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Induction of indoleamine 2,3-dioxygenase: A mechanism of the antitumor activity of interferon γ

(cell proliferation/L-tryptophan/amino acid metabolism/neoplastic cells/cell culture)

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ABSTRACT **The antiproliferative effects of interferon α (IFN-α) and interferon γ (IFN-γ) were found to be cell-dependent. Among the human cell lines examined, IFN-γ had a greater antiproliferative effect against cell lines that exhibited induction of indoleamine 2,3-dioxygenase, such as the KB oral carcinoma or WiDr colon adenocarcinoma, than against those that lacked the enzyme activity, such as the SW480 colon adenocarcinoma or NCI-H128 small-cell lung carcinoma. Induction of this dioxygenase showed a clear temporal relationship with increased metabolism of L-tryptophan and the depletion of this amino acid in the culture medium. While 70–80% of L-tryptophan remained in the medium of IFN-α- or vehicle-treated cells, virtually all of this amino acid was depleted in the medium of the IFN-γ-treated group following 2–3 days of culture. Supplementing the growth medium with additional L-tryptophan reversed the antiproliferative effect of IFN-γ against KB cells in a dose- and time-dependent manner. The antiproliferative effects of IFN-α and IFN-γ on SW480 and NCI-H128 cells, which are independent of the dioxygenase activity, and the inability of added L-tryptophan to reverse the effects of IFN-γ in WiDr cells suggest multiple mechanisms of action of the IFNs. The data show that the antiproliferative effect of IFN-γ through induction of indoleamine 2,3-dioxygenase, with a consequent L-tryptophan deprivation, is an effective means of regulating cell growth.**

Interferons (IFNs) are a family of proteins that exert antiviral, cell growth-regulatory, and immunomodulatory actions (1–4). These effects are probably mediated by changes in expression of genes or RNA and protein synthesis (5–13). Among the proteins, a (2′-5′)oligoadenylate synthetase and a double-stranded-RNA-dependent protein kinase that phosphorylates the α subunit of eukaryotic translation initiation factor eIF-2 are induced by IFNs. Increased activities of these enzymes, stimulation of a (2′-5′)oligoadenylate-activated latent ribonuclease that degrades both mRNA and rRNA (14–18), impairment of 5′ methylation of newly synthesized mRNA (19, 20), and inactivation of the amino acid-accepting ability of certain tRNA species (21) have been proposed to be important mechanisms in the establishment of the IFN-induced antiviral state. Indoleamine 2,3-dioxygenase (IDO) is another enzyme that occurs in a variety of mammalian tissues (22–26) and is induced by viral infection or IFNs in normal and malignant tissues (27–29). Utilizing reduced flavin and superoxide anion, IDO catalyzes the oxygenative decyclization of L-tryptophan to form *N*-formyl-L-kynurenine (30–32). A physiological role of IDO in the establishment of immunomodulatory or cell growth-regulatory actions of IFN-α and IFN-γ has been suggested by the finding that these cytokines markedly elevate L-tryptophan metabolism by a mechanism of induction of the enzyme in human peripheral blood monocytes (33). In addition, participation of IDO in host-cell defense mechanisms against proliferation of intracellular parasites following IFN-γ treatment has suggested another important biological role for this enzyme (34, 35). In light of the known antiproliferative actions of IFNs on either normal or malignant cells (36–44) and of the requirement of L-tryptophan, the least abundant amino acid, for protein synthesis and cellular growth (45–48), we investigated whether the induction of IDO constitutes another mechanism to explain the antiproliferative action of IFNs.

