Transfer of the sFLT-1 Gene in Morris Hepatoma Results in Decreased Growth and Perfusion and Induction of Genes Associated with Stress Response

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ABSTRACT

Purpose: Inhibition of tumor angiogenesis is emerging as a promising target in the treatment of malignancies. Therefore, monitoring of antiangiogenic approaches with functional imaging and histomorphometrical analyses are desirable to evaluate the biological effects caused by this treatment modality.

Experimental Design: Using a bicistronic retroviral vector for transfer of the soluble receptor for the vascular endothelial growth factor (sFLT) hepatoma (MH3924A) cell lines with sFLT expression were generated. In human umbilical vein endothelial cells cultured with conditioned medium of sFLT-expressing hepatoma cells, the inhibitory action of secreted sFLT was determined using a Coulter counter and a thymidine incorporation assay. Furthermore, in vivo experiments were done to measure the effects on tumor growth and perfusion. Finally, the tumors were examined by immunohistochemistry (including computer-assisted morphometry) and DNA chip analysis.

Results: Stable sFLT-expressing hepatoma cells inhibited endothelial cell proliferation in vitro. In vivo, growth and perfusion, as measured by H215O positron emission tomography, were reduced in genetically modified tumors. However, the immunohistochemically quantified microvascularization and macrovascularization, as indicated by CD31- and α-actin-positive area, revealed no significant changes, whereas the number of apoptotic cells was increased in sFLT-expressing tumors, although not significantly. DNA chip analysis of tumors with gene transfer showed an increase of genes related to apoptosis, signal transduction, and oxidative stress.

Conclusion: Our results suggest that sFLT expression inhibits tumor growth and perfusion and enhances expression of apoptosis-related genes in this model. Enhanced expression of genes for signal transduction, stress, and metabolism indicates tumor defense reactions.

INTRODUCTION

Malignant tumors are dependent on angiogenesis, showing a correlation of the microvessel density with the grade of invasiveness, frequency of metastasis, and clinical prognosis (1, 2). For tumor angiogenesis, angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and interleukin 8 need to be produced within tumors either by cancer cells or by infiltrating lymphocytes, macrophages, or fibroblasts (3, 4). Out of these, VEGF may be one of the most important factors for tumor angiogenesis and is suggested as an angiogenic factor, which augments tumor vascularity (5, 6). Second, it may act as a permeability factor to promote the supply of nutrients to the tumor by diffusion (7, 8). In this context, tumor suppression has been achieved in animal experiments by inhibiting VEGF or its receptor using neutralizing VEGF antibodies, blocking VEGF receptor antibodies, antisense oligonucleotides against VEGF, VEGF antisense expression plasmids, VEGF-diphtheria toxin conjugate, and a truncated soluble form of the VEGF receptor that inhibits the functioning of the wild-type (WT) receptor (9–16). Because the abovementioned methods require either a substantial amount of protein or a direct insertion of the molecules into cancer cells, the transfer of antiangiogenic genes into tumor cells or regional expression near tumor sites should be more effective in suppressing tumor growth (17, 18). Several groups have used the gene of the soluble VEGF receptor (sFLRT) in a variety of tumor entities (17, 19–23). sFLT may form a heterodimeric complex with a WT VEGF receptor and function as a dominant-negative receptor (15, 16). Furthermore, sFLT should also be secreted from infected cells into the blood stream and should reach most sites of angiogenesis within the tumor and sequester VEGF from receptors on the target cells, thereby achieving an effective suppression of tumor growth.

Because intervention with angiogenesis should result in functional consequences with respect to blood supply, monitoring of antiangiogenic approaches with functional imaging and histomorphometrical analyses are desirable to deliver information about the biochemical and physiologic effects caused by this
treatment modality. Positron emission tomography (PET) with
\(^{15}\)O-labeled water has been used in several trials that investigated
potential antiangiogenic effects of razoxane, endostatin, or
combretastatin A4 phosphate (24–26). Tumors responding to
treatment showed a dose-dependent reduction in tumor perfusion
and blood volume (24–26). However, in renal carcinomas no
significant differences of perfusion, blood volume and fractional
volume of distribution were seen prior and after treatment with
razoxane. Furthermore, no change was seen during tumor
progression, too (24).

In the following experiments, stable Morris hepatoma
(MH3924A) cell lines expressing human sFlt (sFLT-MH3924A)
were established. In vitro the effect of conditioned medium from
sFLT-MH3924A on the proliferation of human umbilical vein
cells (HUVEC) was measured. Furthermore, in rats, bearing WT-
MH3924A or genetically modified hepatomas, tumor growth,
and tumor perfusion were determined. These functional data
were combined with histomorphometrical investigations of the
vascularization, apoptosis, proliferation, oxidative stress, and
inflammation as well as gene array analysis of the expression
pattern in cells and tumors.

