Expression of Indoleamine 2,3-Dioxygenase in Tumor Endothelial Cells Correlates with Long-term Survival of Patients with Renal Cell Carcinoma

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Abstract

Purpose: The inflammatory enzyme indoleamine 2,3-dioxygenase (IDO) participates in immune tolerance and tumor immune escape processes by degradation of the essential amino acid tryptophan and formation of toxic catabolites. Here, we analyzed the role of IDO in tumor growth and disease progression in patients with clear cell renal cell carcinoma (RCC).

Experimental Design: Expression of IDO mRNA was analyzed by quantitative reverse transcription-PCR in 55 primary and 52 metastatic RCC, along with 32 normal kidneys. Western blot and immunohistochemistry analyses were used to semiquantitatively determine IDO proteins in a subset of tumor samples, in RCC cell lines, and microvessel endothelial cells. IDO expression was correlated with expression of the proliferation marker Ki67 in tumor cells and survival of patients with tumor.

Results: More than 75% of the clear cell RCC in comparison to normal kidney contained elevated levels of IDO mRNA, which correlated with their IDO protein content. Low IDO mRNA levels in primary tumors represented an unfavorable independent prognostic factor (hazard ratio, 3.8; \( P = 0.016 \)). Unexpectedly, immunohistochemical analyses revealed that IDO is nearly exclusively expressed in endothelial cells of newly formed blood vessels and is virtually absent from tumor cells, although RCC cells could principally synthesize IDO as shown by \textit{in vitro} stimulation with IFN-\( \gamma \). A highly significant inverse correlation between the density of IDO-positive microvessels and the content of proliferating Ki67-positive tumor cells in primary and metastatic clear cell RCC was found (\( P = 0.004 \)).

Conclusions: IDO in endothelial cells might limit the influx of tryptophan from the blood to the tumor or generate tumor-toxic metabolites, thus restricting tumor growth and contributing to survival.

Renal cell carcinoma (RCC) represents 3% of all tumors. In 2006, RCC was diagnosed in nearly 40,000 patients in the United States, and 13,000 died from this disease (1). At the time of diagnosis, 30% of the patients clinically present with metastases and an additional 30% of the patients will develop metastases during the subsequent course of their disease (2). Once metastasis has occurred, the prognosis of patients with RCC is very poor because RCC is largely refractory to radiation therapy and chemotherapy.

However, RCC represents one of the few immunogenic tumors (3). Consequently, the current first line therapy for patients with metastasized RCC involves nonspecific immunotherapy with interleukin-2 and/or IFN-\( \alpha \) cytokines with or without 5-fluorouracil treatment albeit with only 5% to 10% durable responses (4, 5). Immune escape mechanisms may be responsible for the limited success of immunotherapies in patients with RCC (6, 7).

Indoleamine 2,3-dioxygenase (IDO; EC 1.13.11.52) catalyzes the rate-limiting initial step in the catabolism of the essential amino acid L-tryptophan by formation of kynurenine derivatives. IDO is induced by IFN-\( \gamma \) in a variety of cells and seems to
To be a key player in the innate immune system mediating inhibition of intracellular pathogens by tryptophan depletion and/or generation of toxic metabolites (8, 9). Furthermore, antigen-presenting cells, like subsets of dendritic cells and macrophages, could mediate immunoregulatory functions via IDO through cell cycle arrest in T cells (10).

Overexpression of IDO in otherwise immunogenic murine tumor cells prevents rejection by T cells and treatment of tumor cells with IDO-specific small interfering RNA postponed tumor formation in mice suggesting an immunosuppressive function in tumors (11, 12). Cancer cells of a number of human malignancies constitutively express IDO (11). Therefore, IDO might also play a role in tumor immune escape in humans. Indeed, patients with IDO-positive ovarian, colorectal, endometrial, and esophageal tumors have a worse prognosis (13–16). Based on these observations, it has been suggested that IDO inhibitors should be used to support tumor therapies (17, 18). On the other hand, IDO has also been shown to correlate well with good prognosis in patients with hepatocellular carcinoma and non–small cell lung cancer tumors (19, 20). This has been attributed to the presence of IDO-positive tumor-infiltrating leukocytes which might express IDO due to the existence of a Th1 cytokine milieu in the tumor (20).