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MATERIALS AND METHODS

Cell Culture. Human cell lines HL-60 (promyelocytic leukemia), KG-1 (myelogenous leukemia), CCRF-SB (B-cell leukemia), CCRF-CEM (T-cell leukemia), MOLT-4 (T-cell leukemia), THP-1 (monocytic leukemia), U-937 (histiocytic lymphoma), NCI-H69 and NCI-H128 (small-cell lung carcinomas), A-427 (lung carcinoma), MRC-5 (normal fetal lung), HEp-2 (larynx carcinoma), KB (oral carcinoma), MCF7 (breast adenocarcinoma), SW480 (colon adenocarcinoma), WiDr (colon adenocarcinoma), and I407 (normal embryonic intestine) were obtained from the American Type Culture Collection. The cells ($0.3–1 \times 10^6$) were grown in 60-mm plastic culture plates (Costar, Cambridge, MA) containing 6 ml of culture medium consisting of 25 mM Hepes-buffered RPMI 1640 (GIBCO) with 10% heat-inactivated fetal bovine serum, penicillin (50 units/ml), and streptomycin (0.05 mg/ml; Hazleton Research Products, Lenexa, KS) in the presence of IFN-α (interferon α-n1: Wellferon, Burroughs Wellcome, Research Triangle Park, NC), IFN-γ (Interferon Sciences, New Brunswick, NJ) or their vehicle (phosphate-buffered saline; GIBCO). Prior to the various experimental manipulations, cells were preincubated at 37°C for 2 hr in an atmosphere composed of 5% CO_2 in air and then were further incubated for 2–8 days; the culture medium was replenished on day 4 of the incubation period. Cell viability was determined by Trypan blue (Sigma) exclusion. Before the determination of IDO activity, cell number was determined in triplicate with a Coulter counter (Coulter) after harvesting with vigorous trituration in phosphate-buffered saline containing 1 mM EDTA.

Assays. Assays for IDO in the cells, using L-[*ring*-2-^{14}C]tryptophan (1.8 GBq/mmol; Research Products International, Mt. Prospect, IL), and for L-tryptophan or L-kynurenine (Sigma) levels in the culture medium, using HPLC on a 5-μm Bio-Sil ODS column (Bio-Rad). with fluorescence detection (excitation/emission wavelengths of 285/340 nm and 380/480 nm, respectively), were performed as described by Ozaki *et al.* (31, 32). Protein concentration was determined with the Bradford reagent (Bioanalytical Systems, West Lafayette, IN). All data were expressed as mean ±

Abbreviations: IFN, interferon; IDO, indoleamine 2,3-dioxygenase.
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SEM and were analyzed by analysis of variances, exponential regression analysis, or a two-tailed Student's *t* test.

RESULTS

A study of the relationship between the antiproliferative effects of IFNs and the induction of IDO was initiated by screening various human neoplastic cell lines for their ability to express the enzyme in culture. Among the cell lines examined, substantial increases in the activity of IDO in response to IFN-γ treatment (1000 units/ml) for 2 (or 4) days were observed in THP-1, MRC-5, HEp-2, KB, WiDr, and I407 (Table 1). No IDO activity was detected in the vehicle-treated control cultures. When the same cells were similarly incubated for 2 days with IFN-α (5000 units/ml), IDO activity was not significantly different from the control values (0–0.2 nmol per hr per mg of protein). These high doses of IFN-α (5000 units/ml) and IFN-γ (1000 units/ml) have been determined to be sufficient for maximal induction of IDO in human peripheral blood monocytes (33); however, neither IFN-α nor IFN-γ significantly induced IDO in the following cell lines: HL-60, KG-1, CCRF-SB, CCRF-CEM, MOLT-4, U-937, NCI-H69, NCI-H128, A-427, MCF7, and SW480.

The KB and WiDr cell lines, both of which respond to IFN-γ with induction of IDO activity, and the SW480 and NCI-H128 cell lines, in which IDO is not inducible, were further investigated for their responses to IFNs. KB and WiDr cells, with substantially elevated IDO activity, showed markedly greater responses to the antiproliferative action of IFN-γ (Fig. 1 *A* and *B*) than those cell lines (SW480 and NCI-H128) in which the enzyme was not inducible (Fig. 1 *C* and *D*). Whereas IFN-α (54% suppression of growth relative to the day 8 control group; $P < 0.001$) was more effective than IFN-γ against SW480 cells (33% suppression; $P < 0.01$), both cytokines were almost without effect against NCI-H128 cells. Thus, the differences in the response of the cells to the antiproliferative actions of IFN-α or IFN-γ appeared to be cell-dependent and, in the case of IFN-γ, related to the ability of the cells to express IDO.

