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Overexpression of Vascular Endothelial Growth Factor-A165 Enhances Tumor Angiogenesis but not Metastasis during β-Cell Carcinogenesis[1]

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ABSTRACT

The pivotal role of vascular endothelial growth factor (VEGF-A) in the regulation of angiogenesis, in particular in the onset and maintenance of tumor angiogenesis, has been demonstrated repeatedly in experimental model systems and, more recently, in clinical trials. Experimental evidence has also suggested that up-regulated expression of VEGF-A may cooperate with other genetic or epigenetic changes to induce or accelerate tumor progression to invasive and metastatic cancers. Here we report the generation of transgenic mouse lines that express human VEGF-A165 under the control of the rat insulin promoter in the β cells of pancreatic islets of Langerhans (Rip1VEGF-A). These mice do not exhibit detectable changes in islet development, vascularization, or physiology. Intercrosses of these mice with a transgenic mouse model of pancreatic β cell carcinogenesis (Rip1Tag2) result in an earlier onset of tumor angiogenesis and with it accelerated tumor growth and mortality. The transition from benign tumors (adenoma) to malignant tumors (carcinoma) is modestly accelerated; however, tumor metastases are not observed. Our findings indicate that in β-cell tumorigenesis, overexpression of VEGF-A165 accelerates the onset of tumor angiogenesis and with it tumor progression but is not sufficient to induce tumor metastasis.

INTRODUCTION

VEGF (or VEGF-A)[4] is a key regulator in the *de novo* formation of blood vessels during embryonic development (vasculogenesis) and in the formation of blood vessels from preexisting blood vessels (angiogenesis) in a number of physiological and pathological settings (1, 2). VEGF-A is the founder member of a family of growth factors, including VEGF-A, B, C, D, E, and placental growth factor. It is a dimeric, disulfide-bonded protein with molecular weights between M_r 32,000 and M_r 42,000. Alternative splicing of the mRNA encoding VEGF-A generates six different isoforms (206, 189, 183, 165, 145, 121 amino acids) with varying heparin affinities (3). Among the various family members, VEGF-A is a potent angiogenic factor. By binding with high affinity to its two tyrosine kinase receptors that are expressed on endothelial cells, flt-1 and flk-1/KDR (VEGFR-1 and -2, respectively), VEGF-A is able to stimulate endothelial cell proliferation, migration, differentiation, and to induce angiogenesis *in vitro* and *in vivo* (1, 2). A third VEGF receptor (flt-4; VEGFR-3) is almost exclusively expressed in the lymphatic endothelium of adult tissue, and signal transduction through this receptor is predominantly stimulated by VEGF-C and D, resulting in lymphangiogenesis (4–6).

The importance of VEGF-A in physiological angiogenesis (*e.g.*, postnatal growth, corpus luteum formation) has been clearly established (7), and VEGF-A is now also being explored as a therapeutic tool to induce angiogenesis and the formation of new collateral vessels in peripheral and myocardial ischemia (8–10). The pivotal role of VEGF in tumor angiogenesis has been demonstrated repeatedly in a large variety of experimental systems (10–12). Inhibition of VEGF function, *e.g.*, by neutralizing antibodies to VEGF-A or VEGFR-2, by soluble VEGFR constructs, or by specific inhibitors of VEGF receptor signaling, has unequivocally demonstrated the requirement of VEGF-A for tumor angiogenesis and, thus, for tumor growth. Currently, several of these therapeutic approaches are in early clinical trials aimed at the prevention of tumor angiogenesis (8, 10).

It is thought that the process of angiogenesis is also required for metastatic dissemination, because an increase in vascular density will allow easier access of tumor cells to the circulation. Indeed, induction of angiogenesis precedes the formation of malignant tumors, and increased vascularization appears to correlate with the invasive properties of tumors and, thus, with the malignant tumor phenotype (12, 13). However, although angiogenesis appears to be a rate-limiting event in tumor growth and metastatic dissemination, it remains to be determined whether angiogenesis is causally involved in promoting the transition from a well-differentiated, noninvasive to an invasive, malignant tumor phenotype, *i.e.,* a direct connection between the induction of angiogenesis and the progression to tumor malignancy has not been demonstrated. A first answer to this question came from a xenograft model of multistage skin carcinogenesis (HaCaT) in which expression of VEGF-A/VEGFR-2 correlated with tumor progression and in which inhibition of VEGF-A function prevented progression to tumor malignancy (14). Moreover, overexpression of VEGF-A in a number of different tumor cell lines caused tumor malignancy on xenograft transplantation into immunocompromised mice (15–17). However, a causative connection between tumor angiogenesis and tumor metastasis has not been demonstrated.