We reasoned that the immunogenic RCC tumors should especially benefit from the presence of IDO. Therefore, we investigated IDO expression in primary clear cell RCC tumors, clear cell RCC metastases from different organs, and normal tissue from tumor-bearing kidneys. Interestingly, patients with high IDO mRNA contents in their tumors exhibited longer survival. Surprisingly, immunohistochemical analyses revealed that IDO is nearly exclusively expressed in endothelial cells of predominantly newly formed blood vessels. The frequency of IDO-positive microvessels inversely correlated with the content of proliferating tumor cells in primary and metastatic clear cell RCC. Therefore, IDO in endothelial cells might limit the influx of the essential amino acid tryptophan from the blood to the tumor cells or generate tumor-toxic metabolites, thus restricting tumor growth.

### Materials and Methods

#### Patients

We retrospectively analyzed 55 unselected clear cell RCC primary tumors and 52 metastases samples from patients who underwent surgical tumor resection between 1990 and 2005, and were subsequently followed for a median of 26 months. Follow-up data were available for 52 patients with primary and 48 patients with metastatic RCC. For 10 patients (8 with follow-up), both primary and metastatic tumors were analyzed. Clinical and histopathologic data are summarized in Table 1.

#### Tissue samples

Clear cell RCC samples and tumor-free tissue from tumor-bearing kidneys were obtained from radical nephrectomies. Samples were snap-frozen within 30 min of surgery and stored in liquid nitrogen. Paraffin-embedded tumor tissues were retrieved from the archives of the Institute of Pathology, Ludwig-Maximilians-University Munich, Munich, Germany. For paraffin embedding, standard fixation and dehydration procedures were used. The study was approved by the local ethics committee; all patients gave written consent.

#### Cell culture

The cell lines A498 and HEK293 were obtained from the American Tissue Culture Collection. The cell line RCC26 was kindly provided by Schendel et al. (21). The cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1 mmol/L of sodium pyruvate, 4 mmol/L of L-glutamine, 100 μg/mL of streptomycin, 100 units/mL of penicillin (complete RPMI; all from Life Technologies). Primary human dermal microvessel endothelial cells (HDMEC; PromoCell) were grown for three passages in Endothelial Cell Growth Medium MV Cells (PromoCell).

#### Immunohistochemical procedures and estimation of IDO- and Ki67-positive cell density

Sections from paraffin wax–embedded tissue were deparaffinized in Roti-Histol (Carl Roth GmbH), rehydrated and microwaved for 30 min in 0.1% boric acid or Target Retrieval Solution at pH 9 (DakoCytomation). Cytocentrifuge preparations of HDMECs were obtained by sedimentation at 100 × g for 5 min using a Cytopsin 2 centrifuge (Shandon). Cells were fixed in acetone for 10 min at room temperature. Sections and cytocentrifuge cell preparations were treated with

### Table 1. Correlation of IDO expression in primary RCC and clinicopathologic variables

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients, no. (%)</th>
<th>High IDO mRNA expression, no. (%)*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases</td>
<td>55 (100)</td>
<td>10 (18)</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
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<tr>
<td>≤ 60</td>
<td>27 (49)</td>
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</tr>
<tr>
<td>&gt; 60</td>
<td>28 (51)</td>
<td></td>
<td></td>
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<tr>
<td>Gender</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33 (60)</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>Female</td>
<td>22 (40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1/2</td>
<td>22 (40)</td>
<td></td>
<td>0.15</td>
</tr>
<tr>
<td>T3/4</td>
<td>33 (60)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>41 (75)</td>
<td></td>
<td>0.21</td>
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</tr>
<tr>
<td>M0</td>
<td>34 (62)</td>
<td></td>
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<tr>
<td>M1</td>
<td>21 (38)</td>
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<td>Histologic grade</td>
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</tr>
<tr>
<td>G3</td>
<td>28 (51)</td>
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</table>

