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Prognostic Value of Indoleamine 2,3-Dioxygenase Expression in Colorectal Cancer: Effect on Tumor-Infiltrating T Cells

Gerald Brandacher,[1] Alexander Perathoner,[1,5] Ruth Ladurner,[1] Stefan Schneeberger,[1] Peter Obrist,[6] Christiana Winkler,[3,4] Ernst R. Werner,[3] Gabriele Werner-Felmayer,[3] Helmut G. Weiss,[1] Georg Göbel,[2] Raimund Margreiter,[1,5] Alfred Königsrainer,[1] Dietmar Fuchs,[3,4] and Albert Amberger[5]

Abstract

Purpose: The pathologic interactions between tumor and host immune cells within the tumor microenvironment create an immunosuppressive network that promotes tumor growth and protects the tumor from immune attack. In this study, we examined the contribution of the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) on this phenomenon.

Experimental Design: Expression of IDO was analyzed in colorectal cancer cell lines by reverse transcription-PCR and functional enzyme activity was assessed by high-pressure liquid chromatography. Semiquantitative immunohistochemistry was used to evaluate IDO expression in the tissue samples of 143 patients with colorectal carcinoma, and was then correlated with the number of tumor-infiltrating T cells and clinical variables.

Results: *In vitro* IDO expression and functional enzyme activity in colorectal cancer cells was found to be strictly dependent on IFN-γ stimulation. Immunohistochemical scores revealed IDO-high expression in 56 of 143 (39.2%) tumor specimens, whereas 87 of 143 (60.8%) cases showed low IDO expression levels. IDO-high expression was associated with a significant reduction of CD3+ infiltrating T cells (46.02 ± 7.25) as compared with tissue samples expressing low IDO (19.42 ± 2.50; $P = 0.0003$). Furthermore, IDO-high immunoreactivity significantly correlated with the frequency of liver metastases ($P = 0.003$). Kaplan-Meier analysis showed the crossing of survival curves at 45 months. By multivariate Cox's analysis, IDO-high expression emerged as an independent prognostic variable (<45 months, $P = 0.006$; >45 months, $P = 0.04$).

Conclusion: IDO-high expression by colorectal tumor cells enables certain cancer subsets to initially avoid immune attack and defeat the invasion of T cells via local tryptophan depletion and the production of proapoptotic tryptophan catabolites. Thus, IDO significantly contributes to disease progression and overall survival in patients with colorectal cancer.

Colorectal cancer is the most common gastrointestinal malignancy and one of the leading causes of cancer-related deaths worldwide (1). Five-year overall survival rates range from 90% for stage I to 75% and 50% for stage II and III patients, respectively (1). There are several factors, such as tumor invasion or frequency of lymph node or distant metastasis that limit the prognosis of the disease. Recent studies have elucidated some of the underlying molecular mechanisms contributing to tumor progression. Therefore, the ability of certain tumors to actively create a state of immunologic tolerance towards tumor-associated antigens seem to be of particular clinical relevance (2). Tumor antigen–specific immune tolerance is initiated by a constitutive interaction between tumors and the patients' immune system, and is controlled by various modifications to the immune response present in the tumor environment (3). Nevertheless, the exact mechanisms by which such unresponsiveness to malignant cells is generated or maintained are not fully understood. The first evidence for a tumoral immune resistance mechanism based on tryptophan degradation was provided by Uyttenhove et al. in a murine model, in which they showed that the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) reduces antitumoral T cell attack (4).

IDO is widely distributed in mammals and is inducible preferentially by IFN-γ. IDO degrades the essential amino acid tryptophan to form *N*-formyl kynurenine, which, depending on cell type and enzymatic repertoires, is subsequently

Authors' Affiliations: [1]Department of General and Transplant Surgery, [2]Department of Medical Statistics, Informatics, and Health Economics, and [3]Division of Biological Chemistry, Biocenter, Innsbruck Medical University; [4]Ludwig Boltzmann Institute of AIDS Research; [5]Tyrolean Cancer Research Institute, Innsbruck; and [6]Institute of Pathology, Wagner-Jauregg Hospital, Linz, Austria

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Note: G. Brandacher and A. Perathoner contributed equally to this study.

R. Ladurner and A. Königsrainer are currently at the Department of General and Transplant Surgery, Medical University of Tübingen, Tübingen, Germany.

Requests for reprints: Gerald Brandacher, Department of General and Transplant Surgery, Innsbruck Medical University, Anichstrasse 35, A-6020 Innsbruck, Austria. Phone: 43-512-5042-2603; Fax: 43-512-5042-2605; E-mail: gerald.brandacher@uibk.ac.at.