MATERIALS AND METHODS

Cell Culture and Generation of Recombinant Cells. Cells were cultured in RPMI 1640 (Life Technologies,
Eggenstein, Germany) supplemented with 292 mg/L glutamine,
100,000 IE/L penicillin, 100 mg/L streptomycin, and 20% FCS.
The cell lines were cultured at 37°C, in an atmosphere of 95% air
and 5% CO2.

sFLT Gene Transfer and Northern Blots. For transfer
of the sFLT gene, a bicistronic retroviral vector was used
consisting of the sFLT gene (cloned from pBacPAK8sFLT-1,
obtained from H. Weich 27) and the hygromycin resistance gene
cloned downstream of the elongation factor 1a (EF1a) promoter.
To ensure simultaneous expression of the genes coding for the
sFLT and for the hygromycin resistance and stabilization of the
mRNA a synthetic intron and an internal ribosomal entry site
from encephalomyocarditis virus was inserted between the genes
(28, 29).

For transient packaging of the retroviral particles, a
lipofection of the transient packaging cell line BOSC23 was
done. After 2 days, the medium was centrifuged to remove
detached BOSC23 cells and used for the infection of MH3924A
cells in the presence of 8 µg polybrene/mL over night. The cells
were treated with 425 µg/mL hygromycin for 4 weeks until
resistant cell lines were established. These were further incubated in hygromycin-containing medium.

Total RNA was isolated using Trizol reagent (Roche,
Mannheim, Germany) and mRNA expression was assessed by
Northern blots using radioactive labeled probes delivered by
digestion from the sFLT plasmid.

Measurement of Cell Proliferation. Human umbilical
vein endothelial cells (HUVEC) were isolated from umbilical
cords as previously described (30) and cultured in supplemented
RPMI 1640 [with 292 mg/L glutamine, 100,000 IE/L penicillin,
100 mg/L streptomycin, 20% FCS, and 4 ng/mL basic FGF (bFGF)]. To determine the effects of sFLT on HUVEC
proliferation, HUVECs were cultured in conditioned medium
from WT-MH3924A or sFLTMH-3924A cells in 6-well plates.
The medium consisted of 75% medium 199 (Life Technologies;
supplemented with 20% FCS, 2 mmol/L glutamine, 100 IU/mL
penicillin, 100 IU/mL streptomycin, and 2 ng bFGF/mL) and
25% medium from WT-MH3924A or sFLTMH-3924A cells.
The cells were cultured in the absence of growth factors or in the
presence of 20 ng/mL VEGF or 20 ng/mL bFGF. Proliferation of
the endothelial cells was measured after 5 days by incorpora-
tion of \((\text{methyl-}^3\text{H})\)-thymidine (Amersham-Buchler, Ismaning,
Germany) in DNA as previously described (31). The experiment
was repeated twice.

Animal Studies. ACI rats and RNU (nude) rats were
supplied by Charles Rivers Laboratories (Kingston, NY). All
animal studies were done in compliance with the national laws
relating to the conduct of animal experimentation. The in vitro
doubling time of the cell lines used was 18.7 and 17.7 hours for
WT-MH3924A and sFLT-MH3924A cells, respectively. After
inoculation of WT or sFLT cell suspensions (2 × 10^6 cells per
animal) into the thigh of ACI or RNU rats, tumor growth was
measured using calipers. PET studies of tumor perfusion were
done in separate animals weighing 200 to 250 g. Perfusion in
tumors with diameters between 10 and 13 mm was examined
after i.v. bolus injection of 70 to 150 MBq H2O\(^{15}\) in 0.3 mL by
dynamic PET (20 × 3 seconds, 6 × 10 seconds, 4 × 15 seconds,
6 × 30 seconds) acquired in two-dimensional mode using a
matrix of 256 × 256 on an ECAT HR+ (Siemens CTI,
Knoxville, TN) scanner (pixel size, 2.277 × 2.277 × 2.425 mm;
transaxial resolution, 4.3 mm). A transmission scan
was done for 10 minutes before tracer administration with three
rotating germanium pin sources to obtain cross sections for
attenuation correction. After iterative reconstruction (OSEM,
8 subsets, 16 iterations) the dynamic PET data were evaluated as
described earlier using the PMOD software package (32).
Time-activity curves were created using volumes of interest. A volume
of interest consists of several regions of interest over the target
area. Irregular regions of interest were drawn manually.
Perfusion studies with \(^{15}\)O-water can be evaluated using a one-
tissue-compartment model (33). For the input function, we used
the mean value of the volumes of interest data obtained from the
heart. Regions of interest were defined at 3 to 6 seconds after
bolus injection in at least three consecutive slices (2.4-mm
thickness) surrounded by two slices showing the heart in each
direction. This approach was based on the findings of Ohtake
et al. demonstrating that the input function can be retrieved from
the image data with acceptable accuracy (34). The heart weight
and heart volume of a 250-g rat are 1.0 g and 1.2 mL,
respectively (35). Furthermore, the size of the heart was
determined in our rats showing a median value of 1.0 × 1.3 cm.
This results in an estimated recovery error of 10%, which can be
neglected for the purposes of this study. The transport constant
\(K_t\) and the rate constant \(k_e\) were calculated using a one-tissue-
compartment model for \(^{15}\)O-water based on a method imple-
mented in the PMOD software taking into account the vascular
fraction, which is associated with the volume of blood
exchanging with tissue in a volume of interest.