*Above the 80th percentile.
by standard procedures to block endogenous peroxidase activity. Slides were incubated for 20 min in PBS, containing 3% bovine serum albumin (Sigma-Aldrich) or 2.5% horse serum to reduce nonspecific binding prior to addition of monoclonal mouse antibodies against IDO (35 μg/mL, ref. 22), CEA and CEACAM1 (4/3/17, Genovac GmbH; 10 μg/mL), CD31 (clone IC70A; 17 μg/mL), CD45 (clone 2B11 and PD7/26; 7 μg/mL), Ki67 (clone MIB-1; 0.8 μg/mL), α-smooth muscle actin (clone HHF35, 1:200) all from DakoCytomation and CD34 (QBEnd/10, 8 μg/mL; Ventana Medical Systems S.A.), podoplanin (clone 18H5, 1:20; Acris). Immunohistochemical staining with the latter three antibodies was done using the Benchmark XT slide preparation system (Ventana Medical Systems S.A.) according to the manufacturer’s specifications. For all other stainings, antibodies diluted with PBS were incubated at room temperature for 1 to 2 h. Slides were rinsed thrice in PBS and subsequently incubated either with rabbit anti-mouse immunoglobulin conjugated with horseradish peroxidase (1:100; DakoCytomation) as secondary antibody or ImmPRESS Reagent (Vector Laboratories) followed by staining with 0.03% diaminobenzidine (CD34, α-smooth muscle actin) or 3-amin-9-ethyl-carbazole (Sigma-Aldrich), 0.03% H2O2 in sodium acetate buffer (0.1 mol/L; pH 5.2) for 15 min. Slides were counterstained with hematoxylin. To estimate the density of IDO- and Ki67-expressing microvessels and tumor cells, respectively, at least 25 high-power magnification fields (×400) randomly distributed over the tumor area of the tissue sections were evaluated using an ocular grid.

Isolation of peripheral blood T cells. Peripheral blood mononuclear cells were separated by centrifugation through a Ficoll/Hypaque (Pharmacia) density gradient (density = 1.077 g/mL). Cells from the interphase of the gradient were harvested, washed twice with PBS and CD4+ and CD8+ T cells were positively selected by MACS sorting (Miltenyi Biotec).

Cocultures and cytokine stimulation. RCC cells at a density of 0.5 × 10^5 cells per well were cocultured for 3 days with 5 × 10^5 purified CD4+ and CD8+ T cells from a healthy donor in complete RPMI 1640 in 24-well plates. Every 3rd day, half of the medium was replaced with fresh medium. Cells were harvested and IDO mRNA expression was analyzed after removal of lymphocytes by MACS using anti-CD45 antibodies. Cytokine stimulation of RCC cells was done by growing subconfluent cultures in six-well plates with or without 50 ng/mL of IFN-γ (PeproTech) for 3 days. The reversibility of IFN-γ action was measured after incubation of the cells for 3 days with IFN-γ as above, replacement of the IFN-γ–containing medium with fresh medium (day 0) and retrieval of samples at the indicated time points for quantitative reverse transcription-PCR (RT-PCR) analyses.
RNA isolation and real-time RT-PCR. Total mRNA was isolated from 5 \times 10^5 cells, 10 to 20 10-μm tissue cryosections or 30 mg of tissue pulsedized under liquid nitrogen using the RNeasy Mini Kit (Qiagen). The RNA yield was quantified photometrically and the quality-assessed by capillary electrophoresis (Agilent 2100 Bioanalyzer and RNA 6000 Pico Kit, Agilent Technologies Deutschland). Total RNAs from human normal tissues were purchased from Chemicon International and BioCat GmbH. One microgram of total RNA (RNA integrity number > 5) was used for complementary DNAs (cDNA) syntheses by reverse transcription using the Reverse Transcription System (Promega). The RNA integrity number provided by the Agilent system allows a quantitative estimate of the RNA quality (23). cDNAs were amplified by quantitative PCR with the LightCycler 2.0 System (LightCycler FastStart DNA MasterFluorescent SYBR Green I Kit; Roche). Human β-actin cDNA was quantified using the LightCycler Primer Set (SearchLC Research). The RNA yield was quantified photometrically and the quality-assessed by capillary electrophoresis (Agilent 2100 Bioanalyzer and RNA 6000 Pico Kit, Agilent Technologies Deutschland). Total RNAs from human normal tissues were purchased from Chemicon International and BioCat GmbH. One microgram of total RNA (RNA integrity number > 5) was used for complementary DNAs (cDNA) syntheses by reverse transcription using the Reverse Transcription System (Promega). The RNA integrity number provided by the Agilent system allows a quantitative estimate of the RNA quality (23). cDNAs were amplified by quantitative PCR with the LightCycler 2.0 System (LightCycler FastStart DNA MasterFluorescent SYBR Green I Kit; Roche). Human β-actin cDNA was quantified using the LightCycler Primer Set (SearchLC Research). The relative amounts of IDO cDNA were determined from 1 to 20 of the reverse transcription reactions using the sense primer 5′-GGTCATGGAGATGTCCGTAA-3′ and the antisense primer 5′-ACCAA-TAGAGAGACCAGGAAGAA-3′ (11) and the following conditions: initial denaturation step, 95°C for 10 min; denaturation, 95°C for 10 s; annealing, 60°C for 10 s; extension, 72°C for 16 s; 35 cycles. We used the default setting of the LightCycler instrument to determine the crossing point (number of cycles needed to obtain a certain amount of PCR product). Only a crossing point of < 31 could be determined under these conditions. Smaller cDNA levels were set to 31 cycles resulting in normalized amounts of < 10 (see formula below). Relative amounts of β-actin and IDO cDNA were calculated as follows: relative amount of β-actin cDNA = 2^(ΔCt) β-actin \times 10^7; relative amount of IDO cDNA = 2^(ΔCt) IDO cDNA \times 10^7. The factors 10^7 and 10^4 were chosen arbitrarily. Analysis of the PCR products by agarose gel electrophoresis showed single DNA fragments with expected sizes. The relative inter assay standard deviation for the determination of the β-actin and IDO cDNA content by quantitative PCR using pooled primary RCC and normal kidney samples was 18% and 23%, respectively.