Table 1. Induction of IDO in human neoplastic cell lines treated with IFN-α or IFN-γ

	IDO activity, nmol per hr per mg of protein		
Cell line	Control	IFN-α	IFN-γ
HL-60	0	0	0
KG-1	0	0	0
CCRF-SB	0	0	0
CCRF-CEM	0	0	0
MOLT-4	0	0	<0.1
THP-1	0	0	15.3 ± 0.5
THP-1*	0	0	63.7 ± 2.4
U-937	0	0	0
MRC-5	0	0	131.1 ± 6.1
NCI-H69	0	0	0
NCI-H128	0	0	0
A-427	0	0	0
HEp-2	0	<0.2	121.6 ± 4.3
KB	0	<0.1	102.3 ± 5.8
MCF7	0	0	<0.3
I407	0	<0.1	68.2 ± 3.7
SW480	0	0	0
WiDr	0	0	79.7 ± 4.9

The various human cell lines were incubated in culture medium with IFN-α (5000 units/ml), IFN-γ (1000 units/ml), or vehicle (control). IDO activity was determined at day 2 (or 4) of the culture period. An asterisk (*) indicates the activity level at day 4.

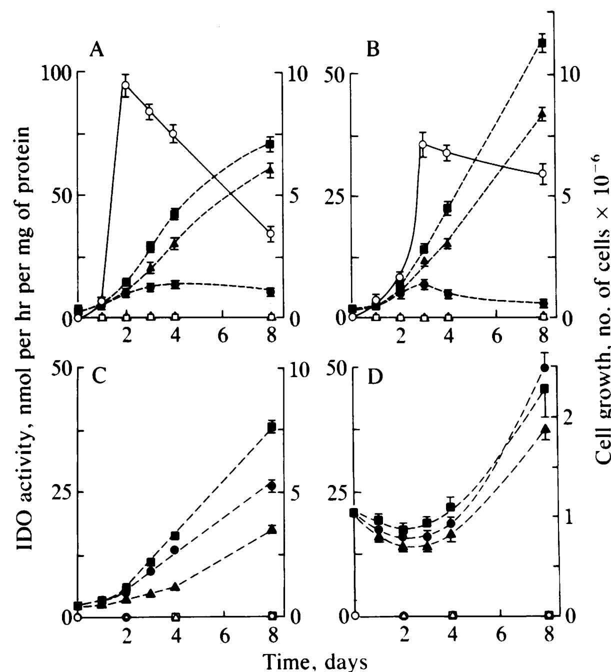

FIG. 1. Growth and IDO activity of neoplastic cells treated with IFN-α or IFN-γ. KB (*A*), WiDr (*B*), SW480 (*C*), or NCI-H128 (*D*) cells were incubated in culture medium containing IFN-α (5000 units/ml), IFN-γ (1000 units/ml), or their vehicle for 8 days. Cell growth (filled symbols) and IDO activity (open symbols) in the IFN-α (triangles), IFN-γ (circles), or vehicle (squares) were determined from the same samples. Neither IFN-α nor vehicle treatments induced IDO in the cells examined.

The culture medium from the study described above (Fig. 1 *A* and *B*) was saved and analyzed for changes in the concentrations of L-tryptophan and its relatively stable metabolite L-kynurenine, the deformylated form of the primary product of IDO (*N*-formyl-L-kynurenine). The results of the studies on the culture medium from the initial half of the 8-day incubation period clearly demonstrate that L-tryptophan is utilized in all of the cell lines or treatments examined. However, the cells in which IDO was induced by IFN-γ exhibited a much greater reduction in L-tryptophan levels and a greater elevation of L-kynurenine in the culture medium than those cells treated with IFN-α or vehicle (Fig. 2). For example, while almost 80% of the L-tryptophan remained in the IFN-α- or vehicle-treated KB cells, virtually all of this essential amino acid was depleted in the IFN-γ-treated group on the second day of the incubation period,

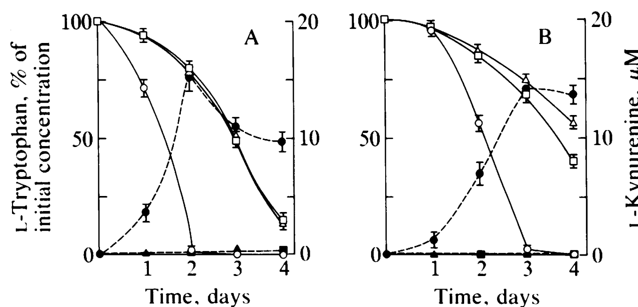

FIG. 2. Effects of IFN-α or IFN-γ on the utilization of L-tryptophan and formation of L-kynurenine in the culture medium. Culture medium from the initial 4-day incubation period (of the study described in Fig. 1) for KB (*A*) or WiDr (*B*) cells was analyzed for L-tryptophan (open symbols) and L-kynurenine (filled symbols). Triangles, IFN-α group; circles, IFN-γ group; squares, vehicle group.