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converted to finally form niacin (5). More recently, it has been proposed that activation of IDO is also critically involved in the regulation of immune responses, to establish immune tolerance in pregnant mice on their fetuses, or to induce T cell unresponsiveness (6, 7). Cell proliferation of alloreactive T cells is thereby arrested in the G_1 phase of the cell cycle via local tryptophan deprivation and the accumulation of toxic, proapoptotic catabolites (8).

Recently, functional inactivation of tumor-reactive T cells was analyzed as another important mechanism of tumor immune evasion (9). In addition, there is accumulating evidence showing a positive correlation between the number of tumor-infiltrating lymphocytes (TIL) and increased patient survival in breast (10), ovarian (11), prostate (12), and colorectal carcinomas (13). These studies showed that TILs in direct contact with tumor cells recognize tumor antigens, undergo clonal expansion, display tumor-specific cytolytic activity, and that their presence correlates with clinical outcome (10). Whether IDO-mediated tryptophan depletion is able to affect the quantity of local TILs is currently unknown.

Because the biological role, prognostic value, and clinicopathologic utility of IDO in colorectal cancer have not been addressed thus far, we investigated intratumoral IDO expression in 143 patients with colorectal cancer. Furthermore, we tested the hypothesis that the level of IDO expression correlates with the presence of TILs and thereby may contribute to disease progression and clinical outcome.

Table 1. Clinical and histopathologic patient data

Variables	Number of cases (%)
Number of patients	143 (100)
Age (y)	
≤60	43 (30.1)
>60	100 (69.9)
Gender	
Male	76 (53.1)
Female	67 (46.9)
Tumor stage	
1	29 (20.3)
2	24 (16.8)
3	78 (54.5)
4	12 (8.4)
Nodal status	
N0	83 (58.0)
N1-3	60 (42.0)
Histologic grade	
I	32 (22.4)
II	98 (68.5)
III	13 (9.1)
Tumor size (cm)	
<5	72 (50.3)
≥5	59 (41.3)
Missing	12 (8.4)
Site of tumor	
Caecum	16 (11.2)
Ascending colon	12 (8.4)
Transverse colon	12 (8.4)
Descending colon	4 (2.8)
Sigmoid colon	52 (36.4)
Rectum	42 (29.4)
Missing	5 (3.5)
Liver metastases	
M0	90 (62.9)
M1	53 (37.1)

Materials and Methods

Patients. We retrospectively analyzed 143 nonconsecutive and unselected tumor samples of patients who underwent surgical resection of the primary colorectal carcinoma between March 1994 and June 2002 which were subsequently followed for a mean (±SD) of 51.8 ± 31.5 months. Basic patient demographic data are summarized in Table 1.

Chemicals. Unless otherwise indicated, all reagents were purchased from Sigma (Vienna, Austria). Cell culture reagents were obtained from PAA Laboratories (Linz, Austria). IFN-γ was purchased from R&D Systems (Wiesbaden, Germany).

Cell lines and culture. *In vitro* experiments were conducted with different human colon cancer cell lines, HRT-18, HCT-15, and Caco-2, which were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 supplemented with antibiotics and 10% FCS according to standard procedures. In order to estimate IDO expression, 7×10^5 cells were seeded into cell culture flasks (Greiner, Frickenhausen, Germany) and cultured for 24 hours. Medium was then removed and cells were grown in medium with or without IFN-γ (750 units/mL) for 48 hours.

Measurement of IDO activity in cultured cells. Tryptophan and kynurenine concentrations were analyzed in cell culture supernatants by reversed-phase high-pressure liquid chromatography following precipitation of protein with trichloroacetic acid. Tryptophan was measured by its native fluorescence detection at 285 nm excitation wavelength and 365 nm emission wavelength (14). Kynurenine was detected by UV absorption at the 360 nm wavelength in the same chromatographic run. Finally, the kynurenine to tryptophan ratio (kyn/trp) was calculated by dividing kynurenine concentrations (nmol/L) by tryptophan concentrations (nmol/L).

Reverse transcription-PCR. To examine IDO transcription in human colon cancer cell lines, total RNA was isolated using TRI-Reagent (MBI, Minneapolis, MN) and reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). Thirty cycles of PCR were done using Taq polymerase (Promega) and specific primer pairs for IDO designed from the published sequence in the National Center for Biotechnology Information database (accession no., NM_002164) forward primer 5′-GGCAAAGGTCATGGAGATGT-3′, reverse primer 5′-GCTTGCAGGAATCAGGATGT-3′. Reverse transcription-PCR of glyceraldehyde-3 phosphodehydrogenase was used as an invariant endogenous control. PCR products were visualized under UV light after gel electrophoresis in 2% agarose containing ethidium bromide.