Tissue Preparation and Immunohistochemistry. Rat
tumors were fixed with immunohistochemistry zinc fixative (BD
Biosciences, Heidelberg, Germany) or 4% paraformaldehyde
(and routinely embedded in paraffin) or shock-frozen in liquid
Data Analysis and Statistics. Results are presented as mean ± SD. Statistical comparisons between groups were done by the Mann Whitney U-Wilcoxon rank sum W test or by the unpaired Student’s t test using the SIGMASTAT program (Jandel Scientific, Erkath, Germany). P ≤ 0.05 was considered statistically significant.

RESULTS

Inhibition of Proliferation of Human Umbilical Vascular Endothelial Cells Cultured with Conditioned Medium of sFLT-MH3924A. The successful transfer of the sFLT gene into MH3924A (sFLT-MH3924A) and the overexpression of this gene was verified by Northern blotting in different hygromycin-resistant cell lines (data not shown). Of these, the cell line with the highest signal as evaluated by the sFLT/β-actin ratio was chosen for further experiments. Next, we investigated the effects of culture with conditioned medium from WT-MH3924A and sFLT-MH3924A on proliferation of HUVECs. The proliferation was measured by (methyl-[3H]-thymidine incorporation into the DNA of HUVECs after perchloric acid extraction. The acid-insoluble fraction in this assay represents nucleic acids and proteins, whereas the acid-soluble fraction represents unbound radioactivity in acid-soluble molecules, which are not in DNA and proteins. In the presence of conditioned medium from WT-MH3924A cells, bFGF and VEGF caused a 2-fold and 1.5-fold increase in thymidine uptake in the acid-insoluble fraction compared with the value obtained without these growth factors. Using conditioned medium from sFLT-MH3924A cells, a 60% (without growth factor), 89% (with bFGF), and 83% (with VEGF) decrease of thymidine (P < 0.001) uptake in the acid-insoluble fraction occurred compared with the uptake in the presence of the corresponding WT-MH3924A medium. The uptake in the acid-soluble fraction decreased by 71%, 95%, and 92%, respectively (P < 0.001; Table 1).

Inhibition of Tumor Growth and Perfusion in sFLT-MH3924A Tumors In vivo: Positron Emission Tomography and Immunohistomorphometry of Vascularization. After inoculation, we examined the growth of WT-MH3924A or sFLT-MH3924A tumors in ACI and athymic (nude) RNU rats, including PET measurements and histologic analyses of vascularization. RNU rats were used to exclude immunologic effects as possible causes for growth inhibition.

Table 1  Proliferation of HUVECs cultured with conditioned medium from WT-MH3924A or sFLT-MH3924A cells

<table>
<thead>
<tr>
<th>Conditioned medium</th>
<th>Acid-insoluble fraction (Bq/105 cells)</th>
<th>Acid-soluble fraction (Bq/105 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-MH3924A cells</td>
<td>Without growth factor 2,689.1 ± 58.22</td>
<td>333.6 ± 46.72</td>
</tr>
<tr>
<td></td>
<td>bFGF 5,287.82 ± 380.4</td>
<td>270.7 ± 3.62</td>
</tr>
<tr>
<td></td>
<td>VEGF 3,975.17 ± 197.3</td>
<td>264.43 ± 13.27</td>
</tr>
</tbody>
</table>

NOTE. Incubation of HUVECs with conditioned medium from sFLT-MH3924A cells significantly inhibited their proliferation as indicated by diminished (methyl-[3H]-thymidine uptake (Bq/105 cells) in the insoluble fraction after bFGF or VEGF treatment with P < 0.001.
At 18 days after inoculation in RNU rats, \textit{in vivo} growth of sFLT-MH3924A tumors was reduced by 43\% in comparison with WT-MH3924A (Fig. 1). Similar results were obtained with ACI rats (data not shown).