Western blot analyses. Cell and tissue lysates were generated from ≥1 \times 10^6 cells washed twice with PBS or 10 to 20 10-μm tissue cryosections using 100 μL of lysis buffer [50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 0.5% NP40, 0.1% SDS, and protease inhibitor cocktail tablets (Complete Mini, Roche Diagnostics GmbH)]. HEK293 cells (3–4 \times 10^5/well) were transiently transfected for 24 h with a human IDO expression plasmid (pCMV-SPORT6-IDO; RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH) in a six-well plate using FuGene6 Transfection Reagent (Roche Diagnostics GmbH). Thirty or 40 μg of total protein were size-fractionated by electrophoresis in 4% to 12% NuPage Novex Bis-Tris SDS polyacrylamide gels (Invitrogen GmbH) and transferred to a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences Europe GmbH). IDO was visualized by luminescence (ECL Kit, Amersham) using Hyperfilm ECL (Amersham) after incubation with anti-human IDO antibody (2 μg/mL) and horseradish peroxidase–coupled sheep anti-mouse IgG. β-Actin was detected after removal of the IDO antibody by incubation with 100 mmol/L of 2-mercaptoethanol, 2% SDS, 62.5 mmol/L of Tris-Cl (pH 6.7) for 30 min at 60°C with a mouse anti-human β-actin monoclonal antibody (0.4 μg/mL; clone AC-74; Sigma-Aldrich) by luminescence as above.

Results

IDO mRNA and protein in primary RCC and metastases. To determine the content of IDO mRNA in 55 clear cell RCC primary tumors, 52 clear cell RCC metastases, and in 32 normal kidney samples, we used gene-specific quantitative RT-PCR. The IDO cDNA levels of the various tissues were normalized by their β-actin cDNA content. The mean β-actin cDNA content of tumor tissues was found to be about twice as high as that of normal renal tissues (Fig. 1A). Both primary and metastatic tumor tissues exhibited similar broad IDO mRNA expression levels covering three 10logs (Fig. 1B). On average, 24- and 32-fold higher IDO cDNA levels were measured in primary tumors and metastases, respectively, in comparison to tissue from normal kidneys. No significant differences were detected between primary tumors and metastases and for RCC metastases found in the adrenal, lymph node, and lung. No IDO mRNA or low levels were observed in liver, brain, and breast metastases (Fig. 1B). The mean of IDO mRNA levels in primary and metastatic RCC tissues is as high as that found for spleen. Maximal tumor IDO mRNA levels were higher as found in placental tissue where IDO is known to be involved in local immunoregulation, indicating that in some RCC tumors, IDO levels might be of functional relevance (Fig. 1B and C). IDO mRNA could also be detected in additional normal tissues (placenta > spleen > small intestine > lung > uterus > duodenum).

To investigate whether IDO transcripts are accompanied by the presence of IDO protein, primary RCC and RCC metastases from different organs were assessed by Western blot analyses using an IDO-specific monoclonal antibody (22). The antibody detected a 40 to 42 kDa peptide in HEK293 cells transiently transfected with an IDO expression vector consistent with the size of the IDO protein (Fig. 2). Bands of the same size were detected in extracts of primary and metastatic RCC tumor
tissues. Their intensity was found to correlate with the corresponding IDO mRNA levels (Fig. 2).