Immunohistochemistry. Immunostaining was done on paraffin-embedded sections (4-6 μm) and cytospins of tumor cell lines were fixed in 10% formalin in PBS: after deparaffinization and rehydration, the sections were treated with 0.3% hydrogen peroxide (and incubated with 10% bovine serum albumin) to block nonspecific staining. Incubation with proteinase for 15 minutes at 37°C was used for antigen-retrieval on the IDO tissue sections, whereas the IDO CD3 costaining sections were boiled (86°C) for 15 minutes in 1 mmol/L EDTA solution (pH 8.0) in a water bath. The primary antibodies were rabbit anti-IDO polyclonal antibodies (AB5968, Chemicon, Hampshire, United Kingdom) and were used at a dilution of 1:300. The sections were incubated with the antibody at 4°C overnight. After

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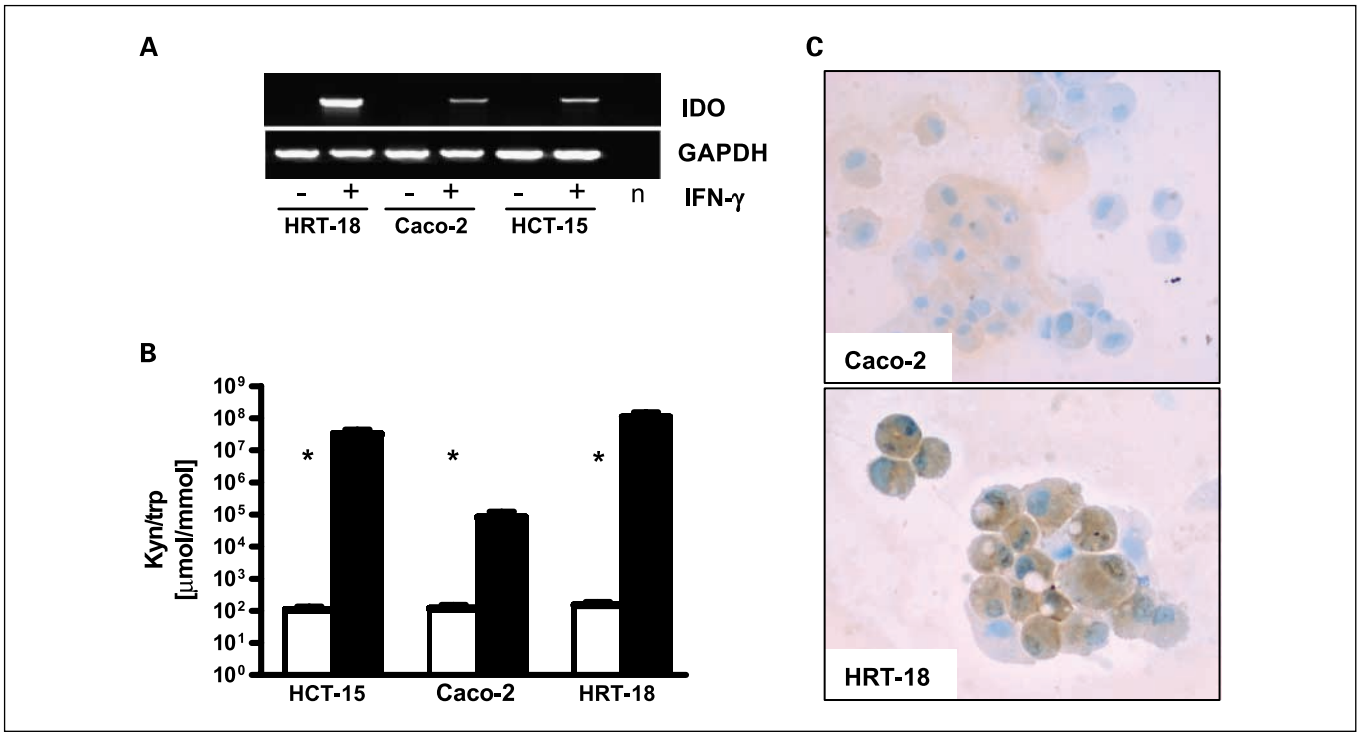

Fig. 1. *A,* IDO expression in colon carcinoma cell lines HRT-18, Caco-2, and HCT-15 with/without stimulation by IFN-γ assessed by reverse transcription-PCR. *B,* functional IDO enzyme activity in cell culture supernatants in terms of their ability to catabolize its substrate tryptophan to form kynurenine by means of high-pressure liquid chromatography. The kynurenine to tryptophan ratio (kyn/trp) is indicating IDO activity (*, $P < 0.001$). *C,* IFN-γ-treated CaCo cells with low IDO activity showing low immunostaining scores (*top*) and HRT-18 cells with highest IDO enzyme activity revealed IDO-high immunostaining scores (*bottom*). Spearman correlation coefficient: $r = 0.572$; $P < 0.01$ (original magnification, ×500).

washing in TBS, they were incubated with biotinylated swine anti-rabbit IgG (Dako, Copenhagen, Denmark) at a dilution of 1:500, and detected with an ABC peroxidase Kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine as a substrate.

