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Paneling Human Thyroid Cancer Cell Lines for Candidate Proteins for Targeted Anti-Angiogenic Therapy

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Abstract Tumor angiogenesis is believed to result from an imbalance of pro- and anti-angiogenic factors, some of which are candidates for targeted therapy. Such therapy has raised hopes for patients with undifferentiated thyroid carcinomas, who are facing a grave prognosis with a survival of only months. In this study, in vivo growth of xenografted human thyroid carcinomas unexpectedly responded quite differently to neutralizing anti-vascular endothelial growth factor (VEGF) antibody. In particular, lasting inhibition as well as accelerated growth occurred after treatment. Consequently, a panel of anti-angiogenic factors was addressed in a representative sample of thyroid carcinoma lines. VEGF, fibroblast growth factor (FGF-2), and endostatin were demonstrated by Western blotting and EIA, whereas PDGF-A, PDGF-B, and IL-6 were negative. Quantification of VEGF, FGF-2, and endostatin revealed a wide range of concentrations from 500 to 4,200 pg/ml VEGF, 5 to 60 pg/ml FGF-2, and 50 to 300 pg/ml endostatin, not related to a particular histologic thyroid carcinoma background. Angiostatin (kringles 1–3) was detected in all, but one of the cell lines. Finally, aaATIII was confirmed in FTC133 cells. These data highlight the complex regulation of angiogenesis in thyroid carcinoma cell lines and suggest that the array of angiogenic factors differs markedly between individual cell lines. For the first time, angiostatin, endostatin, and possibly also aaATIII are identified as novel candidate regulators of angiogenesis in thyroid carcinoma cells. J. Cell. Biochem. 98: 954–965, 2006. © 2006 Wiley-Liss, Inc.

Key words: thyroid cancer; angiogenesis; VEGF; FGF-2; angiostatin; endostatin; aaATIII

Although the majority of thyroid carcinomas can be treated by established protocols with favorable prognosis, the poorly differentiated and anaplastic thyroid carcinomas often escape standard treatment. In these patients survival may be limited to months, thus innovative strategies of treatment are urgently needed [Ain, 1999; McIver et al., 2001; Sherman, 2003]. New hope arises from preclinical studies using targeted anti-angiogenic therapy in thyroid cancer [Turner et al., 2003]. Angiogenesis is a prerequisite for tumor growth as well as metastatic spread and describes the recruitment of blood vessels by a growing primary tumor or a metastasis. It is initiated by factors intrinsic to the tumor cells that induce migration and proliferation of endothelial cells [Folkman, 1990, 2002; Hanahan and Folkmann, 1996].

Among the increasing number of angiogenic factors, vascular endothelial growth factor (VEGF) has been particularly well studied in benign as well as malignant thyroid conditions. VEGF is an endothelial cell specific mitogenic growth factor secreted by tumor cells that has been shown to function as an autocrine regulator of angiogenesis in a number of thyroid cancer cell lines [Viglietto et al., 1995; Soh et al., 1997]. We have recently reported, that TSH induces VEGF via the protein kinase c (*PKC*) pathway and that VEGF is also regulated by other growth factors [Hoffmann et al., 2004]. Experimental as well as clinical studies suggest

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that VEGF may be the dominant angiogenic factor in thyroid cancer [Soh et al., 1997; Klein et al., 2001; Lennard et al., 2001]. Several reports have recently outlined the potential of neutralizing antibodies to VEGF (Avastin®) or inhibitors of VEGF-receptor tyrosine kinases (STI571, PTK787) to inhibit the growth of experimental thyroid cancer [Soh et al., 2000b; Bauer et al., 2002; Podtcheko et al., 2003; Schoenberger et al., 2004].

However, in our own pilot study using a neutralizing anti-VEGF antibody, the results of which are being presented in this article for the first time, we found heterogeneous inhibition of growth in four follicular thyroid cancer xenotransplants. While some of the tumors had sustained inhibition of growth following discontinuation of treatment, others grew with an accelerated pattern, suggesting the institution of alternate pathways of angiogenesis. Following reports on the phenomenon of tumor cell 'escape' from anti-angiogenic monotherapy [Kerbel et al., 2001; Viloria-Petit et al., 2001; Cao, 2004a], a number of recent publications suggest angiogenesis to be the result of a balance of pro- and anti-angiogenic factors rather than the function of a dominant single factor [Turner et al., 2003; Pawelec, 2004]. There is accumulating evidence that increased angiogenesis of thyroid cancer may also be the result of a shift of this balance of pro- and anti-angiogenic factors. For instance, in a clinical series of papillary thyroid cancer, elevated serum levels of fibroblast growth factor (FGF-2) have been demonstrated [Pasieka et al., 2003] and FGF-2 has previously been shown to be a pro-angiogenic factor in a number of different tumors [Trojan et al., 2004]. Aberrant expression of PDGF receptors was suggested to be involved in the development of anaplastic thyroid carcinomas and PDGF receptor tyrosine kinase inhibitor STI571 was shown to enhance the effect of Taxol® in experimental anaplastic thyroid cancer [Heldin et al., 1988; Pietras et al., 2002]. In another clinical series, IL-6 expression, another positive regulator of angiogenesis, has been related to aggressive behavior in thyroid cancer [Kurebayashi et al., 2003]. Moreover, endostatin, a 20-kDa fragment of collagen XVIII, known as a potent endogenous inhibitor of angiogenesis, has been shown to reduce tumor growth and metastasis and to downregulate signaling pathways in human microvascular endothelium associated with angiogenic activity [Abdollahi et al., 2004]. In an experimental setting, recombinant endostatin was shown to inhibit growth of follicular thyroid cancer xenografts [Ye et al., 2002]. Finally, it has been suggested that angiostatin, a 38-kDa internal fragment of plasminogen, which has previously been demonstrated in various other cancers, may as well be presented by thyroid carcinoma cells [Kisker et al., 2001; Ramsden et al., 2002; Cao, 2004].