Because sFLT has been described as a strong inhibitor of angiogenesis, we expected a significant difference in tissue perfusion in tumors expressing this gene. Therefore, dynamic PET measurements with H\textsuperscript{15}O were done. The diameters of WT and sFLT-expressing tumors were not significantly different. The pharmacokinetic analysis of the PET data showed that tumor perfusion, represented as the transport rate $K_1$ (in mL/C\textsubscript{2}mL tissue/C\textsubscript{0}1/minute/C\textsubscript{0}1), was significantly decreased ($P = 0.035$) in sFLT-MH3924A ($0.31 \pm 0.07$) in comparison with WT-MH3924A ($0.43 \pm 0.10$; Fig. 2A). Similar changes were obtained for the $k_2$ value with $0.26 \pm 0.09$ and $0.73 \pm 0.24$ and for sFLT and WT tumors, respectively ($P = 0.005$; Fig. 2B). The vascular fraction showed no difference (Fig. 2C), whereas the fractional volume of distribution increased significantly ($0.65 \pm 0.26$ for WT and $1.26 \pm 0.24$ for sFLT-expressing tumors with $P = 0.001$; Fig. 2D).

The most commonly used vascular marker, $\alpha$-actin, is expressed by mural cells of most arterioles and venules but not in capillaries (37). In tumors of ACI rats, the macrovascularization, as indicated by the $\alpha$-actin immunoreactive area (measured in the center and in the periphery of the tumors), is similar in sFLT-MH3924A and WT-MH3924A tumors (Fig. 3A). Furthermore, we found that microvascularization, as determined by CD31 immunoreactivity, is unchanged in sFLT-MH3924A tumors in comparison with WT-MH3924A (Fig. 3B). The vascularization identified by CD31 immunoreactivity was twice higher in the periphery in comparison with the center in both tumor types.

sFLT Gene Transfer, Induction of Apoptosis, and Changes of Markers Associated with Oxidative Stress or Inflammation: Terminal Deoxynucleotidyl Transferase–Mediated Nick End Labeling, CD11b, and Cyclooxygenase 2.

Next, we examined \textit{in situ} apoptosis, inflammation, necrosis, proliferation, and markers of oxidative stress inducing cells in WT-MH3924A and sFLT-MH3924A tumors by histomorphometrical analyses. Immunohistochemical quantifications revealed that in sFLT-MH3924A tumors the density of apoptotic cells shows a 17\% (nonsignificant with $P = 0.1$) increase (Table 2). The proliferation rate as identified by proliferating cell nuclear antigen staining is also not significantly changed in comparison with the WT-MH3924A tumors (Table 2). In addition, the

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Tumor growth after transplantation of WT-MH3924A, or sFLT-MH3924 cells in RNU rats. Points, mean ($n = 5$); bars, SD. sFLT-expressing tumors revealed showed a significantly decreased $K_1$ ($P = 0.035$) and $k_2$ ($P < 0.005$).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{PET. Tumor perfusion [$K_1$, $k_2$, vascular fraction ($VB$), and fractional volume of distribution ($DV$) values] measured of WT-MH3924A, or sFLT-MH3924A in ACI rats; two-dimensional acquisition (one-tissue compartment). sFLT-expressing tumors showed a significantly decreased $K_1$ ($P = 0.035$; A) and $k_2$ ($P < 0.005$; B). The vascular fraction showed no difference (C), whereas the fractional volume of distribution increased significantly ($P = 0.001$; D).}
\end{figure}
necrotic area was unchanged in sFLT-MH3924A in comparison with WT-MH3924A with 21.9 ± 4.1% in WT and 21.7 ± 5.4% in sFLT-expressing tumors.

The percentage of CD11b immunoreactive macrophages, as major source of oxidative stress, decreased from 14.1 ± 1.0% in WT-MH3924A to 7.1 ± 1.0% in sFLT-MH3924A with $P = 0.001$. As a marker for inflammation, we used COX-2 and found that in sFLT-MH3924A tumors the percentage of COX-2 immunoreactive cells were significantly reduced to 18% ($P = 0.036$). MIF staining showed a 26% increase with $P < 0.001$ (Table 2).

**Effects of sFLT Gene Transfer on Expression of Other Genes In vitro and In vivo: Gene Arrays.** To assess general effects of sFLT gene transfer on the tumor cells in *vito* and in *vivo* the gene expression pattern was studied using arrays. Because the transfer of selection markers such as the hygromycin gene and the selection procedure may cause changes in the genetic program, we analyzed the expression pattern in the tumor cell lines as well as in the tumors obtained after transplantation of these cell lines. In general, we found changes in the expression of genes related to signal transduction, cellular matrix, and apoptosis (Table 3).

### DISCUSSION

Transfer of antiangiogenic genes leading to local production of the therapeutic agent at the tumor site has been suggested as a new strategy of tumor therapy. In this study, we examined the effects of *sFLT* gene transfer into rat hepatoma on tumor growth, perfusion/vascularization, and apoptosis/inflammation/proliferation.

*In vitro* a significant inhibition of thymidine incorporation into the DNA of HUVECs was seen, when conditioned medium of sFLT producing cells was added, thus indicating an inhibition of endothelial cell proliferation. These data are consistent with recently published results showing that adenoviral transfer of the *sFlt-1* gene in human embryo kidney 293 cells as well as a sFLT-expressing 293 embryonic kidney cell line (293-Flt1-3d) inhibited the *in vitro* growth of VEGF-stimulated HUVECs (38, 39).