Double immunohistochemistry for IDO and CD3. Double immunohistochemical analysis for the detection of CD3+ T cells on the IDO-stained tissue sections was done with mouse anti-CD3 monoclonal antibodies (Neomarkers, Fremont, CA). After washing in TBS, the antibody reaction was amplified with rabbit anti-mouse antibodies (Dako) and alkaline phosphatase anti-alkaline phosphatase mouse monoclonal antibodies (Dako; both 1:50) according to the alkaline phosphatase anti-alkaline phosphatase method, and detected with Fast Red as substrate. All tissue sections were washed in water, counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany), and coverslipped.

Quantification method. Immunoreactivity was semiquantitatively estimated as previously described (15). The total IDO immunostaining score was calculated as the sum of a proportion score and an intensity score. The proportion score reflects the estimated fraction of positively stained infiltrating cells (score 0, none; score 1, <10%; score 2, 10-50%; score 3, 51-80%; score 4, >80%). The intensity score represents the estimated staining intensity (score 0, no staining; score 1, weak; score 2, moderate; score 3, strong) giving a total score ranging from 0 to 12. We defined IDO overexpression as a total score >4.

An association between IDO expression patterns and the appearance of local tumor-infiltrating T cells was investigated by counting CD3+ cells manually in three to six high-power fields. Areas with the most abundant distribution were selected and intratumoral T cells were then graded, as +, ++, or +++ (0-19, 20-40, or >40 T cells per high-power field, respectively).

Statistics. Parametric distributed data are presented as mean ± SD, nonparametric data are presented as median ± range. The relationship between IDO-high expression and CD3 expression was tested by an unpaired Student's t test. The associations between IDO expression, tumor, and clinical variables were calculated with the χ^2 test. The primary end point in this study was overall survival. Thus, both univariate and multivariate methods for survival analysis were used. For univariate survival analysis, the log-rank test for censored survival data was used and the survival curves were calculated according to the Kaplan-Meier method. Follow-up time was censored if the patient was lost to follow-up.

For multivariate survival analyses, an extended (time-dependent) Cox proportional hazard regression model with constant—but different—hazard ratios of IDO expression within two time intervals (overall survival <45 and >45 months) was used. The time point of 45 months was used because of the crossing survival curves yielded by the univariate Kaplan-Meier analysis. The expression of IDO was adjusted for established prognostic variables of colorectal cancer (age, gender, histologic grade, tumor staging, nodal status, and frequency of liver metastases).

Statistical analyses were done with SPSS 10.0 for Windows (SPSS, Chicago, IL).

Results

IDO expression in colorectal cancer cell lines. We first tested whether different human colon cancer cell lines express IDO mRNA constitutively by means of reverse transcription-PCR. However, none of the tumor cell lines tested (HRT-18, HCT-15, and Caco-2) showed IDO mRNA expression constitutively without IFN-γ stimulation.

However, after stimulation with IFN-γ (750 units/mL) for 48 hours, all cell lines revealed a strong induction of IDO gene expression (Fig. 1A).

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IFN-γ stimulation and induction of IDO gene expression and enzyme activity. To confirm that IDO gene expression was associated with the presence of a functionally active protein, enzymatic assays by means of high-pressure liquid chromatography were used.

The activity of IDO from the different cell lines was analyzed in terms of their ability to catabolize tryptophan to form kynurenine.

Tryptophan was massively degraded leading to a significant increase in kynurenine concentrations, the first product of tryptophan catabolism, in IFN-γ-stimulated cells as compared with untreated cell lines (all $P \leq 0.001$). Kyn/trp (mean ± SD) in unstimulated HCT-15 cells was 112 ± 24 mmol/mol, in Caco-2 cells, it was 123 ± 31 mmol/mol, and in HRT-18 cells, it was 156 ± 34 mmol/mol. IFN-γ stimulation massively increased IDO enzyme activity resulting in kyn/trp of $86 \times 10^3 \pm 36 \times 10^3$ mmol/mol in Caco-2, $3.3 \times 10^7 \pm 1 \times 10^7$ mmol/mol in HCT-15, and $1.12 \times 10^8 \pm 3.98 \times 10^7$ mmol/mol in HRT-18 cells (Fig. 1B).

These results suggest that all tumor lines that expressed IDO upon stimulation with IFN-γ also contained functionally active IDO. In order to connect the functional activity of IDO to the level of IDO expression by immunohistochemistry, cytospins of CaCo-2 and HRT-18 cells were stained for IDO. The amount of IDO enzyme activity in IFN-γ-stimulated tumor cells thereby correlated significantly with immunostaining scores ($r = 0.572$, $P < 0.01$; Spearman rank correlation; Fig. 1C).