These incidental reports suggest a rather complex regulation of angiogenesis in thyroid carcinoma. However, until now single anti-angiogenic factors in individual thyroid carcinoma cell lines have been addressed. A paneling of several of these factors involving a number of different thyroid cell lines has not yet been reported. This prompted us to formally evaluate a panel of pro- and anti- angiogenic factors in thyroid carcinoma cell lines. We were particularly interested to determine which of those angiogenic factors, that are potential targets for anti-angiogenic therapy, would be expressed in thyroid carcinoma cell lines, whether histiotypic arrays of factors would be presented and whether such arrays would be related to malignant behavior.

MATERIALS AND METHODS

Cell Lines Used for In Vitro and In Vivo Analysis

Three follicular (FTC133, FTC236, FTC238) and a Hürthle cell thyroid cancer cell line (XTC) were used for in vivo experiments. We have previously documented that XTC and FTC cells express VEGF in vitro [Hoffmann et al., 2004]. All of these cell lines are tumorigenic in the nude mouse. In addition to these cell lines, six previously well-described thyroid cancer cell lines were analyzed in vitro TPC1 (papillary), HTC+ and HTC− (follicular), C 643, Hth74, and KAT 4 (anaplastic). All cell lines were screened for the presence of positive and negative regulators of angiogenesis, resulting in a panel of 10 cell lines. BxPC3, a pancreatic cancer (ATTCC, Rockeville, MD), was employed as a positive control for generation of endostatin, angiostatin, and aaATIII [Kisker et al., 2001].

Animal Procedures

Five- to six-week-old female, pathogen-free BALB/c NCR-Nu congenitally dysthymic mice were purchased (Harlan Winkelmann, Germany), and housed in sterilized cages on laminar air

flow benches at 24°C and a 12/12 hour light/dark cycle. Mice were fed an autoclaved standard chow (Altromin®) and allowed to adapt prior to experiments. All procedures were monitored and approved by local ethics committee and federal authorities and conducted in accordance to the guidelines for the welfare of animals in experimental neoplasia. Growth of thyroid cancer xenografts (FTC133, 236, 238 and XTC) was determined with subcutaneous implants of 3×10^6 vital cells to groups of 12 animals per cell line. After 2 weeks of graft growth, animals were given i.p. injections of either 75 μg anti-VEGF antibody mA461 (Genentech, South San Francisco, CA) in 100 μl of PBS or PBS alone every 3 days (six animals per condition and cell line) for a total of 21 days. After commencement of therapy, half of the animals were killed and tumorgrafts were processed for morphometric analysis of angiogenesis after paraffin embedding. Remaining animals were followed with continued assessment of tumor volume every 3 days using calibrated micrometer calipers according to (length $\times$ width $\times$ high) $\times$ 0.5236 [Zielke et al., 1998].

Morphometry of Tumor Vascularization

Quantitation of angiogenesis was achieved by evaluating vascular surface densities (VSDs) of the tumors according to a protocol previously reported [Barth et al., 1996]. The method uses stereologic assessment of contiguous tumor sections, analyzing number of CD31 positive vessels and area they cover, as well as the areas of tumor cells and stroma, allowing to calculate the area covered by microvessels in a given tumor volume (VSD, 1/mm). Twenty random areas were analyzed per tumor (Leica Q500 MC/MPS 60-System, Wetzlar). Immunostaining for CD31 used affinity purified polyclonal goat anti-mouse CD31/PECAM-1 antibody (M-20, 1:100–1:400, SantaCruz Biotechnology, Santa Cruz, CA).

Cell Culture, Cell Viability, and Cell Number

All cell lines were maintained as monolayer cultures in full growth medium (FGM), DMEM/HAM's F-12 supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biochrom, Berlin, Germany) in a 37°C, 5% CO2 incubator [Hoffmann et al., 2004]. Cells used for experiments were detached by cold trypsin/EDTA and seeded into culture flasks or dishes according to the experiment. Cell viability was tested by tryphan blue exclusion and cells were counted by use of a "Neubauer" chamber. During experiments, cell numbers were determined using the MTT vital colorimetric cell quantification assay as described [Hoffmann et al., 2004] and optical densities (OD) were read at 570 nm with a reference filter of 630 nm (Emax, Molecular Devices, Munich, Germany).

Panel of Angiogenesis-Related Factors

Thyroid cancer cell lines were screened for the following established pro-angiogenic factors: VEGF, FGF-2, PDGF-A, PDGF-B, IL-6 and anti-angiogenic factors: angiostatin, endostatin, and aaATIII. Screening was done either by Western Blot analysis of total cell lysate for VEGF, FGF-2, PDGF-A, PDGF-B, IL-6 and conditioned medium (CM) incubated with plasminogen or ATIII for angiostatin and aaATIII and by use of commercially available EIA/ELISAs for VEGF, FGF-2, and endostatin. During Western blot analysis, staining intensities were estimated by a single investigator using a semiquantitative descriptive scale.

