Indoleamine 2,3-Dioxygenase-1 Expression is Changed During Bladder Cancer Cell Invasion

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Hellen Joyce Sousa Pereira Santos[1], Luiz Henrique Gomes Matheus[1], Aline Silva[1], Stephanie Vanin Dalmazzo[1], Andressa Assunção Santos[1], Letícia Rafaela Alves Rubens Santos[1], Diego Mota Souza[1], Sabrina Thalita Reis[2], Ivan Pereira Nascimento[3] and Humberto Dellê[1]

[1]Postgraduate Program in Medicine, Universidade Nove de Julho (UNINOVE), São Paulo, Brazil. [2]Laboratory of Medical Investigation (LIM55), Urology Department, University of São Paulo Medical School, Sao Paulo, Brazil. [3]Laboratório Especial de Desenvolvimento de Vacinas, Instituto Butantan, São Paulo, Brazil.

ABSTRACT: The severity of the bladder carcinoma (BC) is directly linked to cell invasion and metastasis. Indoleamine 2,3-dioxygenase-1 (IDO-1) is an INF-γ-induced immunomodulating enzyme that has been linked to the cancer cell invasiveness. Because IDO1 is variable among the tumors, we analyzed its expression in the BC invasion using BC mice models and cell culture. MB49 cells were orthotopically or ectopically inoculated in C57Bl6 mice to evaluate IDO1 by immunohistochemistry. For in vitro experiments, expression of IDO1 and INF-γ was evaluated in grade-1 (RT4) and in grade-3 (T24) BC cell lines. Invading and non-invading T24 cells were separated using the Matrigel/Transwell system, of which total RNA was extracted immediately or after 2 weeks of subculture. Finally, IDO1 was silenced in T24 cells to verify its role on cell invasiveness. In both animal models, IDO1 was differentially expressed between non-invading and invading cells. In cell culture, T24 cells expressed more IDO1 than RT4 cells, independently of the INF-γ expression. IDO1 was differentially expressed between non-invading and invading T24 cells, a difference that was lost by long-time subculture. IDO1 silencing resulted in diminished cell invasiveness. In conclusion, IDO1 expression is changed during bladder carcinoma invasion, playing an important role in this process.

KEYWORDS: Bladder cancer, indoleamine 2,3-dioxygenase, cancer invasiveness, metastasis

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CORRESPONDING AUTHOR: Humberto Dellê, Molecular Innovation and Biotechnology Laboratory, Postgraduate Program in Medicine, Universidade Nove de Julho (UNINOVE), Rua Vergueiro, 235, 2° subsolo, São Paulo, SP 01504-001, Brazil. Email: phdelle@gmail.com

Introduction

Bladder cancer (BC) is the most common malignancy of the urinary tract, being the fourth most common cancer in men and the ninth in women.[1] Approximately 70% to 80% of BC cases are non-muscle-invasive, but a significant portion of those patients recurs and progresses to muscle-invasive form (15%) despite transurethral resection and adjuvant intravesical therapies. Muscle-invasive BC is a more aggressive disease, being associated with a 5-years survival rate of 60% and 10% for patients with localized disease and metastases, respectively.[2] In these cases, radical cystectomy is often necessary. Neoplastic cell invasion through the basement membrane annunciates a sequence of events that culminate in metastatic dissemination, with life-threatening consequences. Invasiveness and metastasis are dependent on multiple mechanisms, including the epithelial-mesenchymal transition (EMT) and production of extracellular-matrix-degrading enzymes.[3]

Indoleamine 2,3-dioxigenase 1 (IDO1) is the first and rate-limiting enzyme in the degradation of tryptophan. Its trajectory as an immunomodulatory enzyme emerged from Munn et al's[4] study in which IDO1 was described as a pivotal molecule in protecting the embryos against the maternal immune attack. Although IDO1 is produced by many cell types of the immune system, including dendritic cells, macrophages, and regulatory T cells, other cell types may express IDO constitutively or induced by mediators. Because IDO1 is overexpressed in several types of cancer, it has been strongly linked to cancer immunoediting.[5] In addition to their immunomodulatory properties, IDO1 has been correlated to other biological functions as such as cancer invasiveness and metastasis.[5]

Regarding BC, few studies were conducted in order to understand the role of IDO1. In human BC, immunostaining for IDO1 was predominantly found in muscle-invasive tumors compared to non-muscle-invasive tumors and was positively correlated to tumor progression.[6] In parallel, we demonstrated that T24 human transitional carcinoma cell line responds to TGF-beta1 effecting EMT with an altered IDO1 expression, suggesting a possible involvement of IDO1 in bladder tumor invasiveness.[7] More recently, IDO1 was correlated to EMT markers and poorer survival in the human early stage BC.[8] Not all bladder tumors produce IDO1 and in an IDO1 producing tumor, not all cells express IDO1. Although IDO1 expression is classically induced by INF-γ and other cytokines, what moves neoplastic cells to express IDO1 in the absence of inflammatory conditions as such as cell culture remains unclear. It is possible that in the same tumor, IDO1-positive and -negative subpopulations emerge through the genome instability or the expression of IDO1 varies according to the cell behavior.