Immunostaining for IDO. Because colon tumor cell lines cultured *in vitro* may not be representative of the exact state of malignant cells *in vivo*, primary colorectal cancer specimens from all 143 patients were stained for IDO by immunohistochemistry, and then scored and analyzed. Liver metastases were available for staining and analysis in 53 of 143 cases. In 31 patients, both primary tumors and corresponding synchronous ($n = 19$) and metachronous ($n = 12$) liver metastases were available.

Primum. IDO expressing tumor cells were found in all 143 of 143 cases of human colon carcinomas analyzed. By visual estimation, tumors were grouped into two categories, "IDO-high expression" and "IDO-low expression" according to a proportion and intensity score (see Materials and Methods; Fig. 2A and B). IDO was highly expressed in 56 of 143 (39.2%) tumor specimens, whereas 87 of 143 (60.8%) cases showed low IDO expression levels. By contrast, in normal

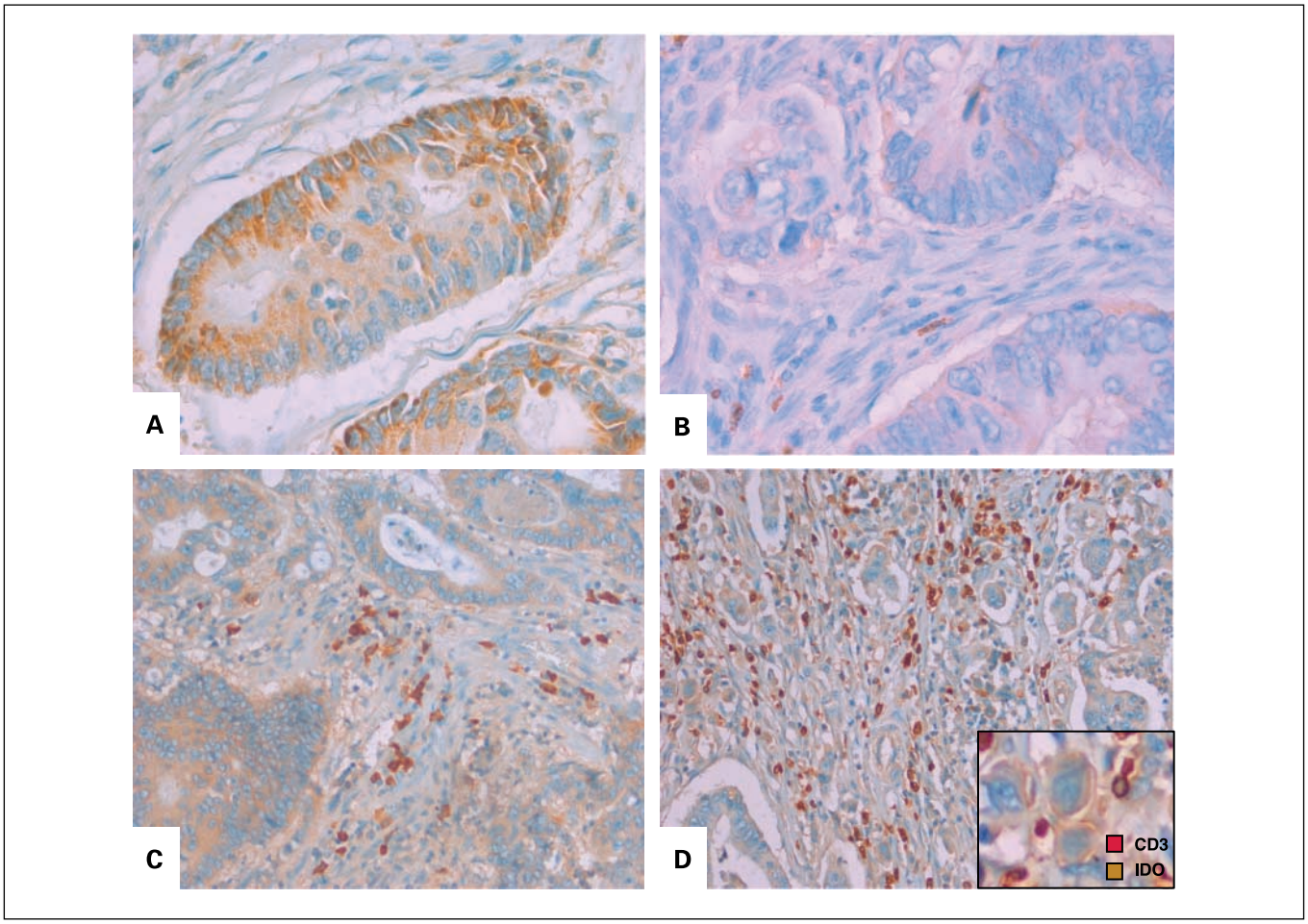

Fig. 2. IDO expression in colorectal carcinoma shown by immunohistochemistry. *A-D,* moderately differentiated adenocarcinomas of the colon characterized by invasive malignant glands surrounded by desmoplastic tumor stroma. Cytoplasmatic IDO-positive staining was found in the malignant gland cells, which was most pronounced at the luminal surface. *A,* example of IDO-high expression; *B*, IDO-low expression (*A* and *B*; original magnification, ×400). *C* and *D,* double immunostainings for IDO and CD3. IDO-high expressing tumors exhibited a significantly lower proportion of intratumoral CD3+ cells (*C*) as compared with IDO-low expressing tissue samples in (*D*). Inset, higher magnification of IDO-positive malignant cells and CD3+ cells along the invasive margin (*C* and *D*; original magnification, ×250).

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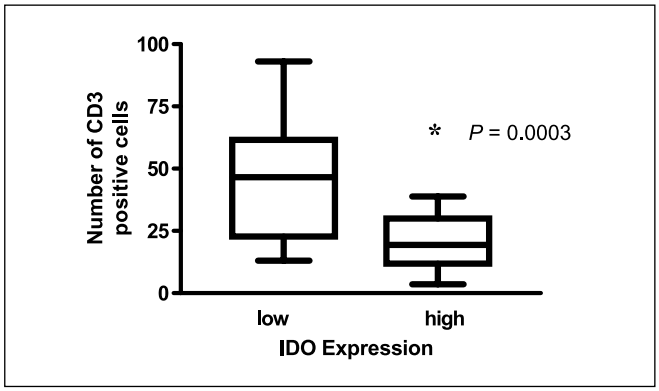

Fig. 3. IDO expression patterns in correlation with the number of tumor-infiltrating CD3+ T cells in human colorectal carcinoma in tumors with IDO-high (n = 20, total score >4; see text) or IDO-low (n = 11, total score <4; see text) expressing cancer cells.

nonmalignant colon tissue adjacent to cancers, IDO showed absent to weak staining patterns.

In addition, apart from tumor cells, we found a substantial number of IDO-positive cells within the tumor stroma classified as antigen-presenting cells such as macrophages and dendritic cells (data not shown).