Western Blot Analysis of VEGF, FGF-2, IL-6, PDGF-A, and PDGF-B

Cells were grown in 75-ml culture flasks, washed with PBS, detached by trypsin/EDTA and total cell lysates were prepared using RIPA buffer containing PMSF, sodium-orthovanadate, and aprotinin as protease inhibitors. After denaturation in sample buffer (1 mM Tris-HCl, glycerol, β-mercaptoethanol, SDS, bromophenol blue), total cell lysates (adjusted to 5×10^5 cells /lane) were analyzed by SDS–PAGE (12% SDS for VEGF, 15% SDS for PDGF-A, PDGF-B, and 17% SDS for FGF-2). Proteins were transferred to nitrocellulose membranes (Hybond extra C, Amersham, Piscataway, NJ). Detections were carried out using specific primary antibodies (VEGF A-20, PDGF-A H-77, PDGF-B H-55, and FGF-2 H-138, SantaCruz Biotechnology) and horse-raddish peroxidase conjugated secondary antibodies (SantaCruz). Proteins were detected using the enhanced chemoluminescence detection system with Luminol reagent (SC-2048, SantaCruz) and ECL-Hyperfilm (Amersham). The appropriate recombinant proteins were used as positive controls at all times.

Generation of Angiostatin

To obtain CM, cells were grown in 75-ml flasks (Greiner, Solingen, Germany) until 80% confluent, washed with PBS and switched to serum-free H5 medium (DMEM/HAM's F-12, supplemented with 10 mg/ml bovine insulin, 5 μg/ml transferrin, 2 ng/ml G-H-L-acetate, 10 ng/ml somatostatin, and 0.36 ng/ml hydrocortisone, Sigma, Roedermark, Germany). After 48 h of incubation, CM were collected and centrifuged at 5,000g for 10 min. Then plasminogen (P-5661, Sigma) was incubated in CM at a concentration of 1 μg/100 μl at 37°C in a roller bottle incubator (Bachofer, Reutlingen, Germany) for 48 h. Aliquots of this incubation were stored at −80°C until further analysis was done by Western blotting. CM from BxPC3, known to generate angiostatin under these conditions, was generated in an identical manner and served as a positive control [Kisker et al., 2001].

Generation of Cleaved aaATIII

CM was prepared as described above for angiostatin. For generation of aaATIII, antithrombin III (A-2221, Sigma) at concentrations of 0.25–3 μg ATIII/100 μl was incubated with CM at 37°C in a roller bottle. For purification, aliquots of ATIII-incubated samples of CM containing 500 μg protein were applied to 500–1,000 μl heparin sepharose beads (Biorad, Hercules, CA) equilibrated in application buffer (0.01 M KH_2PO_4, 0.15 M NaCl/PBS) and incubated for 45 min with slight agitation. Thereafter, sepharose beads were allowed to sediment for 15 min and the supernatant was removed. Then 500 μl of elution buffer (application buffer,1.5 M NaCl) was added to the pellet and the affinity eluate collected after 15 min. This step was repeated three times. Aliquots of CM, ATIII-CM, and affinity eluate were then probed for aaATIII, a cleaved 53-kDa fragment of ATIII, by Western blotting [Kisker et al., 2001].

Western Blot Analysis of Angiostatin and aaATIII

For detection of angiostatin and aaATIII, aliquots of the incubated CM (adjusted to 50 or 100 μg of protein) were run on 8% and 12% SDS–PAGE gels. Following nitrocellulose transfer, membranes were probed using polyclonal rabbit anti-human plasminogen antibody AXL204 (Westbury, NY), monoclonal mouse anti-human angiostatin antibody Ab-2, clone Angio 53 1–188 (Oncogene, Cambridge, MA), and monoclonal mouse anti-human ATIII antibody A-5816 (Sigma). CM of BxPC3, with established ability to produce angiostatin and aaATIII, preparations of kringles 1–3 and kringles 1–4.5, as well as native ATIII were used as positive controls. Plain H5 medium, incubated with plasminogen or ATIII, served as negative controls.

Quantification of VEGF, FGF-2, and Endostatin

For quantitative analysis of VEGF, FGF-2, and endostatin, cells were plated on six chamber multiwell plates (2×10^5 cells/well) and allowed to adhere for 24 h in FGM. Thereafter, FGM was changed or replaced by H5. After 48 h of continued incubation, supernatants were collected as CM and aliquots were stored at −80°C. At the same time, cell numbers were determined for each condition using the MTT assay. Quantitative analysis of VEGF protein in CM was carried out using the DuoSet ELISA Development System for human VEGF (DY293, R&D Systems, Minneapolis, MN). Secretion of FGF-2 was analyzed using an ultra-sensitive human FGF-2 ELISA (HSFB75, R&D Systems). Quantification of endostatin was done using a human endostatin EIA, which allows to discriminate free endostatin (EL-EP, Cytimmune, College Park, MD). All immunoassays were performed following manufacturers' instructions.