**Tumor endothelial cells are responsible for IDO expression in RCC.** Immunohistochemistry with the IDO-specific monoclonal antibody was used to identify the cell population responsible for IDO production. Surprisingly, endothelial cells within the tumor tissues represented the most prominent IDO-positive cell population in both primary RCC (7 out of 17) and

Fig. 3. IDO is mainly expressed in endothelial cells in RCC primary tumors and metastases. Sections from paraffin-embedded tissues from primary RCC (A-G, I, J, P-S), a lung metastasis (H) and lymph node RCC metastases from two different patients (K-O) were incubated with anti-IDO (A, D, H, I, K, M, P, R), anti-CD34 (B, G, O), anti-CEACAM1 (C, E, N), anti-α-smooth muscle actin (α-actin; F), or anti-Ki67 antibodies (L, O, S) followed by a peroxidase-labeled anti-mouse immunoglobulin antibody and staining with 3-amino-9-ethyl-carbazole (red; A, C, D, E, H-N, P-S) and diaminobenzidine (brown; B, F, G, O), respectively. The tissue sections were counterstained with Mayer’s hemalum. IDO expression is observed in regional clusters of endothelial cells of small and large vessels in primary and metastatic RCC (A, D, and H; filled arrowheads and inset in A). Not all blood vessels which can be identified by the pan-endothelial cell marker CD34 in parallel sections expressed IDO (open arrowheads in A, compare with B and G). In parallel sections, newly formed and mature vessels were identified by detection of CEACAM1 and α-smooth muscle actin, respectively (filled arrowheads in E). Endothelial cells of large vessels do not express CEACAM1 (C and E; open arrowheads). A subset of focally infiltrating leukocytes, identified by staining for the leukocyte marker CD45, at the tumor periphery expresses IDO (I and J). Tumor cells and not endothelial cells at the periphery of two RCC lymph node metastases express IDO (K and M; arrows and insets). In one case, the IDO-positive tumor cells are next to lymphatic tissue with germinal centers containing a large fraction of proliferative B cells identified by the proliferation marker Ki67 (K and L). IDO-positive tumor cells lie next to the microvessels surrounding the tumor nodules (M-O). Inverse expression of IDO (P and R) and Ki67 (Q and S) in parallel sections of primary RCC. Note the presence of only a few Ki67-positive nuclei (arrow) in the IDO-positive tumor. gc, germinal center; li, leukocyte infiltrate; s, stromal septum; t, tumor. Bars, 200 μm (A-H, K-S), 100 μm (I and J; inset in A), and 50 μm (insets in K and M).
RCC metastases from the adrenal gland (1 of 1), lymph node (1 of 5), and lung (3 of 4). Their identity was proven by staining with anti-CD31/PECAM1 and anti-CD34 monoclonal antibodies which specifically detect endothelial cells in RCC (Fig. 3A and B; data not shown). The absence of staining with an antipodoplanin monoclonal antibody ruled out a lymphatic origin of these vessels (data not shown; ref. 24). Both small and large blood vessels were IDO-positive (Fig. 3A and D). However, IDO-positive blood vessels, mostly a minority of all tumor blood vessels, tended to cluster in certain regions of the tumors. These regions were often separated by strands of stromal tissue rich in collagen and other extracellular matrix proteins as revealed by Elastica van Gieson staining or at the tumor periphery with no evidence of preferential CD45-positive leukocyte infiltration (Fig. 3A; data not shown). In general, tumors with IDO-positive endothelial cells exhibited a high microvascular density (data not shown). In addition to the focal staining of vascular endothelial cells, occasionally, small fractions of CD43-positive leukocyte infiltrates at the periphery of tumors stained positive for IDO (Fig. 3I and J). With the exception of lymph node metastases (see below), no IDO-positive tumor cells were detected within primary and metastatic tumor samples. No IDO-positive cells could be identified in nontumorous adjacent renal tissue (data not shown).

To further characterize the IDO-positive blood vessels, we used CEACAM1 and α-smooth muscle actin as additional markers which allows the discrimination of endothelia, newly formed by neoangiogenesis, from mature blood vessels (25, 26). The CEACAM1 antibody used in this study (4/3/17) is specific for CEACAM1 in the absence of the closely related CEACAM5/CEA, which is not present in kidney and RCC tissues (27). Interestingly, CEACAM1 showed the most similar expression pattern in comparison to that found for IDO in being also restricted to microvessels of certain areas of the tumor. However, CEACAM1 expression was observed in endothelia of additional tumor areas which were negative for IDO. IDO-positive endothelial cells were almost always positive for CEACAM1 with the exception of few larger mature vessels which did not express CEACAM1. The latter could be shown to represent more mature, stabilized vessels which can be identified by the presence of vessel-covering pericytes using their α-smooth muscle actin content as a marker (Fig. 3D-G; refs. 26, 28). CEACAM1 has been reported to be predominantly expressed in newly formed tumor endothelial cells but also in microvessels of the normal kidney (27, 29).