Furthermore, *in vivo* an inhibition of tumor growth in animals with sFLT-MH3924A tumors was observed. These data are in line with results by Ye et al. showing that inoculation of 293-Flt1-3d cells at a site remote to a follicular thyroid carcinoma tumor transplant significantly inhibited the growth of these tumors (39). As expected, we found a reduction of tumor perfusion (decreased $K_{1}$ and $k_{2}$ values), measured by $^{15}$O dynamic PET, in sFLT-MH3924A tumors. The fractional volume of distribution increased significantly, indicating changes in the membrane permeability. Because the diameters of WT and sFLT-expressing tumors were not different, this difference is not due to partial volume effects. However, the histologic findings revealed that the CD31-immunoreactive area, as a marker for endothelial cells and microvascularization, is not significantly changed in sFLT-expressing tumors in comparison with WT hepatoma. The area of α-actin immunoreactive smooth muscle cells, as a marker for larger vessels (e.g., arterioles), was also comparable between WT-MH3924A and sFLT-MH3924A. These data are consistent with the fact that the vascular fraction as measured by PET remained unchanged. This may be due to the fact that the measurement of perfusion is more sensitive for the detection of changes

### Table 2  Histochemical analyses of Morris hepatomas transplanted into ACI rats: apoptosis, proliferation, oxidative stress, and inflammatory cells

<table>
<thead>
<tr>
<th>Variable</th>
<th>WT-MH3924A</th>
<th>sFLT-MH3924A</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUNEL-positive cells/mm²</td>
<td>414.31 ± 27.92</td>
<td>482.74 ± 31.81</td>
<td>NS</td>
</tr>
<tr>
<td>% PCNA-positive cells</td>
<td>24.86 ± 1.52</td>
<td>21.68 ± 4.61</td>
<td>NS</td>
</tr>
<tr>
<td>% CD11b-positive cells</td>
<td>14.09 ± 2.25</td>
<td>7.08 ± 2.22</td>
<td>0.001</td>
</tr>
<tr>
<td>% COX-2-positive cells</td>
<td>10.68 ± 3.44</td>
<td>1.93 ± 0.56</td>
<td>0.03</td>
</tr>
<tr>
<td>% MIF-positive cells</td>
<td>69.30 ± 5.01</td>
<td>87.44 ± 3.12</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**NOTE.** Mean ± SD ($n = 6$).

**Abbreviation:** NS, not significant.
Table 3  Changes in gene expression in sFLT-expressing hepatomas

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tumor ratio</th>
<th>Cell ratio</th>
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<tbody>
<tr>
<td>clone p12.3 tenascin</td>
<td>10.45</td>
<td>0.34</td>
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<tr>
<td>laminin</td>
<td>8.87</td>
<td>1.70</td>
</tr>
<tr>
<td>tenascin-C</td>
<td>4.18</td>
<td>1.58</td>
</tr>
<tr>
<td>fibronectin, exons 2b and 3a</td>
<td>3.62</td>
<td>2.39</td>
</tr>
<tr>
<td>tenascin-X</td>
<td>2.70</td>
<td>0.63</td>
</tr>
<tr>
<td>ICE-like cysteine protease (Lice)</td>
<td>4.39</td>
<td>1.04</td>
</tr>
<tr>
<td>cyclin-dependent kinase 2-δ</td>
<td>4.08</td>
<td>2.12</td>
</tr>
<tr>
<td>Ca²⁺-independent phospholipase A2</td>
<td>3.28</td>
<td>0.29</td>
</tr>
<tr>
<td>Tumor necrosis factor receptor</td>
<td>2.74</td>
<td>2.00</td>
</tr>
<tr>
<td>α-fodrin (A2A)</td>
<td>2.42</td>
<td>1.31</td>
</tr>
<tr>
<td>LIM kinase 1</td>
<td>2.39</td>
<td>1.78</td>
</tr>
<tr>
<td>caspase 6 (Mch2)</td>
<td>2.29</td>
<td>2.23</td>
</tr>
<tr>
<td>TNF-α converting enzyme</td>
<td>2.20</td>
<td>1.08</td>
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Signal transduction and stress