RESULTS

Tumor Cell Proliferation and VEGF Secretion In Vitro

In vitro tumor cell proliferation of the follicular thyroid cancer cell lines was evaluated by determining cell numbers via the colorimetric MTT assay and is presented as population doubling time. Within the FTC cell lines, there was a trend towards more rapid in vitro growth of the metastatic variant FTC238 compared to the primary tumor cell line FTC133 ($P < 0.05$, *t*-test). The amount of VEGF released into CM was highest in FTC133 cells, and reduced to almost zero in the originally lung metastatic variant FTC238. XTC cells secreted intermediate levels of VEGF (Table I).

TABLE I. Synopsis of the In Vitro and In Vivo Parameter of the Thyroid Carcinoma Cell Lines Used for In Vivo Experiments With Neutralizing Anti-VEGF Antibody

	XTC	FTC133	FTC236	FTC238
Parameter in vitro				
PDT (d)	4.62 ± 0.4	2.9 ± 0.38	2.6 ± 0.44	2.43 ± 0.4*
VEGF (pg/ml)	469 ± 93	1,513 ± 314	783 ± 118	<10
Parameter in vivo				
During treatment				
Tumor volume (% of ctrl)	41*	92	88*	32*
tvd2 (treated vs. ctrl)	5.8 vs. 7.5*	6.2 vs. 6.4	5.2 vs. 5.6	0.7 vs. 1.8*
VSD (% of ctrl)	57.5*	79.4*	54.2*	48.0*
VEGF (% of ctrl)	45.4*	72.3	38.5*	43.8*
After treatment				
Tumor volume (% of ctrl)	210.0	n.d.	74.0	14.0
tvd 2	3	n.d.	7	7

Synopsis of various endpoints evaluated in thyroid cancer cells in vitro and in vivo. Three follicular cancer cell lines (FTC133, 236, and 238 and a Hürthle cell tumor line (XTC)) were employed.
ctrl, control tumors are xenografts to nude mice not receiving anti-VEGF antibody; tvd2, in vivo tumor volume doubling time (d); standard deviations <20% mean values for all of the in vivo parameter.
*$P < 0.05$ (Student *t*-test).

Effects of Anti-VEGF Antibody on the Growth of Human Thyroid Cancer Xenografts

After induction of thyroid carcinoma xenografts to nude mice, these were treated with i.p. injections of either 75 μg anti-VEGF antibody mA461 or PBS every 3 days (six animals per condition and cell line) for a total of 21 days. Half of the animals were sacrificed and the other half followed for post-treatment observation. A significant reduction of tumor volume was registered in three of the four cell lines, that is, XTC, FTC236, and FTC238. In FTC cells, reduction of tumor volume ranged between 68% (FTC238) and only 8% (FTC133) as compared to untreated xenografts, respectively (Table I). A reduction of VSD and VEGF expression, as well as an increase of in vivo tumor volume doubling time was observed at magnitudes concordant with the reduction of tumor volume. No upregulation of VEGF occurred.

However, tumors responded quite differently to the discontinuation of anti-VEGF treatment with the neutralizing antibody. For example, a sustained suppression of in vivo tumor growth was observed in the FTC238 cell line. The average sizes of these tumors that had received anti-VEGF treatment were only some 14% of untreated controls 24 days after discontinuation of the treatment. In contrast, in the originally more differentiated XTC cell line, a dramatic increase of the size of the tumors that had received anti-VEGF therapy was registered. At the end of the observation period, these tumors had increased in size to more than 200% over the untreated control tumors (Fig. 1). These divergences could not be explained by the VEGF angiogenic system alone. It is therefore, that a paneling of pro- and anti-angiogenic factors with previously reported relevance in thyroid cancer was attempted.

Immunoblot Analysis

Expression of angiogenic factors VEGF, FGF-2, PDGF, IL-6, angiostatin, and aaATIII was determined by standard Western blot analysis as described in the method section.

Expression of Pro-Angiogenic Factors VEGF, FGF-2, PDGF, IL-6

Western blot analysis determined that all of the thyroid cancer cell lines presented VEGF and FGF-2 at quantities readily detectable by immunoblotting. Generally, the staining intensities of VEGF and FGF-2 paralleled the quantities of secreted proteins as determined by EIS/ELISA quantification. There were few exceptions, one of which concerned the anaplastic Hth74 cell line. In this particular cell line, VEGF was inconsistently detectable at very small levels. Also in the KAT 4 cell line, established from an anaplastic thyroid cancer, FGF-2 was not expressed. None of the cell lines had PDGF-A, PDGF-B, or IL-6 expression detectable by immunoblotting, despite extensive efforts to titrate the detection system and optimize test conditions as evidenced by appropriate controls.