We have used the Rip1Tag2 transgenic mouse model of multistage tumorigenesis to determine whether VEGF-A function is not only involved in the onset and maintenance of tumor angiogenesis but also in the modulation of tumor invasion and metastatic dissemination during the growth of endogenous tumors. In these mice, SV40 T antigen is expressed under the control of the rat insulin promoter resulting in the development of β-cell tumors in the pancreatic islets of Langerhans (18). Several tumor stages can be distinguished, including β-cell hyperplasia, adenoma, and carcinoma (19, 20). Notably, hyperplastic islets can be classified into two types: nonangiogenic islets that do not affect cocultured endothelial cells and angiogenic islets that are able to induce proliferation, migration, and endothelial tube formation *in vitro*, suggesting that soluble factors are involved in the onset of tumor angiogenesis (21). The expression of one of these factors, VEGF-A, is only moderately up-regulated during Rip1Tag2 tumorigenesis and, once secreted, is sequestered in the ECM. However, sequestered VEGF-A is released from the extracellular matrix concomitantly with the onset of angiogenesis by MMPs (22, 23). Consistent with these findings, inhibition of VEGF function by specific inhibitors of MMPs or VEGFR signal transduction (23), or by soluble VEGFR-1 (24) represses tumor angiogenesis in these mice.

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[4] The abbreviations used are: VEGF or VEGF-A, vascular endothelial growth factor; MMP, matrix metalloprotease; BrdUrd, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling; ECM, extracellular matrix.

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Recently, our laboratories have demonstrated that the expression of the lymphangiogenic factor VEGF-C during Rip1Tag2 tumorigenesis results in increased lymphangiogenesis and lymph node metastasis (25). We now set out to investigate whether up-regulated expression of VEGF-A, and with it increased tumor angiogenesis, would also promote tumor invasion and metastasis. We generated transgenic mice expressing human VEGF-A165 in β cells of pancreatic islets (Rip1VEGF-A). These mice were then intercrossed with Rip1Tag2 mice, and changes in tumor development were analyzed. The results indicate that forced expression of VEGF-A165 in Rip1Tag2 transgenic mice accelerates the onset of tumor angiogenesis and tumor progression but does not result in tumor metastasis.

MATERIALS AND METHODS

Transgenic Mouse Lines. Rip1VEGF-A transgenic mice were generated according to standard procedures (26). The transgene was constructed by cloning the cDNA encoding the 165 amino acid isoform of human VEGF-A between the ~695-bp *Bam*HI/*Xba*I fragment of the rat insulin gene II promoter (Rip1; Ref. 18) and a 2154-bp genomic DNA fragment containing human growth hormone introns and polyadenylation signal. Genotypes were confirmed by Southern blot and PCR analysis. PCR screening of Rip1VEGF-A heterozygotes was performed using a pair of primers specific for the human VEGF-A cDNA (forward: 5′-CCTAGAGGAAGGCATCCAAACGC; reverse 5′-GCCCGTAGTTCTTGAGTAGTGCG), starting from standard tail or toe genomic DNA preparations. PCR cycles were: 94°C, 3 min (×1); 94°C, 1 min, 56°C, 1 min, and 72°C, 1 min (×34); and 72°C, 3 min (×1). PCR products were analyzed in 1% agarose gels. Generation and phenotypic characterization of Rip1Tag2 mice was as described previously (18). Glucose (5% w/v) was added to the drinking water of all of the tumor-bearing mice, beginning at 10 weeks of age, to counteract hypoglycemia that resulted from insulinoma development.

Immunoblotting. Rip1VEGF-A mice were sacrificed at 8–10 weeks of age. Expression of the transgene was assessed by immunoblotting. Protein was isolated and precipitated from whole mouse pancreas with TRIzol Reagent (Life Technologies, Inc., Vienna, Austria) according to the manufacturer's recommendations. A total of 80 μg of protein was resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and VEGF-A was visualized with a monoclonal antibody recognizing human VEGF-A (R&D Systems, Minneapolis, MN) and the ECL system (Amersham, Buckinghamshire, United Kingdom). Recombinant human VEGF-A (10 ng; PreproTech Inc., London, United Kingdom) was used as a positive control.