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tumor ratio</th>
<th>Cell ratio</th>
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<tbody>
<tr>
<td>SOD gene</td>
<td>5.14</td>
<td>0.46</td>
</tr>
<tr>
<td>PSD-95/SAp90-associated protein-4</td>
<td>3.90</td>
<td>1.77</td>
</tr>
<tr>
<td>G-protein beta-subunit 6</td>
<td>3.88</td>
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<tr>
<td>protein kinase PASK</td>
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<tr>
<td>c-fos</td>
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<tr>
<td>adenylyl cyclase type VI</td>
<td>3.19</td>
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</tr>
<tr>
<td>MEK5alpha-1 (MEK5)</td>
<td>3.12</td>
<td>1.72</td>
</tr>
<tr>
<td>heat shock protein 27</td>
<td>3.07</td>
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</tr>
<tr>
<td>complement component C3</td>
<td>3.02</td>
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<tr>
<td>scaffold attachment factor B</td>
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<tr>
<td>phosphatidylinositol 4-kine</td>
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<td>c-jun for transcription factor AP-1</td>
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<td>S-adenosylmethionine synthetase</td>
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<tr>
<td>putative protein kinase C regulatory protein</td>
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<td>1.25</td>
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<tr>
<td>ischemia-responsive 9-kDa protein</td>
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</tr>
<tr>
<td>tyrosine hydroxylase</td>
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<tr>
<td>insulin-like growth factor I receptor</td>
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<td>0.73</td>
</tr>
<tr>
<td>pre-pro-complement C3</td>
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<td>0.31</td>
</tr>
<tr>
<td>nuclear serine/threonine protein kinase</td>
<td>2.09</td>
<td>2.33</td>
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<tr>
<td>inositol polyphosphate 4-phosphatase</td>
<td>2.09</td>
<td>0.83</td>
</tr>
<tr>
<td>insulin-like growth</td>
<td>2.08</td>
<td>0.59</td>
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<tr>
<td>factor-binding protein (IGF-BP3)</td>
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</tr>
<tr>
<td>Smad1 protein (Smad1)</td>
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<td>0.80</td>
</tr>
<tr>
<td>nucleosin</td>
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<td>0.80</td>
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Metabolism/synthesis

<table>
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<tr>
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<th>Tumor ratio</th>
<th>Cell ratio</th>
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<tbody>
<tr>
<td>Dihydroorotate</td>
<td>5.15</td>
<td>2.95</td>
</tr>
<tr>
<td>lecithin-cholesterol acyltransferase</td>
<td>4.45</td>
<td>2.54</td>
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<tr>
<td>branched chain α-ketoacid dehydrogenase E1-α subunit</td>
<td>3.83</td>
<td>0.22</td>
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<tr>
<td>putative peroxisomal 2,4-dienyl-CoA reductase (DCR-AKL)</td>
<td>3.51</td>
<td>1.47</td>
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<tr>
<td>stearyl-CoA desaturase 2</td>
<td>2.80</td>
<td>0.42</td>
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<tr>
<td>fatty acid translocase/CD36</td>
<td>2.69</td>
<td>0.63</td>
</tr>
<tr>
<td>hexokinase II</td>
<td>2.66</td>
<td>0.49</td>
</tr>
<tr>
<td>mevalonate kinase</td>
<td>2.54</td>
<td>0.69</td>
</tr>
<tr>
<td>fatty acid translocase/CD36</td>
<td>2.12</td>
<td>0.79</td>
</tr>
<tr>
<td>steroid 5 α-reductase</td>
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<td>1.20</td>
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</table>

Miscellaneous

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tumor ratio</th>
<th>Cell ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>plasminogen activator inhibitor 2 type A</td>
<td>3.63</td>
<td>2.00</td>
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<td>DNA binding protein C/EBP</td>
<td>3.02</td>
<td>1.69</td>
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<tr>
<td>GABA-A receptor delta-subunit</td>
<td>2.76</td>
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<td>transcription factor GATA-1</td>
<td>2.70</td>
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<tr>
<td>gap junction protein, connexin (CXN-37)</td>
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<tr>
<td>transcription factor</td>
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<tr>
<td>myosin regulatory light chain isoform C</td>
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Table 3  Continued

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tumor ratio</th>
<th>Cell ratio</th>
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<tbody>
<tr>
<td>dynel light intermediate chain 53/55</td>
<td>2.45</td>
<td>0.79</td>
</tr>
<tr>
<td>hepatic nuclear factor one (HNF1)</td>
<td>2.28</td>
<td>1.03</td>
</tr>
<tr>
<td>NonO/p54nrb homologue</td>
<td>2.28</td>
<td>2.43</td>
</tr>
<tr>
<td>skeletal muscle α-tropomyosin and fibroblast tropomyosin 1</td>
<td>2.24</td>
<td>1.00</td>
</tr>
<tr>
<td>dithiolethione-inducible gene-1 (DIG-1)</td>
<td>2.22</td>
<td>2.72</td>
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<tr>
<td>silencer factor B</td>
<td>2.20</td>
<td>0.70</td>
</tr>
<tr>
<td>receptor for advanced glycosylation end products (RAGE)</td>
<td>2.15</td>
<td>0.79</td>
</tr>
<tr>
<td>protein tyrosine phosphatase</td>
<td>2.11</td>
<td>0.68</td>
</tr>
<tr>
<td>nuclear serine/threonine protein kinase</td>
<td>2.09</td>
<td>2.33</td>
</tr>
</tbody>
</table>

NOTE. Ratios of modified versus WT tumors and cells are shown.