We noted that out of a total of 27 RCC tumors, two lymph node metastases (out of five analyzed) contained a small fraction of IDO-positive tumor cells, mostly at the tumor margins. There, the tumor cells were in close contact with lymphoid tissue with highly proliferative lymphocytes in germinal centers, as shown by Ki67 staining (see arrows and insets in Fig. 3K and L), and in the neighborhood of newly formed microvessels visualized by staining with anti-CEACAM1.
Antibodies (Fig. 3N and O). In the areas with IDO-positive tumor cells, no IDO-positive endothelial cells could be identified but elsewhere in one of the two lymph node metastases some endothelial cells were IDO-positive but associated tumor cells were not (Fig. 3M and O; data not shown).

We hypothesized that the tryptophan-degrading enzyme IDO in endothelial cells might reduce the influx of the essential amino acid tryptophan into the surrounding tumor tissue and thus diminishing protein synthesis possibly coupled with a decrease in tumor cell proliferation. To test this hypothesis, we identified proliferating tumor cells positive for the proliferation marker Ki67 and, in adjacent sections, the density of IDO-positive blood vessels in the tumor. A highly significant inverse correlation between the mean numbers of IDO-positive microvessels and Ki67-positive tumor cells per high-power magnification field measured across the tumors was noted (Chi-square test; \( P = 0.004 \); Figs. 3P-S and 4).

**IDO mRNA levels in primary and metastatic RCC correlate with longer survival.** Because ~25% of the patients with RCC showed IDO mRNA levels as low as that found in normal kidney, and another 25% of the patients exhibited IDO mRNA levels higher than those of normal spleen (Fig. 1), we wanted to know whether patients with low and high IDO mRNA contents differed in the progression of their disease. We analyzed overall survival in relation to IDO mRNA levels of primary tumors and metastases. Surprisingly, patients with a high IDO mRNA content in either their primary tumors or metastases exhibited a trend of longer overall survival in comparison to patients with lower levels. Various cutoff values of IDO mRNA levels have been tested to separate patients with different disease outcomes.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Categories</th>
<th>Hazard ratio (confidence interval)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDO mRNA in primary tumors (50th percentile)</td>
<td>Age (y) ( \leq 60 )</td>
<td>1.5 (0.7-3.2)</td>
<td>0.281</td>
</tr>
<tr>
<td>IDO mRNA in primary tumors (50th percentile)</td>
<td>Age (y) &gt;60</td>
<td>1.1 (0.5-2.4)</td>
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<td>Gender Male</td>
<td>1.2 (0.5-2.9)</td>
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<tr>
<td>IDO mRNA in primary tumors (50th percentile)</td>
<td>Gender Female</td>
<td>1.1 (0.4-3.2)</td>
<td>0.795</td>
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<tr>
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<td>Tumor stage ( T_{1/2} )</td>
<td>6.0 (2.3-15.7)</td>
<td>0.0003</td>
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<td>IDO mRNA in primary tumors (50th percentile)</td>
<td>Tumor stage ( T_{2/4} )</td>
<td>3.7 (1.5-8.9)</td>
<td>0.004</td>
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<tr>
<td>IDO mRNA in primary tumors (50th percentile)</td>
<td>Lymph node metastasis Yes</td>
<td>2.2 (1.0-4.9)</td>
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<td>IDO mRNA in primary tumors (50th percentile)</td>
<td>Lymph node metastasis No</td>
<td>1.4 (0.7-3.1)</td>
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<td>IDO mRNA in primary tumors (50th percentile)</td>
<td>Metastasis Yes</td>
<td>1.6 (0.7-3.7)</td>
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<td>IDO mRNA in primary tumors (50th percentile)</td>
<td>Metastasis No</td>
<td>1.2 (0.5-2.7)</td>
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<tr>
<td>IDO mRNA in primary tumors (50th percentile)</td>
<td>Histologic grade ( G_{1/2} )</td>
<td>1.5 (0.6-3.9)</td>
<td>0.738</td>
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<td>IDO mRNA in primary tumors (50th percentile)</td>
<td>Histologic grade ( G_{3} )</td>
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<td>IDO mRNA in primary tumors (50th percentile)</td>
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<tr>
<td>IDO mRNA in primary tumors (50th percentile)</td>
<td>IDO mRNA High</td>
<td>3.8 (1.3-11.4)</td>
<td>0.016</td>
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</table>