Metastasis. In 31 patients, specimens from both primary tumors and respective synchronous (n = 19) or metachronous (n = 12) liver metastases were available. IDO was highly expressed in 20 of 31 (64.5%) of this subgroup of patients, whereas a total of 11 of 31 (35.5%) tissue samples showed low IDO expression patterns. Furthermore, expression of IDO was detected in all cases of metastases and showed the same expression score as the primary tumor.

Double immunohistochemistry for IDO and CD3. To determine whether IDO expression patterns have an effect on the presence of TILs, we did double immunostainings for IDO and CD3 in 31 CRC tissue samples (Fig. 2C and D). IDO expression thereby correlated inversely with the number of infiltrating T cells. IDO-low expressing tumors exhibited a significantly higher proportion of intratumoral CD3+ cells (mean ± SE, 46.02 ± 7.25) as compared with IDO-high expressing tissue samples (mean ± SE, 19.42 ± 2.50; P < 0.0003; Fig. 3). CD3+ T cells were mainly distributed along the invasive margin and in the tumor stroma (Fig. 2C and D). This distribution pattern was similar to that of lymphocytes by conventional H&E staining. These results suggest that IDO-overexpressing tumors are able to reduce T cell proliferation locally.

Association of IDO expression with clinicopathologic variables. IDO-high expression in tumor tissue was significantly correlated with the frequency of liver metastases (P < 0.003). Patients with IDO-low expressing primary tumors were free of metastases in 71.3%. By contrast, only 50% of patients with IDO-high expressing tumors were without metastases. In

Table 2. Expression of IDO in correlation with clinicopathologic variables

Variable	IDO low			IDO high		
	All cases (n)	(n = 87)	(60.8%)	(n = 56)	(39.2%)	Significance (P)*
Gender	143					
Male	76	46	60.5	30	39.5	
Female	67	41	61.2	26	38.8	0.935
Age at surgery (y)	143					
<60	43	21	48.8	22	51.2	
≥60	100	66	66.0	34	34.0	0.054
Tumor size (cm)	131					
<5	72	41	56.9	31	43.1	
≥5	59	36	61.0	23	39.0	0.638
Tumor differentiation	143					
G1	32	22	68.8	10	31.3	
G2	98	57	58.2	41	41.8	0.566
G3	13	8	61.5	5	38.5	
pT stage	143					
pT1	29	23	79.3	6	20.7	
pT2	24	14	58.3	10	41.7	0.036
pT3	78	43	55.1	35	44.9	
pT4	12	7	58.3	5	41.7	
Nodal status	143					
pN0	83	54	65.1	29	34.9	
pN1-3	60	33	55.0	27	45.0	0.224
Liver metastases	143					
No	90	62	68.9	28	31.1	
Yes	53	25	47.2	28	52.8	0.003

*Probability, P, from χ^2 test.