Collagen Gel Assay. Islets of Langerhans were isolated from normal control mice, Rip1VEGF-A mice, Rip1Tag2 transgenic mice, and double transgenic Rip1Tag2/Rip1VEGF-A mice at 6 and 9 weeks of age, respectively, as described previously (21, 24). Human umbilical vein endothelial cells were cultured in DMEM supplemented with 20% FCS (Life Technologies, Inc., Gaithersburg, MD), 2 mM glutamine, 40 μg/ml bovine brain extract, 80 units/ml heparin, and antibiotics. Cells were then trypsinized, resuspended in 10% FCS RPMI, and cocultured with tumors in a three-dimensional collagen matrix as described (21). After 2–3 days, the response of endothelial cells to the angiogenic tumors was scored. The experiment was performed three times with approximately 40–50 islets/mouse genotype.

Histology and Immunohistochemistry. Tumor-bearing mice were sacrificed at different ages as indicated. Tumor volumes were calculated from the tumor diameter by assuming that the tumors were spherical. Tumors and pancreata were fixed overnight in 4% paraformaldehyde in PBS and then immersed in a sucrose gradient (15–30% in PBS) over a 12-h period. Samples were then embedded in OCT (Tissue Tek, Torrance, CA) and snap frozen in liquid nitrogen. Sections were analyzed histologically by H&E staining. For BrdUrd labeling, 2 h before sacrifice mice were injected i.p. with 100 μg of BrdUrd (Sigma Chemical Co., St. Louis, MO) per gram of body weight. Sections (10-μm thick) were cut, mounted on silane-coated slides, and immunostained as described (24, 25). For detection of apoptosis, the TUNEL technique was used as described previously (27). Vessel density was determined by CD31 (PECAM-1) or MECA-32 immunostaining (24, 25). Specific antibody staining was visualized using FITC-labeled secondary antibodies or the ABC-Vector horseradish peroxidase kit according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA). Antibodies were antimouse CD31 antibody (1:50; PharMingen, San Diego, CA), and MECA-32 (28) and anti-BrdUrd antibody (1:50; Zymed, San Francisco, CA). Proliferating and apoptotic cells were counted in ≥10 comparable microscopic fields per section.

RESULTS

Expression of VEGF-A in Rip1VEGF-A Single Transgenic Mice. To study the effect of VEGF-A on β-cell carcinogenesis in Rip1Tag2 transgenic mice, we generated transgenic mice overexpressing human VEGF-A165 in the insulin-producing β cells of the pancreatic islets of Langerhans. A cDNA encoding the 165-amino acid isoform of human VEGF-A was cloned between a rat insulin II promoter fragment, and a human growth hormone intron and polyadenylation signal (Fig. 1*A*), thus targeting VEGF-A165 expression to pancreatic β cells. After pronuclear injection of the transgene into 1-cell embryos, several independent transgenic mouse lines were established that exhibited stable transmission of the transgene to their progeny. Rip1VEGF-A transgenic mice were viable, normoglycemic, and fertile. Analysis of transgene expression was examined at the RNA level by reverse transcription-PCR (data not shown). The Rip1VEGF-A mouse line 49 exhibited high levels of human VEGF-A mRNA, and subsequently in this line transgene expression was analyzed at the protein level. Equal amounts of protein from the pancreas of nontransgenic littermates and Rip1VEGF-A single transgenic mice was isolated, and the expression of VEGF-A165 was determined by immunoblotting (Fig. 1*B*). Consistent with the previous findings that normal islets of Langerhans express VEGF-A (22, 23), VEGF-A was detected at low but significant levels in the pancreas of nontransgenic littermate controls. In contrast, ~20-fold higher levels of human VEGF-A165 were detected in Rip1VEGF-A transgenic mice (Fig. 1*B*), indicating efficient expression of the transgene in the pancreas of Rip1VEGF-A transgenic mouse line 49. Subsequently, mouse line 49 was used for additional experimentation.