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In the current study, we demonstrated that human BC cell lines produce different amounts of IDO1 and this is not related to an INF-γ-dependent autocrine regulation. When a muscle-invasive carcinoma cell line is orthotopically or ectopically inoculated in mice models, either IDO1-positive and -negative neoplastic cells are observed inside the tumor mass. In addition, using an in vitro model, we demonstrated that IDO1 expression is essential for cell invasion, but is transiently changed during the invasiveness.

Methods

Animal BC models

MB49 murine cells were used for inducing tumor growth in C57BL/6 female mice by both orthotopic and ectopic inoculations. All protocols were in accordance with the Guidelines for Ethical Care of Experimental Animals from the International Animal Care and Use Committee. All animal procedures were approved by the Nove de Julho University Institutional Animal Care and Use Committee (CEUA-UNINOVE, protocols AN0025-2015 and AN0015-2017).

MB49 murine cells (from Dr. Timothy Ratliff at Purdue University College of Veterinary Medicine were gently shared by Dr. Ivan Nascimento at Butantan Institute, SP, Brazil) were maintained in culture with RPMI 1640 medium supplemented with 10% FBS and antibiotics (50 U/mL penicillin and 0.05 mg/mL streptomycin) under 5% CO_2 atmosphere and 37°C.

For the orthotopic model (n = 3), MB49 cells were trypsinized after reaching approximately 80% confluence and resuspended in PBS at a concentration of 1×10^5 cells/100 µL. Intraperitoneal injection of ketamine (100 mg/kg; Ketamin-S, São Paulo, Brazil) and xylazine (10 mg/kg; Rompun, Bayer, Leverkusen, Germany) was performed. Catheterization was performed with a sterile 24 G polyethylene catheter introduced through the urethra. After urine removal, 100 µL of 1 M silver nitrate ($AgNO_3$) was administered, remaining in the bladder for 10 seconds. The bladder was then rinsed 5 times with PBS buffer to remove acidic residue. After this process, 50 µL of PBS containing 0.5×10^5 MB49 cells were infused, remaining in the bladder for 60 minutes. Confirmation of tumor establishment was performed by the presence of macro hematuria or microhematuria within up to 7 days after the inoculation procedure.

For the ectopic model (n = 3), subcutaneous inoculation was performed on the right thigh muscle of the animals. MB49 cells were acquired accordingly described above. Under anesthesia, a volume of 100 µL of PBS containing 5×10^5 MB49 cells was administered subcutaneously. Tumor mass was confirmed 5 to 7 days after inoculation.

The euthanasia procedure was performed 2 weeks after tumor diagnosis with an anesthetic lethal dose, intraperitoneally (using 4 times the anesthetic dose). Tumor tissues were then properly collected and fixed in a 10% paraformaldehyde solution for hematoxylin and eosin staining (H&E) and immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was used to analyze IDO1 expression in the tumor tissue. Paraffin sections were kept for 30 minutes in an oven at 60°C temperature and then submitted to the process of deparaffinization and rehydration by successive baths in xylol and alcohol. Antigenic recovery was performed using steam-heated EDTA buffer, followed by peroxidase blockage using a 3% hydrogen peroxide solution. To increase nonspecific binding blockade, samples were subjected to a final block consisting of 6% rehydrated milk (Nestlé Brasil LTDA, Sao Paulo, Brazil) and 0.5% bovine serum albumin for 15 minutes. After removal of the excess blockage volume, sections were incubated with anti-IDO1 antibody (ab106134, Abcam, Cambridge, MA, USA) diluted 1:10 in the previous blocking solution and kept overnight in a humid chamber in the refrigerator at 4°C. To complete the sandwich, sections were incubated with EnVision Flex HRP reagents (K0690; Dako Co, Denmark) following manufacturer recommendations. DAB chromogen substrate was used to complete the reaction (K346811; Dako Co, Denmark). As a final step, sections were stained with Harris' hematoxylin.