Table 3

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</thead>
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NOTE. Ratios of modified versus WT tumors and cells are shown.

Induced by sFLT expression than the morphologic assessment of vascularization. In addition, sFLT may induce changes in the vascular permeability (i.e., tight junctions consisting of occludins, claudins, and zona occludens, which contribute to the blood-tissue barrier) or the tumor microenvironment. This interpretation is in line with the observed increase of the fractional volume of distribution.

In this context, we investigated proliferation, apoptosis/necrosis, and the presence of indicators of oxidative stress in the tumors. Immunohistochemical analyses of WT-MH3924 and sFLT-MH3924A tumors showed no difference in the percentage of proliferating cells, as measured by proliferating cell nuclear antigen staining. The percentage of apoptotic cells was increased in sFLT expressing tumors, although not significantly. These data are also in line with a most recent publication showing that sFLT-expressing lung metastases from renal cell carcinoma revealed more apoptotic (TUNEL positive) cells per metastatic tumor nodule than the control group (38).

CD11b+ macrophages are suggested to be the major source of oxidative stress (40) and their cellular infiltration into the tissue was found to correspond to an enhanced tumor growth (41). Vice versa, because the percentage of CD11b+ macrophages is diminished in sFLT-MH3924A tumors, their stimulatory effect on tumor growth may be reduced. Further evidence is obtained by the decrease of COX-2 expression, as an inflammation marker, in sFLT-MH3924A tumors in comparison with WT-MH3924A. The involvement of COX-2 in tumor growth and angiogenesis has been recently shown, because treatment with celecoxib, a COX-2-specific inhibitor, reduced breast cancer progression and microvessel density (42). COX-2 derived prostaglandin E2 stimulated the expression of angiogenic regulatory genes such as VEGF, Flt, Ang2, and Tie-2 in mammary tumor cells isolated from COX-2 transgenic mice. Consequently, COX-2 selective nonsteroidal anti-inflammatory drugs reduced the expression of VEGF and FLT-1 (42, 43). The potential roles of the proinflammatory and carcinogenic cytokine MIF in regulating hepatoma cell migration and the expression of angiogenic factors by hepatoma cells have not been studied yet (44). In colorectal tumors, the immunohistologically determined MIF level is related to their levels of biological aggressiveness (45) and MIF is suggested to act as an autocrine factor that stimulates angiogenesis and metastasis in hepatoma by promoting expression of angiogenic factors and migration of tumor cells (44). Thus, a possible association between overexpression of MIF and hepatocarcinogenesis is suggested (46). However, our
data show that although the percentage of MIF-positive cells is increased in sFLT-MH3924A in comparison with WT-MH3924A tumors, the microvascularization/macrovascularization and proliferation rate are unchanged, but the growth of these tumors is inhibited. Because MIF is a modulator of prooxidative stress-induced apoptosis (47), it may also function in this way in sFLT-MH3924A.

Additionally, changes in gene expression as a result of tumor defense reactions are possible. To assess general escape phenomena of the tumors by changes in the expression pattern, gene array analyses were done. We analyzed the expression pattern in the tumor cell lines as well as in the tumors obtained after transplantation of these cell lines and found changes in a variety of genes related to matrix proteins, apoptosis, signal transduction, stress, or metabolism. A comparison with the results of the gene expression pattern in RNA obtained in vitro (from the tumor cells) showed that most of these genes were elevated only after transplantation of the cells into animals and, therefore, represent changes induced in vivo. A contribution of nontumor cells such as macrophages or endothelial cells to the signal obtained is unlikely for two reasons: immunohistochemistry revealed that CD11b+ macrophages were reduced significantly in sFLT-expressing tumors which excludes a contribution of macrophages. Furthermore, the CD31 immunoreactivity was unchanged which is evidence against a significant contribution of endothelial cells.