Knowing that Rip1VEGF-A mice express increased levels of human VEGF-A165 in their islets of Langerhans, we wished to examine their phenotype in comparison with nontransgenic littermate control mice. Histopathological analyses did not reveal significant differences in islet morphology between Rip1VEGF-A and nontransgenic mice (Fig. 2, *top panels*), and the presence and distribution of cells pro-

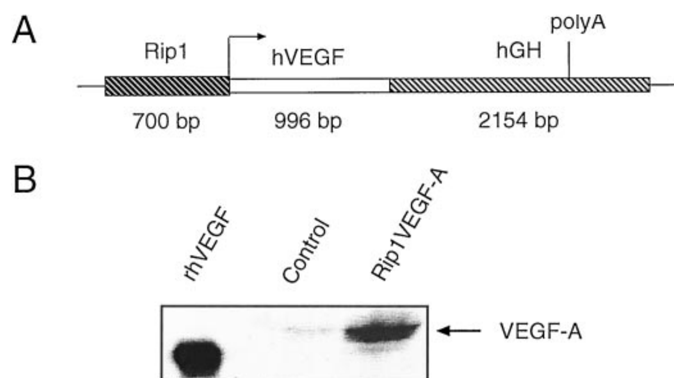

Fig. 1. Expression of human VEGF-A165 in Rip1VEGF-A transgenic mice. *A,* schematic representation of the transgene construct. DNA fragment lengths are given in bp. *Rip1,* DNA fragment containing part of the rat insulin II gene promoter; *hVEGF,* cDNA fragment encoding the 165 amino acid isoform of human vascular endothelial growth factor; *hGH,* DNA fragment containing the human growth hormone intron and polyadenylation sequences; *poly(A),* polyadenylation site. *B,* VEGF-A expression. Expression of VEGF-A in the pancreas of wild-type (*Control*) or Rip1VEGF-A transgenic mice was analyzed by immunoblotting of total pancreatic lysates. Recombinant human VEGF-A (10 ng) served as positive control.

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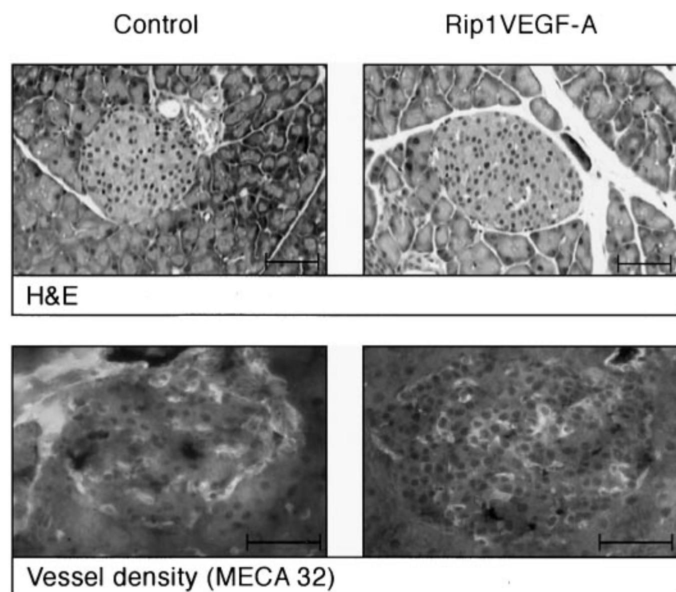

Fig. 2. Overexpression of human VEGF-A165 does not affect islet vascular density in Rip1VEGF-A transgenic mice. Histological sections from pancreata of wild-type (*Control*) and Rip1VEGF-A transgenic mice stained with H&E or immunostained for endothelial cells with the MECA-32 antibody (*Vessel density*); *scale bars,* 50 μm.

ducing insulin, glucagon, pancreatic polypeptide, and somatostatin, as determined by immunohistochemistry, was unaltered regardless of the age of the mice (data not shown). Moreover, the vessel density in islets of Langerhans, as determined by immunohistochemical staining of blood vessels with antibodies against CD31 (not shown) and MECA-32 (Fig. 2, *bottom panels*), was also not significantly affected by the up-regulation of VEGF-A165 in Rip1VEGF-A mice. The average vessel count per microscopic field in islets from both mouse genotypes was not significantly different: 11.09 ± 3.34 in nontransgenic littermate controls compared with 10.83 ± 3.20 in Rip1VEGF-A mice. Similarly, the percentage of islet surface area occupied by vessels as determined by computer-assisted image analysis of MECA-32 stained sections was unaffected in Rip1VEGF-A mice compared with controls (9.12 ± 5.34% for controls and 7.36 ± 2.86% for Rip1VEGF-A mice). These results indicate that up-regulated expression of VEGF-A does not measurably affect islet vascularization.