Processing of Angiostatin From Plasminogen

Western blots of CM incubated with plasminogen, revealed that all of the thyroid cancer cell lines were able to cleave plasminogen to angiostatin. With the exception of Hth74, which

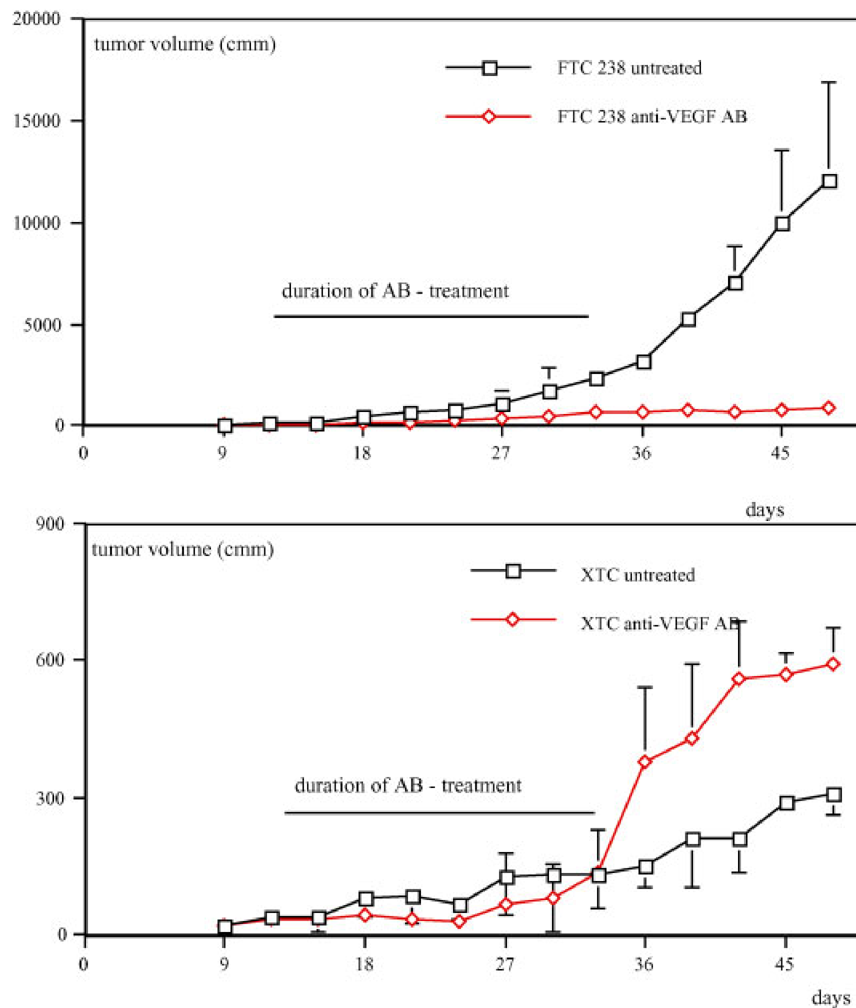

Fig. 1. Follicular thyroid carcinoma xenografts were treated with neutralizing anti-vascular endothelial growth factor (VEGF) AB and tumors followed after discontinuation of treatment for a similar period of time. FTC238 tumors showed lasting inhibitions of tumor growth as compared to untreated tumors, whereas XTC tumors displayed accelerated growth following discontinuation of treatment with neutralizing AB. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

stained positive for angiostatin only once, all of the cell lines stained double positive and, thus had staining intensities comparable to BxPC3, that served as a positive control. Purified plasminogen fragments, that is, kringles 1–4.5 and 1–3, were used as controls allowing us to determine, that the cleaved plasminogen fragment corresponded to the upper band of the kringle 1–3 fragment of plasminogen [O'Reilly et al., 1994]. An example of a typical blot of kringle 1–3 angiostatin, detected in the CM of the HTC cell line, is depicted in Figure 2a.

Processing of aaATIII From Antithrombin III

ATIII was incubated with CM of FTC133 thyroid cancer cells and Western blot analysis was performed to demonstrate the processing from ATIII to cleaved aaATIII by proteolytic activity originating from thyroid cancer cells. These results became available only after purification of ATIII-CM by heparin affinity gels. By use of a human antithrombin antibody, both native ATIII (58 kDA), as well as a second fragment corresponding to cleaved ATIII (aaATIII, 53 kDA) were detected. The FTC133 cell line was the only cell line in which this result was attempted (Fig. 2b).

Quantification of VEGF, FGF-2, and Endostatin by ELISA

Quantitative analysis of cytokines in CM from tumor cells, incubated for 48 h under