and about 60% of the amino acid could be recovered as L-kynurenine. Similarly, while 70–75% of L-tryptophan was unmetabolized in WiDr cells exposed to vehicle or IFN-α, the amino acid was totally depleted in the IFN-γ-treated group by the third day of the incubation period, and 56% was recovered as L-kynurenine. These marked increases in L-tryptophan metabolism (or the formation of L-kynurenine) in both KB and WiDr cells exhibited a clear temporal relationship with the substantial increases in IDO activity (Fig. 1). Thus, the results indicate that dioxygenation of L-tryptophan by IDO is a major pathway for metabolism of this amino acid in IFN-γ-treated KB or WiDr cells.

The apparent relationship between the level of IDO activity and the antiproliferative action of IFN-γ was studied by incubating KB and WiDr cells with various concentrations of IFN-γ (0–1600 units/ml) for 4 days (Fig. 3*A*). The data show that the sensitivity of WiDr cells to IFN-γ was much greater than that of KB cells. While the activity of IDO continued to rise in a dose-dependent manner, the maximum antiproliferative effect of IFN-γ against either KB or WiDr cells was achieved with a dosage of about 1000 units/ml. When percent of maximum cell growth was plotted against the activity of IDO (Fig. 3*B*), the IFN-γ-elicited increases in IDO activity were inversely correlated with cell growth (e.g., KB, $r = 0.98, P < 0.001$; WiDr, $r = 0.97, P < 0.001$).

A study of the effects of various doses of actinomycin D or cycloheximide on the induction of IDO in KB and WiDr cells showed that the transcriptional or translational inhibitor markedly suppressed the increase in IDO activity in either cell line and indicated that the synthesis of mRNA or protein was an obligatory process for induction of IDO (Fig. 4). Although both cell lines responded similarly to cycloheximide, the response of WiDr cells to actinomycin D was far greater than that of KB cells. At a concentration of 1.25 nM (Fig. 4*A*), 95% of the IDO induction was abolished in WiDr cells, whereas only 42% of the enzyme induction was prevented in KB cells. Thus, the data indicate that the sensitivities of WiDr cells to both the IFN-γ and actinomycin D treatments are much greater than those of KB cells (Figs. 3 and 4*A*) and that the resistance to actinomycin D treatment might suggest a relatively stable nature of the transcriptional or posttranscriptional apparatus, including mRNA, for expressing IDO in KB cells.

In order to study whether the antiproliferative action of IFN-γ was mediated through the deprivation of L-tryptophan by IDO, KB and WiDr cells were grown in RPMI 1640 medium containing various concentrations of the amino acid (Fig. 5). The results indicate that L-tryptophan is essential for normal growth of the KB and WiDr cells and that a concentration of 25 μM, the normal L-tryptophan concentration in RPMI 1640 medium, was needed to achieve optimum growth (Fig. 5 *A–D*). During the first 4 days of treatment of KB cells with IFN-γ, L-tryptophan was capable of reversing the antiproliferative effects of IFN-γ in a dose-dependent manner. Complete reversal was observed at a concentration of 800 μM (Fig. 5*A*). However, prolonged treatment (8 days) with both IFN-γ and L-tryptophan indicated that the amino acid was less effective in reversing the antiproliferative effects of the IFN, and with L-tryptophan at 800 μM, cell growth decreased relative to that seen with the amino acid at 400 μM (Fig. 5*B*). In contrast, L-tryptophan supplementation almost completely failed to reverse the antiproliferative effects of IFN-γ against WiDr cells during either the 4- or 8-day incubation (Fig. 5 *C–E*).