Accelerated Rate of Tumor Progression in Rip1Tag2/Rip1VEGF-A Double-Transgenic Mice. To study the effect of increased expression of VEGF-A on tumor progression, we crossed Rip1VEGF-A transgenic mice with Rip1Tag2 mice (18). Tumor development in the resulting single-transgenic Rip1Tag2 and double-transgenic Rip1Tag2/Rip1VEGF-A mice was analyzed. In contrast with Rip1Tag2 mice, which survive up to 14 weeks of age, Rip1Tag2/Rip1VEGF-A double-transgenic mice were found to die at around 10 weeks of age. Determination of the tumor burden revealed that at 10 weeks of age the tumor volumes of double-transgenic Rip1Tag2/Rip1VEGF-A were >10-fold greater than that seen in single-transgenic Rip1Tag2 mice at this age (Fig. 3). In fact, tumor volumes of the double-transgenic mice at 10 weeks of age were comparable with tumor volumes of single-transgenic mice at 14 weeks of age, indicating a common cause of death by tumor burden. These results indicate that up-regulated expression of VEGF-A165 causes accelerated tumor growth in Rip1Tag2 transgenic mice.

Accelerated Onset of Tumor Angiogenesis in Rip1Tag2/Rip1VEGF-A Mice. Knowing that the Rip1Tag2/Rip1VEGF-A mice exhibit accelerated tumor formation, we next wished to investigate whether this was attributable to an earlier onset of angiogenesis as a consequence of up-regulated expression of VEGF-A165. The onset of angiogenesis in Rip1Tag2 transgenic mice can easily be visualized by coculturing isolated islets of Langerhans at different stages of islet tumorigenesis with endothelial cells in a three-dimensional collagen matrix (collagen gel assay; Ref. 21). In the case of an islet that is actively undergoing angiogenesis, endothelial cells respond by chemotactic migration, proliferation, and tube formation, whereas in the case of a nonangiogenic tumor stage or a normal islet, endothelial cells do not respond (as exemplified in Fig. 4, *inset*).

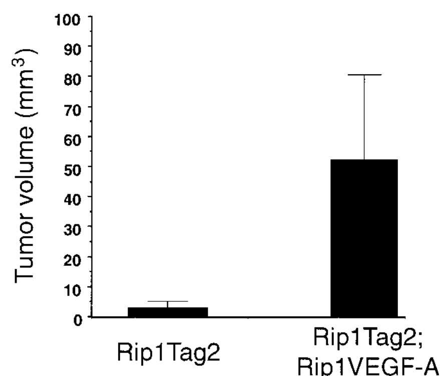

Fig. 3. Accelerated tumorigenesis in Rip1Tag2/Rip1VEGF-A transgenic mice. Tumor volumes in Rip1Tag2 single-transgenic (n = 16) and Rip1Tag2/Rip1VEGF-A double-transgenic mice (n = 18) was determined at 10 weeks of age. Values are averages of the total tumor volume in individual mice; *bars,* ±SD.