When increasing the cutoff levels, the discrimination between the two prognosis groups improved. The 80th percentile was the highest tested to restrict group size imbalance. At this level, \( P \) values of 0.09 and 0.22 were obtained for primary RCC and RCC metastases, respectively (Fig. 5A and B). A similar relation between cutoff percentile and \( P \) value was found when performing multivariate analysis (Cox proportional hazard regression model). Patients with IDO mRNA levels below the 50th percentile had a hazard ratio of 2.2 \( (P = 0.057) \); below the 80th percentile, a hazard ratio of 3.8 \( (P = 0.016) \). IDO mRNA levels below the 80th percentile represent an independent prognostic factor (Table 2).

**Induction of IDO by IFN-\( \gamma \) and activated lymphocytes.** Because endothelial and, in rare circumstances, tumor cells express IDO only in certain areas of RCC tumors, we wanted to know which factors could modulate IDO expression in these cells. Cytokines, like the Th1 cytokine IFN-\( \gamma \), could be produced by, e.g., activated tumor-infiltrating lymphocytes and are able to activate a number of genes. Therefore, we analyzed the capability of IFN-\( \gamma \) to induce IDO in primary HDMEC. Indeed, in the presence of IFN-\( \gamma \) for 2.5 days, the production of IDO protein and IDO mRNA was strongly induced in endothelial cells as shown by quantitative RT-PCR and Western blot analysis, respectively (Fig. 6A and C). Immunocytologic analyses indicate that most endothelial cells are capable of synthesizing IDO (Fig. 6B). To further clarify the unexpected results that in most primary tumors and metastases endothelial cells, rather than tumor cells or tumor-infiltrating leukocytes, express IDO as found for other tumors (13–15, 19, 20), we investigated whether RCC cells are in principle capable of
synthesizing IDO. We analyzed two RCC cell lines, RCC26 and A498, for the presence of IDO mRNA by quantitative RT-PCR and IDO protein by Western blot analysis. No IDO transcripts or IDO protein could be identified in these RCC cell lines. However, 3 days after the addition of IFN-γ, IDO mRNA and IDO protein could be clearly detected in both RCC cell lines at a level similar to that found in IFN-γ–stimulated endothelial cells (Fig. 6A, C, and D, inset). The induction of IDO mRNA was reversible. Three days after the removal of IFN-γ, baseline levels of IDO mRNA were observed in A498 RCC cells (Fig. 6D). Furthermore, the addition of allogeneic CD4+ and CD8+ T cells to both RCC cell lines also induced the expression of the IDO gene, suggesting the possible in vivo induction of IDO mRNA upon attack of tumor cells by tumor-specific cytotoxic T cells or other immune cells (Fig. 6E).

Discussion

Tumors derived from a wide spectrum of tissues and organs have been described to express the tryptophan-catabolizing enzyme IDO (11, 13–15, 19, 20). Previously, either the tumor cells or infiltrating leukocytes were found to be responsible for IDO expression in tumors. In this report, we describe for the first time that the tumor vasculature could be the main source of IDO in tumors.

More than 75% of the analyzed RCC tissues exhibited IDO mRNA levels higher than that found in normal kidney (Fig. 1). The presence of IDO in both primary tumors and metastases of patients with RCC indicates that IDO expression in the tumor vasculature is a stable property of the tumor and is maintained upon metastatic spread. At present, it is unclear how IDO expression is induced in tumor blood vessels. IFN-γ has been identified as the most potent inducer, but other cytokines and inflammatory stimuli like IFN-α, IFN-β, and Toll-like receptor ligands, although to a lesser degree, could also stimulate IDO formation (30). Modulation of IDO expression by interleukin-4, interleukin-6, interleukin-10, transforming growth factor-β, and prostaglandin E2 has also been noticed (31). In mice, systemic expression of IDO in vascular endothelial cells following experimental infection with malaria pathogens has been described. In this system, IFN-γ seems to constitute an indispensable mediator as no induction was observed in IFN-γ–/- mice (32). Thus, two main scenarios involving the active role of the tumor or the host can be envisioned. First, tumor cells directly induce IDO expression in tumor endothelial cells. The factors involved might be connected with
IDO Expression in Endothelial Cells of RCC

