANTES and Il-8 levels in our experiments support this hypothesis, since both agents are proinflammatory mediators that have been described to induce leukocyte infiltration and chemoattraction. Activation of leukocytes and endothelial cells through inflammatory agents leads to increased leukocyte sticking on the vessel wall, which is regarded to be a major prerequisite for leukocyte diapedesis. The results of our study support the close relationship between the angiogenic and inflammatory systems and TBK-1 seems to be a mediator at their

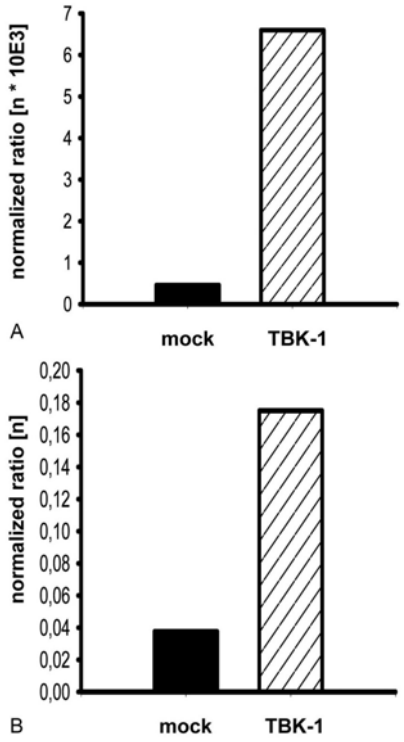

Figure 4. A+B: RANTES (A) and Interleukin 8 (B) expression of tumor cell clones analysed by quantitative PCR.

interface. Angiogenic factors are known to affect leukocyte function and leukocyte recruitment (28). Lee at al postulated that VEGF induces IL-8 expression in human brain microvascular endothelial cells and consequently contributes to leukocyte infiltration (29). On the other hand, leukocytes have often been described to alter angiogenesis. They produce a variety of angiogenic factors like VEGF, PDGF and FGF (30, 31). Tumor associated macrophages (30) have been demonstrated to promote tumor growth directly by releasing angiogenic factors and indirectly by stimulating tumor cells to produce pro-angiogenic agents (32). Based on our data, the TBK-1-IRF3 pathway increases leukocyte-endothelial interactions *in vivo* by altering the inflammatory chemokine system. Therefore, the TBK-1 pathway plays an important role in tumor associated inflammation as well as in tumor angiogenesis. TBK-1 might therefore represent a possible new target for anti-angiogenic therapy.

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Abbreviations: TBK-1: TANK-binding-Kinase 1; IRF-3: Interferon-regulatory-factor 3; NF-kappaB: Nuclear Factor kappaB; HUVEC: human umbilical vein endothelial cells; IL-8: Interleukin-8; RANTES: regulated upon activation, normal T cell expressed and secreted; FIVD: functional intratumoral vessel density; TIVD: total intratumoral vessel density; PDGF: platelet derived growth factor; FGF: fibroblast growth factor; VEGF: vascular endothelial growth factor; CCR-1: CC chemokine receptor type 1; CCR3: CC chemokine receptor type 3, CCR-4; CC chemokine receptor type 4; CC chemokine receptor type 5.

Key Words: Tank-binding Kinase-1, Tumor Angiogenesis, Inflammation, Endothelial Cell Proliferation, Tumor Microcirculation

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