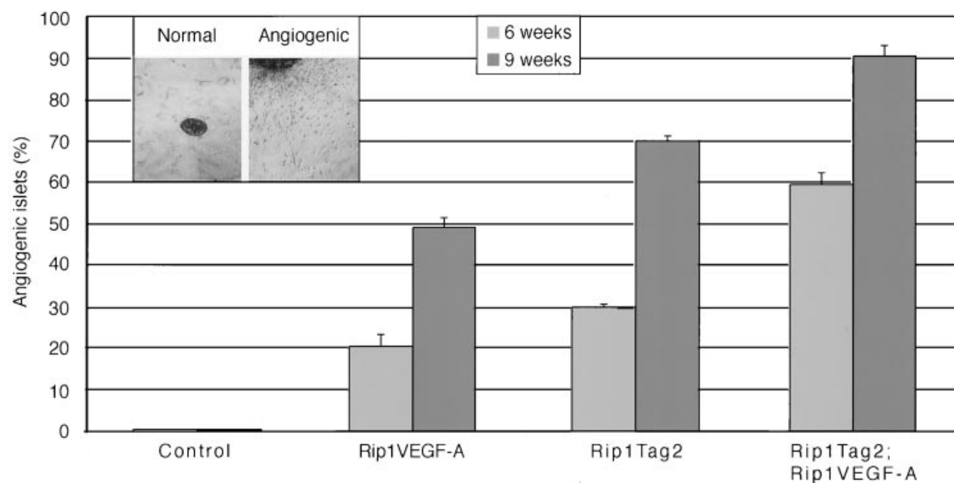

Fig. 4. Accelerated onset of angiogenesis in Rip1Tag2/Rip1VEGF-A transgenic mice. Islets of Langerhans isolated from 6 and 9 week old Rip1Tag2, Rip1Tag2/Rip1VEGF-A, Rip1VEGF-A transgenic mice, and wild-type control mice were cocultured with endothelial cells in a three-dimensional collagen matrix. Representative examples of a normal nonangiogenic islet and an angiogenic islet are shown in the *inset*. The quantitative evaluation of the angiogenic response is depicted in bar graphs. Values are the percentage of angiogenic islets and are pooled from two independent experiments; *bars,* ±SD.

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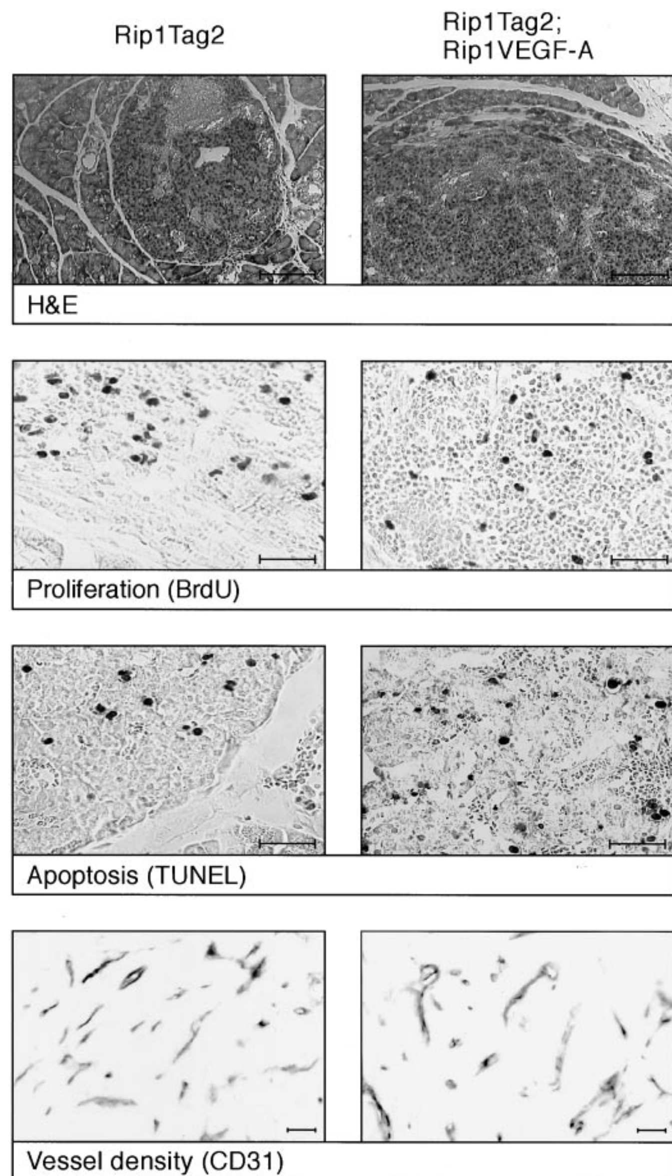

Fig. 5. Histopathological analyses of tumor progression in Rip1Tag2 and Rip1Tag2/Rip1VEGF-A mice. Histological sections from pancreata of Rip1Tag2 or Rip1Tag2/Rip1VEGF-A transgenic mice were stained with H&E, immunostained for incorporation of BrdUrd to determine the number of cells that had progressed through S phase (*Proliferation*), immunostained for apoptotic cells using the TUNEL assay (*Apoptosis*), or immunostained for endothelial cells with antibodies against CD31 (*Vessel density*). *Bars*, 100 μm (*H&E*); 50 μm (*BrdUrd* and *TUNEL*); 10 μm (*CD31*).

Islets of Langerhans were isolated from Rip1VEGF-A mice, from Rip1Tag2 mice, and from Rip1Tag2/Rip1VEGF-A double-transgenic mice at both 6 and 9 weeks of age, and their angiogenic potential was compared using the collagen gel assay (Fig. 4). These experiments reveal that a greater percentage of the islets from the double-transgenic mice are angiogenic compared with Rip1Tag2 transgenic mice. The difference is most striking at 6 weeks, when 60% of the islets of double-transgenic mice are angiogenic as compared with 30% of the islets from Rip1Tag2 mice (Fig. 4). At 9 weeks of age this difference is still apparent although to a lesser extent.