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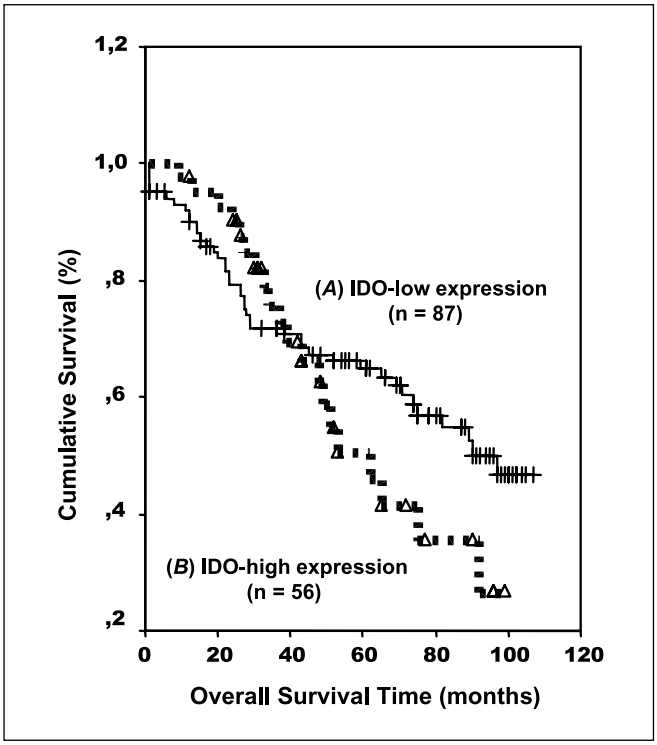

Fig. 4. Kaplan-Meier estimates of overall survival for 143 patients with CRC grouped according to their IDO expression pattern. *A,* 87 cases with IDO-low expression. *B,* 56 cases with IDO-high expression.

addition, IDO expression was significantly associated with the occurrence of synchronous or metachronous liver metastasis.

No correlation was found between IDO expression patterns and tumor stage, tumor size, histologic grade, nodal status, gender, and age (Table 2).

In univariate survival analysis, tumor-node-metastasis classification, nodal status, histologic grade, and frequency of metastases were significant predictors of overall survival, as expected and repeatedly reported in the literature (16).

Survival analysis according to Kaplan-Meier analyses revealed no significant correlation with overall survival for patients with IDO-high or IDO-low expressing primary tumors because survival curves crossed at 45 months (Fig. 4).

By time-dependent multivariate Cox's analysis, however, apart from nodal status, prevalence of liver metastases, histologic grade, and tumor stage, only IDO-high expression proved to be an independent prognostic variable for overall survival within the first 45 months ($P = 0.006$).

After 45 months of follow up IDO-high expression ($P = 0.044$), the prevalence of liver metastases ($P = 0.015$) as well as age ($P = 0.005$), emerged as significant independent variables (Table 3).

Discussion

In this study we showed that IDO expression in colon carcinoma cell lines is dependent on IFN-γ and that high expression of IDO within tumors correlates inversely with the number of TILs and the clinical outcome of colorectal cancer patients.

Our *in vitro* observations thereby contrast previous results by Uyttenhove et al., who claimed constitutive IDO expression in cell lines and as well as in tumor sections (4). Rather, our data confirms and extends earlier investigations in malignant and nonmalignant cell types in which a constitutive degradation of tryptophan could not be found, and where IDO was up-regulated only on treatment with IFNs and/or lipopolysaccharides (17). In cancer patients, significantly accelerated degradation of tryptophan with lowered serum concentrations of tryptophan and increased kynurenine as well as an increased kynurenine to tryptophan ratio (kyn/trp) has been previously recognized (18, 19). This phenomenon could be best explained by IDO expression within the tumors. Therefore, increased kyn/trp concurred with elevated neopterin concentrations, and enhanced tryptophan catabolism as well as increased neopterin levels predicted poor survival (18). Neopterin is released by macrophages upon stimulation with IFN-γ but not by tumor cells. In addition, IFN-γ has been shown as an effector cytokine released by tumor-associated antigen – specific T cells within the tumor microenvironment (20). Thus, these *in vivo* observations further support an essential role of IFN-γ for IDO activation rather than being a constitutive feature in cancer tissue. When we tested the expression of IDO proteins *in vivo* in human tumor samples, we detected colorectal tumor cells expressing IDO in all 143 cases analyzed. These results are in line with previous studies indicating that human tumors frequently express IDO (4). However, 39.2% of tumor specimens revealed IDO-high expression, whereas in 60.8% of tumor specimens, the staining was scored as IDO-low expression, indicating certain colon cancer subsets that differ in their ability to express IDO *in vivo*. The finding that IDO activity significantly correlated with immunostaining scores *in vitro* strongly suggests that the enzyme is active and might exert its immunosuppressive activity in patients classified as having IDO-high expression. However, further prospective studies using fresh tumor tissue are required to confirm this hypothesis.