neangiogenesis because IDO expression is preferentially found in the periphery of tumors where neoangiogenesis is favored (33). Furthermore, in RCC, the expression domain of IDO in endothelial cells is largely overlapping, although more restricted, with that of CEACAM1 which has been shown to be involved in tumor neoangiogenesis (29). Second, tumor-infiltrating activated leukocytes could create a cytokine milieu that allows the expression of IDO in tumor microvessels. Indeed, large numbers of both natural killer cells and cytotoxic T cells can be found in RCC known for its immunogenicity (3, 34). However, no positive correlation between the number of infiltrating CD45-positive leukocytes and IDO expression in tumor endothelial cells was noted in the analyzed tumor samples possibly due to lack of activation of the leukocytes.

RCC tumor cells do not constitutively express IDO as it is found for other tumors (11, 13–15). Possibly, malignancy-associated genetic changes differing among tumor types, like inactivation of the IDO-regulating tumor suppressor Bin1, might be involved in differential expression of IDO in tumors (35). However, induction of IDO in RCC tumor cells is principally possible both in vitro and in vivo as shown by in vitro stimulation of IDO through IFN-γ or activated T cells and the observation of IDO-expressing tumor cells in RCC lymph node metastases (Fig. 3). The observed restriction of IDO-positive RCC tumor cells to lymph node metastases hints towards the involvement of activated immune cells in the induction of the IDO gene in RCC cells. Furthermore, because the induction of IDO expression is rapidly reversible (Fig. 6D), continued stimulation needed for expression might only be possible in the neighborhood of massive accumulation of activated lymphocytes as that found in lymph nodes.

The amount of IDO mRNA in RCC primary and metastatic tumors is comparable to that found in tissues in which functional significance has been shown (Fig. 1; placenta, spleen; refs. 10, 36). There is a close correlation between IDO mRNA and protein levels (Fig. 2). Furthermore, the presence of IDO protein–positive microvessels in primary and metastatic RCC could be clearly shown in more than one-third of the tumors (immunohistochemical score “high expression” in 10 out of 27 analyzed tumors; Fig. 4). Significantly, the observed strong inverse correlation between the density of IDO-expressing microvessels in the tumor and the number of proliferating Ki67-positive tumor cells suggests that IDO in endothelial cells inhibits the proliferation of RCC cells by deprivation of the essential amino acid tryptophan, or by the formation of tumoricidal tryptophan metabolites. This could lead to prolonged survival of patients with IDO-positive RCC tumors as indicated by the survival data presented in this study (Fig. 5; Table 2). Indeed, Ki67 is a prognostic marker for patients with RCC, and low labeling indices of tumor cell nuclei with anti-Ki67 antibodies correlate with longer survival (37). This interpretation could also explain the observation that in RCC, high microvessel (differentiated) density, which seems to be a prerequisite for vascular IDO expression (see Results) significantly correlates with lower tumor grade and longer patient survival (38).

At present, it is not clear whether RCC tumor cell proliferation could indeed be inhibited by low tryptophan concentrations or kynurenine pathway metabolites as found for T cells and intracellular pathogens (39). Furthermore, it still has to be shown that expression of IDO in endothelial cells indeed limits the access of tryptophan to the surrounding tissue. This hypothesis could be validated in a murine model in which (tumor) endothelial cells by intravenous application of IDO-inducing factors or genetic engineering can be induced to constitutively or conditionally produce IDO.

Based on the role of IDO in immune tolerance induction in tumor cells as well as in dendritic cells in tumor-draining lymph nodes, the use of small molecule IDO inhibitors, like 1-methyl-tryptophan, has been suggested for the treatment of cancer patients. Indeed, IDO inhibitors have been shown to enhance the chemotherapy of tumors in murine models (17, 18). Our study indicates that the role of IDO in tumor-host interactions seems to be more complex than anticipated. In certain tumors, the expression of IDO might even be beneficial for the patient by mediating the suppression of tumor growth. Therefore, selective enhancement of IDO expression in endothelial cells, but not in tumor cells, in patients with RCC or induction of de novo synthesis in endothelial cells of other tumors might represent a new therapeutic approach.

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Expression of Indoleamine 2,3-Dioxygenase in Tumor Endothelial Cells Correlates with Long-term Survival of Patients with Renal Cell Carcinoma

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