Islets of Rip1VEGF-A mice also exhibit a positive response in the collagen gel assay although at slightly lower levels as compared with islets from Rip1Tag2 mice (Fig. 4). It has been demonstrated previously that VEGF sequestered in the extracellular matrix of islet cells is released by the activities of matrix metalloproteases (23). Hence, the angiogenic reaction of VEGF-A165 islets in the collagen gel assay suggests that overexpression of VEGF-A may circumvent the need for MMPs and that levels of free VEGF-A are rate-limiting in the angiogenic response. Islets from normal mice did not at all exhibit a positive response in this assay (Fig. 4; Refs. 21, 22).

Together, the results indicate that forced expression of VEGF-A165 during Rip1Tag2 tumorigenesis results in an earlier onset of tumor angiogenesis and, with it, in an acceleration of tumor development and mortality.

VEGF-A Does Not Affect β-Cell Tumor Invasion or Metastasis. To assess potential changes in tumor progression between Rip1Tag2 and Rip1Tag2/Rip1VEGF-A mice, pancreatic sections of mice at 8–10 weeks of age were stained with H&E. These analyses did not reveal a significant histopathological difference between the different genotype tumors (Fig. 5). Also, analysis of the vessel density within tumors from Rip1Tag2 and from Rip1Tag2/Rip1VEGF-A mice by staining with anti-CD31 antibodies did not reveal a significant VEGF-A-dependent difference (Fig. 5; 9.1 ± 0.15 and 12.0 ± 3.1 cells/unit area for Rip1Tag2 and Rip1Tag2/Rip1VEGF-A tumors, respectively). Together with the data of the collagen gel assay experiment (Fig. 4), these results indicate that up-regulated expression of VEGF-A165 predominantly induces an earlier onset of tumor angiogenesis but has no significant effect on blood vessel density in large tumors. These results also suggest that once angiogenesis is under way, new blood vessels form at comparable rates, and tumors grow to similar sizes.

Histopathological classification of the different tumor stages into normal islets, hyperplastic islets, benign tumors (adenomas), and invasive, malignant tumors (carcinomas) with increasing grades of severity (G1-G3) revealed a modest but significant increase in invasive, malignant tumors in Rip1Tag2/Rip1VEGF-A double-transgenic mice as compared with Rip1Tag2 single-transgenic mice (Table 1). However, tumor metastases to local lymph nodes or to distant sites were not observed, neither in Rip1Tag2 nor in Rip1Tag2/Rip1VEGF-A mice.

BrdUrd incorporation experiments did not reveal significant differences in the proliferation of tumor cells in Rip1Tag2 and Rip1Tag2/Rip1VEGF-A tumors (Fig. 5; 13.9 ± 2.9 and 17.0 ± 2.9 cells/unit area for Rip1Tag2 and for Rip1Tag2/Rip1VEGF-A tumors, respectively). Analysis of tumor cell apoptosis by TUNEL staining likewise did not show a significant difference between the two genotypes (Fig. 5; 5.0 ± 2.3 and 8.4 ± 1.95 cells/unit area for Rip1Tag2 and for Rip1Tag2/Rip1VEGF-A tumors, respectively).

DISCUSSION

Using the Rip1Tag2 transgenic mouse model of pancreatic β-cell carcinogenesis, we have assessed the influence of overproduction of VEGF-A on tumor progression. Our data indicate that overexpression of human VEGF-A165 in Rip1Tag2 transgenic mice accelerates tumor progression, because Rip1Tag2/Rip1VEGF-A double-transgenic

Table 1 *Tumor progression*

	Rip1 Tag2[a]	Rip1 Tag2/Rip1 VEGF-A[a]
Normal Islets	45%	43%
Hyperplasia	25%	11%
Adenoma	6%	7%
Carcinoma[b]		
G1	5%	9%
G2	18%	21%
G3	1%	9%

[a] Rip1 Tag2 and Rip1 Tag2/Rip1 VEGF-A transgenic mice were sacrificed at 14 and 10 weeks of age, respectively.

[b] Grades of tumor malignancy: G1, tumors with small invasive edges and residual epithelial organization; G2, tumors that are highly invasive, with nuclear atypia and a low amount of cytoplasm; and G3, anaplastic tumors.