IDO-expressing cells have been considered to create a state of immunologic unresponsiveness towards tumor-derived antigens (21). However, which cells in particular, either tumor cells themselves or host antigen-presenting cells expressing IDO, are responsible for tolerance induction is still unclear. Munn et al., in studies of human malignant melanoma and breast cancer, proposed that IDO-expressing antigen-presenting cells within tumor-draining lymph nodes

Table 3. Time-dependent multivariate Cox's regression ($n = 143$)

Follow up	‹45 Months		›45 Months	
Variable	**HR (95% CI)**	***P* value**	**HR (95% CI)**	***P* value**
IDO expression	0.39 (0.20-0.76)	0.006	2.75 (1.03-7.35)	0.044
Age	—	n.s.	1.06 (1.02-1.13)	0.005
Liver metastasis	—	n.s.	3.78 (1.30-10.98)	0.015

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval for relative risk; *P* value, probability (*P*) from Cox regression model; n.s., not significant.

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constitute the most potent mechanism for inducing immunologic unresponsiveness (22, 23). By contrast, Astigiano et al. identified IDO-positive eosinophil granulocytes but not antigen-presenting cells or tumor cells to exert effective immunosuppressive action in patients with non–small cell lung cancer (24). Very recently, Muller et al. stated that IDO activity in tumor cells is the relevant target for inhibition to overcome immune escape (25). Thus, it seems likely that the identity of IDO-expressing cells might depend on the type of tumor. The functional relevance and clinical importance of these differences of IDO expression need to be determined. However, our observation that distinct expression patterns of IDO in tumor cells of colorectal cancer patients correlate with clinical outcome, further substantiates the importance of the tumor itself as the tolerizing agent. The latter hypothesis is supported by Okamoto et al., who reported a significant relationship between IDO staining patterns within tumor cells and overall survival in patients with serous ovarian cancer (26).

Furthermore, this is the first study to show that in patients with colon cancer, a high degree of IDO expression by malignant cells correlates with a significant reduction in intratumoral CD3+ T cells. Thus, providing further evidence for an IDO-mediated limitation of antitumoral immune responses at the site of colorectal tumors. However, this correlation could be an independent phenomenon, reflecting the immunogenicity of the tumor, but it could also reveal that TILs are actively killed or inactivated by IDO-high expressing tumor cells. Previously, the quantity of TILs has been correlated with a better prognosis in various human tumors including colorectal cancers (10–13, 27). However, the exact mechanisms of how IDO suppresses TILs remain to be elucidated. Conceptually, tumor cells in the early stage may be recognized by the host's immune system, which is accompanied by the formation of IFN-γ. As a consequence, IDO is activated, and tryptophan deprivation restricts T cell proliferation and T cell numbers decline (28, 29). This may cause a selective survival benefit of IDO-high expressing malignant cells. In addition, the restriction of available tryptophan by IDO within the tumor stroma could be crucial for a sufficient immune response toward tumor-associated antigens and contribute to immune evasion (30, 31). We speculate that reduction of TILs could be one underlying mechanism of IDO-mediated immunosuppression in cancer patients.

The concept that the host's immunologic response towards the primary tumor may be related to the metastatic potential (2) is further proven by the fact that IDO expression significantly correlated with the frequency of metastases. Furthermore, we showed that the presence of IDO-high expressing tumors at the time of initial diagnosis correlates with a significantly worse outcome after 45 months. Most interestingly, high expression of IDO during earlier periods of follow up (<45 months) seems to exert protective effects. Human tumorigenesis is a slow process that, like chronic infection, might occur over several years. Tumors thereby lack an acute phase of the immune reaction which is essential for T-cell priming and natural killer cell activation and might thereafter profoundly reshape immune responses (3, 20).

Our data suggests that the initial immunologic response of the host towards the primary tumor may be able to generate tolerizing conditions, favorable in IDO-high expressing patients, which once established, will remain present even after successful surgical resection. In the early course of the disease, high IDO activity is believed to reflect patients with a strong innate immune response accompanied with high IFN-γ levels. However, despite such initial effective, general immune responses responsible for the survival benefit during the first 45 months, IDO-high expression will consecutively negatively affect disease progression and overall survival in the long run.

In summary, IDO expression levels significantly correlate with the quantity of TILs, the presence of distant metastases and overall survival in patients with colorectal cancer. IDO-high expressing tumor cells might enable certain cancer subsets to initially avoid immune attack and then to reduce T-cell priming and the invasion of effector T cells via local tryptophan depletion.

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