In sFLT-MH3924A tumors, several matrix proteins showed an elevated expression, which may be a sign of increased oxidative stress: tenasin-C, tenasin-X, and laminin. Tenascins contribute to extracellular matrix structure and influence the physiology of the cells in contact with the tenasin-containing environment. Tenasin-C expression is regulated by mechanical stress and shows highest expression in connective tissue in inflamed tissues, tumors, and wounds, where it may regulate cell morphology, growth, and migration by activating diverse intracellular signaling pathways (48). Furthermore, an association with stress-induced signaling has been described: in dermal fibroblasts inflammatory cytokines or activation of the c-jun-NH2-kinase/stress-activated protein kinase-1 pathway by the addition of sphinogomyelinase increased tenasin-C expression (49, 50). Laminin is a basement-membrane protein that increases in liver fibrosis. Recent investigations have shown that diffusable CYP2E1-derived oxidative-stress mediators induce synthesis of laminins by a transcriptional mechanism in hepatic stellate cells (51) and activate the lectin complement pathway (52). A further hint for a modulation of stress-relevant variables in sFLT-MH3924A tumors is the increased expression of stress-related genes such as genes of the c-jun-NH2-kinase pathway (jun, fos, G-protein β-subunit 6), mitogen-activated protein kinase kinase kinase-1 (MEKK-1), an upstream activator of the stress-activated protein kinase/c-Jun NH2-terminal kinase pathway, or stress-activated protein kinase-3/p38 pathway (SAP90/PSD-95), which are the most prominent changes in these tumors. c-Jun-NH2-kinase has been described to mediate responses to cardiac hypertrophy, ischemia/reperfusion injury to the heart and kidney, and endothelial cell apoptosis caused by diabetic hyperglycemia (53). Additionally, we found other stress genes to be elevated in sFLT-MH3924A tumors, such as Hsp27, SOD, choline kinase, and insulin-like growth factor I receptors, which have been shown to be increased (e.g., after experimental ischemia; refs. 54, 55). We also found that hsp27 and scaffold attachment factor B (SAF-B) gene expression was increased in sFLT-MH3924A tumors. These data are in accordance with others suggesting an involvement of HAP (hnRNP A1-associated protein), which was shown to be identical to SAF-B, in the cellular response to heat shock, possibly at the RNA metabolism level (56).

In line with an increase of histomorphometrically analyzed apoptotic (TUNEL positive) cells in sFLT-MH3924A tumors, gene array analyses revealed an enhanced expression of factors, which are involved in apoptosis induction like Lice, caspase-6, tumor necrosis factor receptor, and tumor necrosis factor α converting enzyme. Because apoptosis is a fast event, the apoptotic cells are removed quickly which results in difficulties of detection. This may explain that the changes observed in histology were not significant. A link between apoptosis induction in hepatoma cells and activation of Smads, mitogen-activated protein kinases, caspases has been shown recently (57); however, until now there were no data available about the influence of sFLT gene expression and induction of apoptosis by activation of these pathways. Because apoptosis is an energy dependent process and hexokinase II mRNA expression was significantly higher in metastatic liver cancers, glycolysis is suggested to be the predominant energy source under the hypoxic environment (58). In addition, we found an induction of the expression of several genes coding for enzymes involved in metabolism/synthesis of cholesterol/fatty acids/triglycerides possibly indicating a high energy expenditure or cell transformation. Furthermore, cyclin-dependent kinases represent potentially promising molecular targets for cancer therapeutic strategies, because cyclin-dependent kinase 2 seems to be involved in massive tumor cell apoptosis in vitro (59). Thus, our data of enhanced expression of cyclin-dependent kinase 2-4 may underlie the involvement of this enzyme in apoptosis.

For the strategy described in our experiments to be effective, the target cancer cells need to depend on VEGF for tumor angiogenesis. Most authors, using this anti-VEGF approach with sFLT, describe smaller lesions with fewer microvessels, slower tumor growth and a longer survival time of the animals after the transfer of the sFLT gene (17, 19, 20, 23, 39). However, in all studies the tumors did not disappear completely. We here show that transfer of the sFLT gene in MH3924A cells reduces tumor growth and perfusion with no significant change in vascularization. Because tumor angiogenesis is known to be promoted by several cytokines or growth factors (FGF, VEGF, platelet derived endothelial cell growth factor, and interleukin 8) and many cancer cells produce multiple angiogenic factors, the simultaneous targeting of multiple factors may be more effective. Ciafre et al. used a tricistronic retroviral vector to transfer two inhibitors of angiogenesis, NH2-terminal fragment of rat prolactin and a secreted form of human platelet factor 4 (60). Although in vitro endothelial cell locomotion and formation of an endothelial cell tube network were inhibited, in vivo effects on glioblastoma progression were not observed. They suggested to combine even more factors or to combine antiangiogenic factors with radiation or chemotherapy (61–63).
CONCLUSION

Our results suggest that transfer of sFLT in the present tumor model inhibits tumor growth and perfusion and induces changes in the expression of multiple genes related to the matrix, signal transduction, apoptosis and metabolism. Because these changes in the gene expression pattern are observed mainly in tumor tissue and not in vitro they represent reactions of the tumor to its microenvironment. Furthermore, inhibition of endothelial cell proliferation in vitro was much stronger than the inhibition of tumor growth and perfusion in vivo. This suggests that at least some of the changes are part of tumor defense mechanisms for survival in a less permissive microenvironment.

ACKNOWLEDGMENTS

We thank U. Schierbaum and K. Leotta for their help in performing the animal experiments.

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Kerstin Schmidt, Johannes Hoffend, Annette Altmann, et al.


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