Human cell lines

Human bladder cancer T24 cells and RT4 cells (HTB-4 and HTB-2, respectively; American Type Culture Collection-ATCC, Manassas, VA, USA) were acquired from the cell bank of the Federal University of Rio de Janeiro. T24 cells were cultured in McCoy's 5A Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO) and maintained at 37°C with 5% CO_2.

Cell separation

To obtain invasive and non-invasive cell subpopulations (IC and NIC, respectively) from total T24 cells, we used Matrigel® (BD Matrigel™ Basement Membrane Matrix, BD Biosciences, Bedford, MA) and Transwell® 6.5 mm diameter and 8 µm pore size inserts (Costar, Corning Incorporated, Kennebunk, ME, USA). Matrigel was diluted in FBS-free RPMI 1640 medium (1:5) and loaded on Transwell (100 µL per Transwell), remaining in 5% CO_2 atmosphere and 37°C for 30 minutes before cell seeding. For cell seeding, total T24 cells were cultured in FBS-free RPMI 1640 medium (starvation) for 24 hours and then trypsinized (0.25% trypsin-EDTA solution) and suspended in FBS-free RPMI 1640 medium. One hundred microliters containing 5×10^4 T24 cells were plated on top of the Matrigel/Transwell System, which was coupled to a 24-well plate containing RPMI 1640 medium supplemented with 20% FBS

(chemo-attractant solution) into the bottom of the lower chamber. After 24 hours incubation in 5% CO_2 atmosphere at 37°C, the chemo-attractant solution was removed and 0.5 mL of 0.25% trypsin-EDTA solution was added into the bottom of the lower chamber to detach cells from Transwell membrane (IC). Approximately 50 IC were pelleted by centrifugation and re-suspended in RPMI 1640 medium 10% FBS for 2 weeks subculture or immediately underwent RNA extraction. The remained cells on the top of the Matrigel were carefully collected using a 100 μL tip and re-suspended in RPMI 1640 medium 10% FBS for 2 weeks subculture or immediately underwent RNA extraction.

IDO1 gene silencing

To verify the effect of IDO1 on cell invasion, IDO1-specific interference RNA (si-IDO) (Silencer® Select IDO, cod4392420, Thermo Fisher, USA) was used to knockdown the IDO1 gene expression, following manufacturer's protocol. Briefly, T24 cells were seeded into 6-well plate, grown to 80% to 90 % confluence, and were underwent to starvation (FBS-free medium) for 24 hours. After starvation, the cells were incubated for 24 hours with si-IDO or si-RNA negative control (si-Control, Silencer_siRNA4390843, Thermo Fisher, USA) at a final concentration of 30 pmol/mL medium, which were delivered upon cationic lipid (3 μL/mL medium, Lipofectamine 3000, Invitrogen, California, USA). The supernatant was replaced by serum-free RPMI 1640 medium and the cells incubated for more 24 hours. The silencing was confirmed by real-time PCR (48 hours from the first contact to si-IDO or si-Control) in a parallel experiment. Finally, the cells were seed into Matrigel/Transwell system, accordingly described above. After 24 hours incubation, the Transwell inserts were rinsed in PBS and then the cells were fixed in cold methanol (−20°C) for 10 minutes and stained with hematoxylin. All experiments were done in triplicate. Cells present in the Transwell inserts were counted at 400× magnification.

Real-time PCR

Total RNA was extracted from cultured cells using the PureLink® RNA mini kit (12183018A, Invitrogen, California, USA) and PureLink® RNA micro kit (12183016, Invitrogen, California, USA). For cDNA synthesis, SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, California, USA) was used. SYBR Green kit (Invitrogen, California, USA Biosystems, California USA) was used in combination with specific primers for IDO1 (sense 5′GGTCATGGAGATGTCCGTAA3′ and antisense 5′ACCA ATAGAGAGACCAGGAAGAA3′), INF-γ (sense 5′ACTG TCGCCAGCAGCTAAAA3′ and antisense (5′TATTGCA GGCAGGACAACCA3′), and Tata box protein (TBP) as housekeeping (sense 5′TTCGGAGAGTTCTGGGATT GTA3′ and antisense 5′TTCGGAGAGTTCTGGGAT TGTA3′). Triplicate of each sample was heated at 95°C for 5 minutes for denaturation, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 60 seconds and extension at 60°C for 60 seconds. Reactions were performed in the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Ca, USA). Cycle threshold (Ct) was determined for the housekeeping gene (TBP) as well as target genes using the auto baseline and auto threshold conditions. Normalized gene expression data were made using ΔΔCt (ΔCt reference − ΔCt target) and the formula $2^{-\Delta\Delta Ct}$.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics (Version 23.0. Armonk, NY: IBM Corp., USA). For the comparison between the different groups, the unpaired t-Student test was used. All results are represented by the mean ± standard error. Statistical significance was considered from $P < .05$.