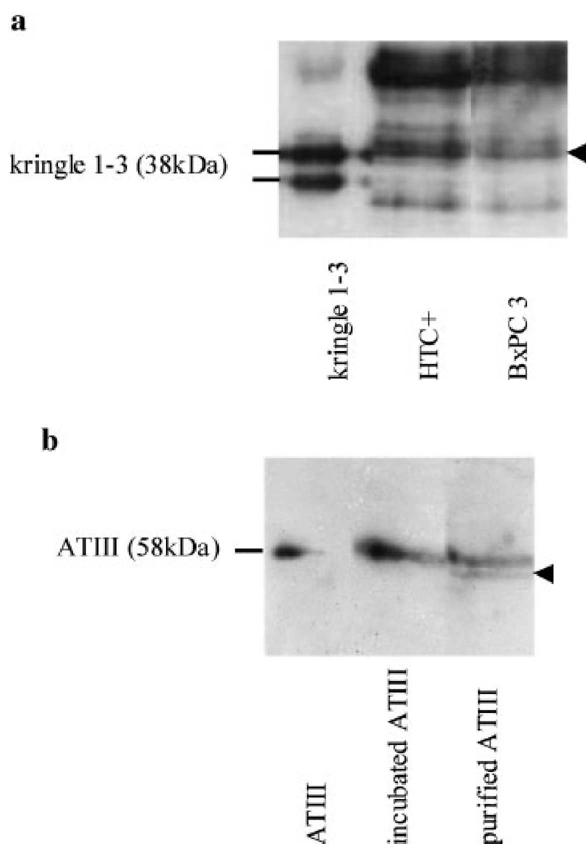

Fig. 2. **a**: Example of a Western blot analysis of angiostatin processed from plasminogen. HTC+, follicular cancer cell line; BxPc3, pancreatic cancer cell line, used as positive control; (→), upper and lower fragment of kringles 1–3; (◀), band corresponding the upper fragment of kringles 1–3 (38 kDa). **b**: Example of a Western blot analysis for cleaved aaATIII (◀) in the FTC133 cell line. The dual band of ATIII and cleaved ATIII (aaATIII) following heparin gel purification of ATIII incubated with conditioned medium (CM) of FTC133 cells is marked. The arrow indicates the minute amount of cleaved 53-kDA aaATIII fragments [ref. Kisker et al., 2001].

either serum-free conditions (H5) or FGM was carried out with commercially available ELISA kits.

Secretion of VEGF

The amounts of VEGF adjusted by cell number ranged from 40 to 3,300 pg/ml (Fig. 3). The highest values were determined in the follicular FTC133 cell line and the anaplastic C643 cell line. The anaplastic Hth74 cells secreted only small amounts of VEGF into the culture medium, which was in agreement with the failure to detect VEGF in this cell line during immunoblot analysis. However, VEGF secretion, although at small quantities, was repeatedly detected when these cells were grown in medium containing fetal calf serum.

Secretion of FGF-2

Detection of basic FGF-2 of thyroid cancer cell lines required the use of a highly sensitive human FGF-2 ELISA. Secretion of FGF-2 was found to be very low for all thyroid cancer cell lines (4–50 pg/ml). In the papillary cell line TPC1, the follicular HTC line, and the anaplastic cell line Hth74, FGF-2 values were close to the limit of detection. However, when these cells were grown in medium containing fetal calf serum, FGF-2 levels increased. In the case of the TPC1, FTC133, and XTC cell lines, this increase was quite exceptional and exceeded 300%. Such an increase of FGF-2 secretion in response to the fetal calf serum did not occur in the two anaplastic cell lines C643 and Kat4 (Fig. 4).

Generation of Endostatin

All thyroid cancer cell lines had the ability to generate endostatin. Endostatin is generated by processing collagen XVIII, which is readily available in the cellular microenvironment. The amounts of endostatin were small, with the notable exception of high levels of endostatin found in the CM of the two most highly differentiated cell lines, namely XTC, a Hürthle cell cancer cell line, and the papillary cancer cell line TPC1. In the majority of cell lines, endostatin levels increased when cells were incubated in the presence of fetal calf serum. As had already been noted for VEGF and FGF-2, there was no particular 'histiotypic' pattern of the magnitude of endostatin generation (Fig. 5).

DISCUSSION

In the present study, xenografted human thyroid carcinomas responded quite differently to neutralizing anti-VEGF antibody, both during as well as after treatment. In particular, lasting inhibition as well as accelerated growth was observed. In order to better define the angiogenetic balance of thyroid carcinoma cell lines, a panel of pro- and anti-angiogenetic factors was, therefore, evaluated in a representative sample of thyroid carcinoma cell lines.

Several recent reports suggest that strategies aiming to affect angiogenesis of undifferentiated thyroid cancers may be promising. Evidence in support of this hypothesis comes from a number of recent studies. For instance, an anti-

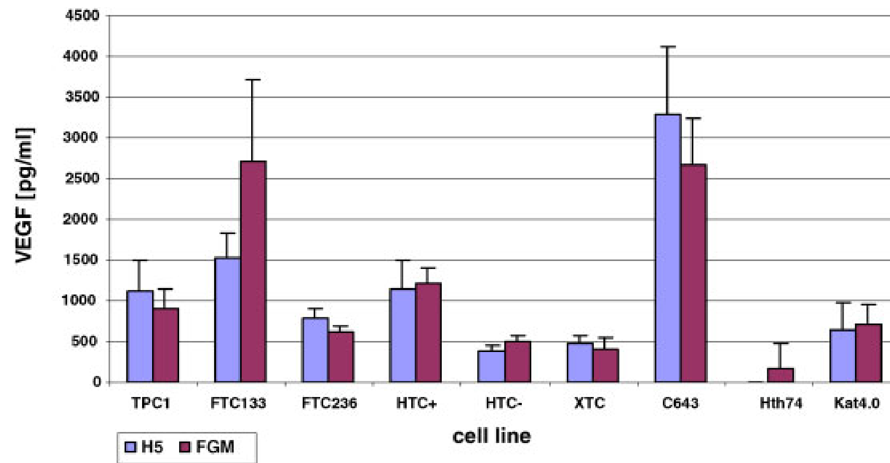

Fig. 3. Secretion of VEGF by thyroid cancer cell lines in vitro. Values are presented as pg/ml and standardized for number of viable cells (2×10^5 cells/well). FGM, full growth medium containing 10% of fetal calf serum; H5 serum-free, defined growth medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

angiogenic effect, induced by farnesyltransferase inhibitor manumycin has been shown to be responsible for the anti-tumor activity in xenografted anaplastic thyroid cancers [Xu et al., 2001]. Also STI571, a tyrosine kinase inhibitor directed against the PDGF receptor, has been shown to exert anti-angiogenic activity and to enhance the effect of Taxol® in experimental anaplastic thyroid cancer [Heldin et al., 1988, Pietras et al., 2002; Podtcheko et al., 2003]. Finally, transfection of undifferentiated follicular thyroid carcinoma cells causing overexpression of endostatin has been shown to inhibit the growth of xenografted tumor cells [Ye et al., 2002]. Moreover, a number of studies including the present one, have documented the growth inhibiting effect of neutralizing antibodies to VEGF or inhibitors of VEGF receptor tyrosine kinases in experimental thyroid cancer [Soh et al., 2000; Bauer et al., 2002, Podtcheko et al., 2003; Schoenberger et al., 2004].