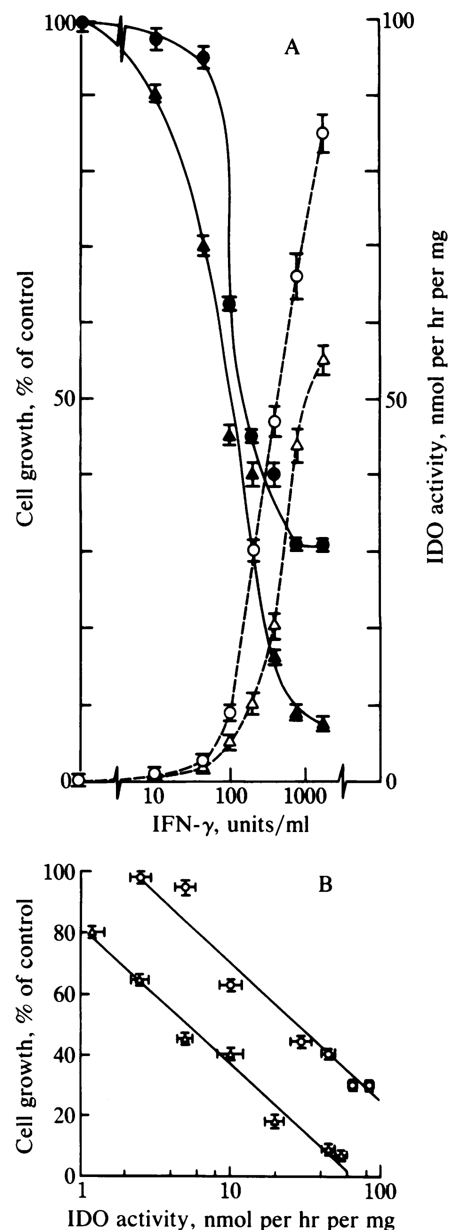

FIG. 3. Relationship between the antiproliferative effects of various doses of IFN-γ and the levels of IDO activity. Cells were incubated in culture medium containing IFN-γ for 4 days. (*A*) Cell growth of KB (●) and WiDr (▲) and the activity of IDO in KB (○) and WiDr (△) were plotted against various concentrations of IFN-γ (0–1600 units/ml). (*B*) Correlation between the inhibition of neoplastic cell growth and the activity of IDO. Percent of maximal cell growth of KB (○) and WiDr (△) was plotted against level of IDO activity.

DISCUSSION

Several mechanisms have been proposed to account for the induction of the antiviral state by IFN (5–21). However, very little is known about the mechanisms by which IFNs inhibit cellular growth. Our results show that IFN-γ can exert a direct antiproliferative effect in neoplastic cells through induction of IDO and consequent deprivation of the essential amino acid L-tryptophan. This inhibition of growth by IFN-γ could be fully or partially reversed by L-tryptophan supplementation, in a dose- and time-dependent manner, thus providing further evidence for a mechanism of regulating cellular growth by IFN-γ through changes in the availability of the least abundant amino acid. These phenomena are consistent with the previous observation that IFN-γ induces high levels of IDO in human peripheral blood monocytes, where the enzyme levels increase more than 100-fold with a marked decrease in L-tryptophan concentration in the culture medium (33). This induction of IDO in

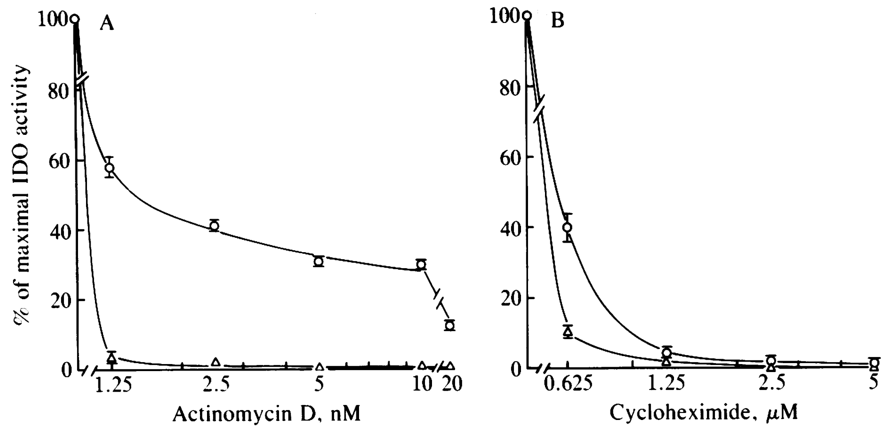

FIG. 4. Actinomycin D and cycloheximide inhibit induction of IDO in neoplastic cells. KB (○) and WiDr (△) cells were incubated with culture medium containing IFN-γ (1000 units/ml) and various doses of actinomycin D (1.25–20 nM; *A*) or cycloheximide (0.625–5 μM; *B*) for 4 days. Percent of maximal IDO activity per 10^6 cells was plotted against the various concentrations of the drugs.