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mice die at ~10 weeks of age with a tumor burden comparable with that of Rip1Tag2 controls at 14–15 weeks of age. Our experimental analysis revealed that the acceleration of tumor progression is predominantly attributable to an earlier onset of tumor angiogenesis. Moreover, progression from benign adenoma to malignant carcinoma was also enhanced in Rip1Tag2/Rip1VEGF-A double-transgenic mice; however, metastases were not observed.

The results suggest that up-regulated expression of VEGF-A facilitates the induction of angiogenesis, likely by overcoming sequestration of VEGF-A in the ECM or by relaxing the need for MMPs to activate ECM-sequestered VEGF-A. However, once angiogenesis is initiated, VEGF-A levels may not be as critical; MMPs are fully active to supply sufficient amounts of VEGF-A, and, hence, vessel densities are at optimum in tumors of both Rip1Tag2 and Rip1Tag2/Rip1VEGF-A mice. Consistent with this notion vessel density in fully developed tumors is not significantly increased by the up-regulated expression of human VEGF-A165. Moreover, other parameters of tumor development, such as tumor cell apoptosis and tumor cell proliferation, are also not affected by increasing VEGF-A expression in Rip1Tag2 transgenic mice.

These results are consistent with a number of reports in which tumor cell lines have been transfected to overexpress VEGF-A and, on xenograft transplantation into immunocompromised mice, exhibited accelerated tumor growth and progression to malignancy (as well as increased vascularity not seen in our studies; Refs. 15–17). In these reports, as in the present study, VEGF-A-dependent formation of tumor metastasis was not observed. Similarly, transgenic expression of VEGF-A in skin resulted in accelerated tumor progression but not in tumor metastasis (29). In contrast, Skobe *et al.* (14) have reported recently a correlation between VEGF-A function and skin tumor malignancy. Subcutaneous implantation of malignant keratinocyte cell lines (HaCaT) caused the formation of invasive malignant tumors correlating with high expression levels of VEGF-A and VEGFR-2. Inhibition of VEGF-A function by blocking antibodies to VEGFR-2 reduced tumor angiogenesis and resulted in loss of progression to tumor malignancy (14). On the basis of these findings and those reported herein, we conclude that VEGF-A-mediated angiogenesis is required but not sufficient for the progression to tumor malignancy and metastatic dissemination.

The lack of tumor metastasis in Rip1Tag2/Rip1VEGF-A double-transgenic mice is unlikely attributable to a general inability of Rip1Tag2 transgenic mice to develop metastases. For example, within a comparable time frame, forced expression of VEGF-C in pancreatic β cells and with it induction of peritumoral lymphangiogenesis was sufficient to promote the formation of metastases in regional lymph nodes of Rip1VEGF-C/Rip1Tag2 double-transgenic mice (25). These results not only reveal a striking difference in the capability of VEGF-A165 and VEGF-C to induce tumor metastasis but also raise the possibility that lymphangiogenesis and blood vessel angiogenesis may exhibit fundamental qualitative differences, *e.g.,* in vessel permeability and in fluid flow or pressure, that may explain the differences in their role in tumor metastasis. The ability of other isoforms of VEGF-A or other VEGF family members to modulate tumor metastasis in Rip1Tag2 mice remains to be determined.

We have also reported previously that interference with the function of the epithelial cell-cell adhesion molecule E-cadherin in Rip1Tag2 β-cell tumors accelerated the transition from adenoma to carcinoma and induced the formation of tumor metastasis (20). Moreover, genetic ablation of the function of the neural cell adhesion molecule during Rip1Tag2 tumorigenesis resulted in the formation of tumor metastases predominantly to the local pancreatic lymph nodes (30). These results indicate that, at least in Rip1Tag2 transgenic mice, changes in the tumor cells themselves, such as the loss of epithelial properties and the gain of invasive potential, may be causative in progression to tumor malignancy and metastasis, and that angiogenesis does not play a causal role.

It is well established that inhibition of VEGF function will interfere with tumor angiogenesis (10–12), and our results indicate that VEGF-A-induced tumor angiogenesis is not sufficient to induce tumor metastasis. However, the question remains whether inhibition of VEGF function will also repress the metastatic dissemination of tumor cells. It is conceivable, for example, that small tumors, which after antiangiogenic therapy have regressed and cuff as dormant tumors around major vessels (31), may still exhibit a malignant, invasive phenotype and disseminate to form metastases. The design of future therapeutic approaches will depend highly on results from experiments addressing these questions.

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