However, in the present study, ambiguous findings were observed during the in vivo experiments with a neutralizing anti-VEGF antibody, as well as after discontinuation of treatment. While one could speculate that the increased susceptibility of FTC238 xenografts to anti-VEGF treatment may be related to the small amount of VEGF secreted by this cell line and thus complete blocking of VEGF, it is still difficult to explain how these cells achieved the fastest in vivo doubling time, largest tumor

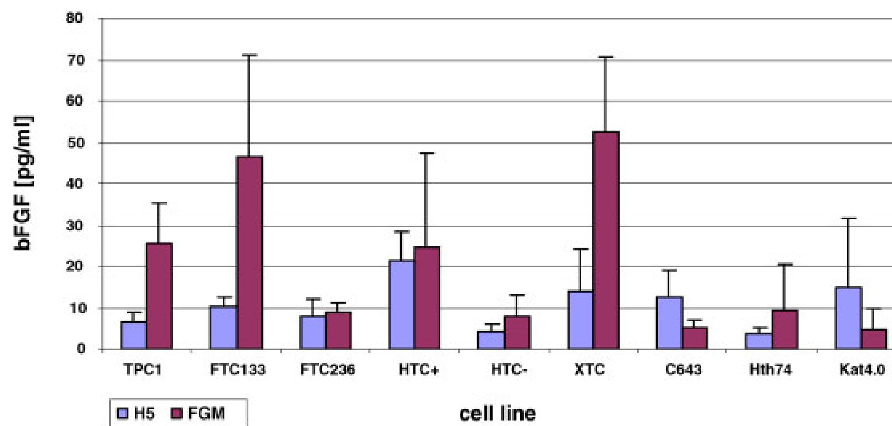

Fig. 4. Secretion of fibroblast growth factor (FGF-2) by thyroid cancer cell lines in vitro. Values are presented as pg/ml and standardized for number of viable cells (2×10^5 cells/well). FGM, full growth medium containing 10% of fetal calf serum; H5 serum-free, defined growth medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

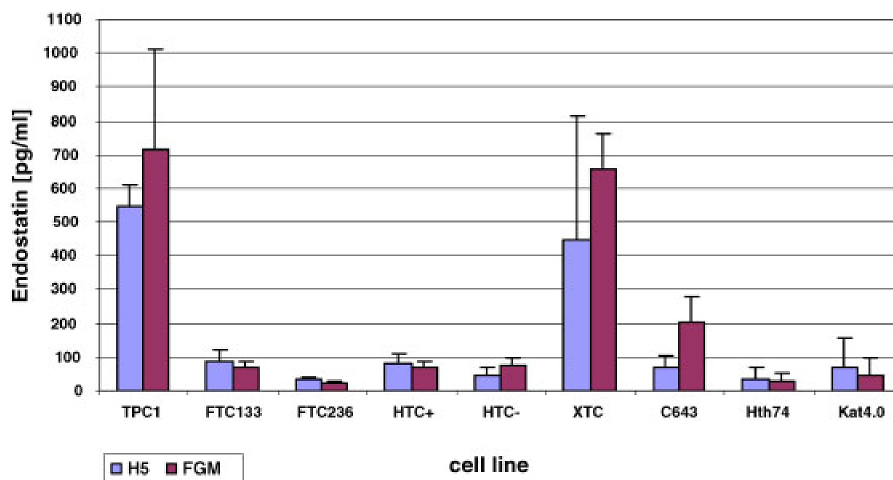

Fig. 5. Generation of endostatin by thyroid cancer cell lines in vitro. Values are presented as pg/ml and standardized for number of viable cells (2×10^5 cells/well). FGM, full growth medium containing 10% of fetal calf serum; H5 serum-free, defined growth medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

volumes and when compared to FTC236, higher VEGF expression in the tumor tissue. Moreover, XTC xenografts had a reduction of VEGF expression and VSD comparable to FTC238 xenografts, but did not show a sustained inhibition of tumor growth persisting for several weeks after discontinuation of the neutralizing antibody. On the contrary, the differentiated XTC cells showed a dramatic increase in tumor growth after cessation of treatment. Of note, an upregulation of VEGF expression was not appreciated in any of the experimental thyroid tumors in response to anti-VEGF treatment.