monocytes may be responsible for the substantial reduction in serum L-tryptophan concentration observed in humans after the administration of IFN-γ (49). Moreover, the significant decrease of L-tryptophan levels in the extracellular fluids may potentiate, or act synergistically with, the direct antiproliferative actions of IFN-γ on tumor cells.

The antiproliferative action of IFN-α or IFN-γ on two tumor cell lines lacking IDO activity (SW480 and NCI-H128), coupled with the inability of added L-tryptophan to antagonize the action of IFN-γ in WiDr cells or in KB cells after prolonged treatment with the cytokine, suggests the presence of diverse mechanisms of action for the IFNs. Their effects appear to be dependent both on the target cells and on the length of treatment. For example, L-tryptophan deprivation by IDO appeared to be sufficient to account for the antiproliferative effect of IFN-γ against KB cells at the early incubation time period, since L-tryptophan supplementation alone could fully restore a normal level of cellular growth. On the other hand, although cell growth was found to be inversely correlated with IDO activity, L-tryptophan

FIG. 5. Effects of L-tryptophan supplementation (5–800 μM) on the growth of neoplastic cells and reversal of the antiproliferative action of IFN-γ. KB cells were incubated in culture medium containing IFN-γ (1000 units/ml; ●) or its vehicle (○) and various concentrations of L-tryptophan for 4 days (*A*) or 8 days (*B*). WiDr cells, treated similarly, were also incubated for 4 days (*C*) or 8 days (*D*); in the inset (*E*), the scale of the IFN-γ-treated group is expanded 5-fold.

deprivation alone seems to be insufficient to account for the effect of IFN-γ against WiDr cells or KB cells after prolonged treatment with the cytokine (Fig. 5). These results suggest the occurrence of irreversible changes in cellular metabolism, which may include alterations in the L-tryptophan-uptake system or susceptibility to the amino acid imbalance or to its toxic oxidative metabolites (50–52). Moreover, the sensitivity of WiDr cells to either IFN-γ or actinomycin D treatment (Figs. 3 and 4*A*) further suggests that alterations in transcriptional and posttranscriptional processes other than the induction of IDO are also important molecular mechanisms that must be considered for the complex actions of IFNs (5–13). The availability of an IDO inhibitor would facilitate a further understanding of the relationship between the IDO-mediated antiproliferative effect(s) and other unknown mechanism(s) of action of IFN-γ.

The physiological significance of IDO has been studied for more than 20 years. Provision of precursors for pyridine nucleotide biosynthesis has been proposed as a primary role of IDO (53–55), similar to that of hepatic tryptophan 2,3-dioxygenase [L-tryptophan:oxygen oxidoreductase (decyclizing), EC 1.13.11.11] (56, 57). However, these enzymes differ in many respects, notably in the characteristics of the reaction mechanisms (31, 32), in the distribution in various mammalian tissues (22–26), and in the response to IFNs (27–29, 33). In contrast, the importance of L-tryptophan for the integrity of mammalian cells has been well documented. Deprivation of L-tryptophan inhibits DNA and protein synthesis, as well as cellular growth of murine L1210 leukemia lymphoblasts, murine LM fibroblasts, and rat liver cells (45–48). These effects are also observed in neoplastic cells with a deficiency of other amino acids, such as asparagine, glutamine, or isoleucine (58–60); however, only the effects of L-tryptophan deficiency on genome replication and cellular growth reflect the antiproliferative action of IFN-γ through induction of IDO. Thus, the present studies not only demonstrate an important biological function of IDO but also an effective mechanism of regulating cellular growth by IFN-γ. Further, they suggest that a prior examination of tumors for their ability to express IDO and a search for other inducers of IDO might facilitate future treatments of malignant tumors with IFNs or other agents.

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