These phenomena cannot be fully explained on the basis of an isolated alteration of VEGF-dependent angiogenesis and tumor growth. We speculated that alternative angiogenic pathways with tumor growth promoting ability may have been upregulated during or after administration of the VEGF antibody. In fact, others have made similar observations and several mechanisms have been offered to explain the 'escape' of tumors during anti-angiogenic (mono)therapy as well as the 'burst' of tumor growth following its discontinuation [Kerbel et al., 2001; Viloria-Petit et al., 2001; Cao et al., 2004b]. These include the institution of alternate pathways of angiogenesis by upregulation of single pro-angiogenic factors including a yet to be characterized hypoxia-inducible factor (HIF-1), downregulation of anti-angiogenic pathways or a shift of the balance of several angiogenic factors resulting in the restoration of angiogenesis [Blagosklonny, 2001; Turner et al., 2003, Pawelec, 2004]. In light of the apparent complexity of the regulation of angiogenesis, we therefore evaluated a panel of topic factors that affect angiogenesis in a larger sample of thyroid carcinoma cell lines.

With respect to known pro-angiogenic factors, we found ubiquitous expression of VEGF and FGF-2 by immumoblotting as well as ELISA. With respect to these two positive regulators of angiogenesis, no histiotypical pattern was revealed. On the contrary, levels of both factors differed markedly from cell line to cell line. The significance of VEGF as a dominant pro-angiogenic factor in thyroid carcinoma has already been outlined, but is further underscored by clinical data suggesting VEGF expression to be a prognostic marker of local and distant metastasis in papillary thyroid cancer [Klein et al., 2001]. The significance of FGF-2 is highlighted by results from two clinical series of patients with papillary thyroid cancer, where serum levels of FGF-2 were elevated twofold as compared to healthy individuals [Pasieka et al., 2003; Vesely et al., 2003]. Much to our surprise, although this had previously been demonstrated, we did not detect appreciable levels of PDGF (as was previously shown by Heldin in C643 cells [Heldin et al., 1988]), nor IL-6 (as had been shown in KTC-2 anaplastic thyroid cancer cells [Kurebayashi et al., 2003]) in none of the cell lines.

With respect to anti-angiogenic factors, this study determined for the first time that thyroid carcinoma cell lines are able to generate angiostatin, endostatin, and also aaATIII. Angiostatin was suggested for gene therapy and is currently under investigation in an ongoing Phase II trial in patients with non-small cell lung cancer (Entremed at www.nci.-nih.gov/clinical_trials). Angiostatin has previously been detected in the serum of primary cultures of non-transformed thyrocytes and in the transformed rat thyrocyte cell line FRTL-5 [Ramsden et al., 2002]. This report describes the detection of angiostatin in a representative sample of human thyroid cancer cell lines. Because all of the cell lines presented angiostatin, it is suggested that angiostatin is yet another member of the growing panel of factors involved in the regulation of angiogenesis by thyroid cancer cells.

Endostatin has been demonstrated to be a potent anti-angiogenic factor by targeting tumor vasculogenesis. Nude mice xenografted the JHH-1 human hepatocellular carcinoma cells that endogenously produce endostatin by proteolytic degradation of collagen XVIII, have been shown to respond to the administration of an anti-endostatin monoclonal antibody with a rapid increase of tumor size [Tsuboi et al., 2004]. The underlying mechanisms of endostatin signaling have recently been elucidated by means of gene profiling and phosphorylation analysis [Abdollahi et al., 2004]. The potential of endostatin as a therapeutic strategy is underscored by promising data of ongoing Phase I studies in advanced solid tumors [Herbst et al., 2002]. The present study has documented for the first time that endostatin is expressed by a number of thyroid cancer cell lines. The highest basal secretion of endostatin was detected in the slow growing and highly differentiated tumor cell lines TPC1 and XTC. None of the undifferentiated follicular or anaplastic thyroid cancer cell lines had elevated levels of endostatin. Although this may be an incidental finding, it may suggest endostatin to be involved in the progression of thyroid malignancies. It should be worthwhile to further elucidate the mechanisms responsible for the regulation of endostatin in thyroid cancer cell lines. In fact, in a previous study, recombinant endostatin and gene transfer have been used to inhibit the growth of xenotransplanted follicular thyroid cancer cells very effectively [Ye et al., 2002].

Finally, this study has demonstrated the presence of aaATIII in the CM of FTC133 thyroid cancer cells. Triple-ATIII is a 53-kDa fragment of antithrombin III and cleaved by proteolysis initiated by the tumor cells. It was first detected in neuroblastoma, lung and pancreatic cancer cell lines [O'Reilly et al., 1999; Kisker et al., 2001], and has been shown to inhibit tumor growth and angiogenesis in experimental pancreatic cancer [Prox et al., 2003]. Detection of aaATIII is demanding and, therefore, was only completed in the FTC133 cell line to document its presence. To this end, it is not clear whether or not aaATIII is consistently presented by thyroid cancer cells.

In conclusion, this report presents a first time paneling of a number of factors involved in regulating angiogenesis in a representative sample of thyroid carcinoma cell lines. The findings highlight the multifaceted regulation of angiogenesis in thyroid cancer cell lines and suggest, that the panel of angiogenetic factors may differ markedly from cell line to cell line and appears to be a characteristic of the individual cell line rather than a histologic tumor type. They also suggest angiostatin, endostatin, and possibly also aaATIII, as novel candidate negative regulators of angiogenesis in thyroid carcinoma. Taken together, the results of this investigation would support the concept that enhanced tumor driven angiogenesis in thyroid cancer may also be a result of an imbalance of several pro- and anti-angiogenic factors, as has previously been demonstrated for a number of other solid cancers.

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