Results

IDO1 expression in BC animal models

Orthotopic and ectopic BC mice models were used to evaluate *in vivo* IDO1 expression, particularly in BC cells. In all animals (3 representative animals per model), MB49 cells proliferated abundantly and a tumor mass was formed as previously described.[9,10] After 2 weeks of inoculation in the bladder, most MB49 cells were installed into the bladder lumen, while many others invaded the bladder wall, destroying the muscle barrier (Figure 1A). IDO1 immunostaining was confirmed in the tumor, specifically in the MB49 cells (Figure 1B). While a strong positivity was observed in the tumor main body, MB49 cells in the tumor front presented a weak or negative immunostaining for IDO1 (Figure 1B). A similar phenomenon was observed in the ectopic model. MB49 cells formed a tumor mass into the subcutaneous space, and a robust number of MB49 invaded the left thigh muscle (Figure 1C and E). MB49 at the tumor edge presented weaker IDO1 staining compared to non-invasive cells (Figure 1D and F).

Expression of IDO1 and INF-γ in RT4 and T24 cells

In order to compare the IDO1 expression between RT4 and T24 cells, real-time PCR was performed. IDO1 expression was markedly increased in T24 cells when compared to RT4 cells, and this increase was not accompanied by INF-γ expression (Figure 2A and B). Incubation of the cells with INF-γ markedly increased IDO1 expression (Figure 2C and D).

IDO1 expression in T24 cells after invasion

IDO1 expression was analyzed after T24 cell invasion. After 24 hours of incubation on Matrigel/Transwell system, total RNA was immediately extracted from NIC and IC cells. As

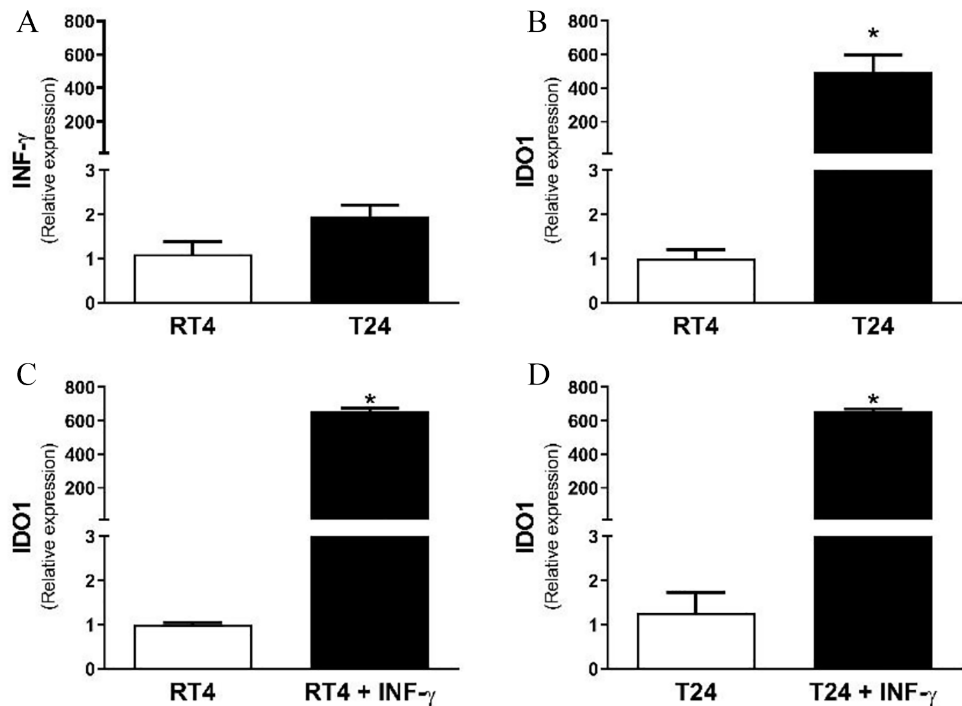

Figure 1. Comparison between bladder carcinoma cell lines RT4 and T24 for expression of INF-γ and IDO1 and the effect of INF-γ on IDO1 expression. (A) INF-γ expression was analyzed by real-time PCR in conventional cell culture. No difference was observed between the two cell lines. (B) RT4 and T24 cells express IDO1 constitutively, but the expression is significantly higher in T24 cells. Incubation (48 h) of RT4 cell (C) and T24 cells (D) with INF-γ significantly increased IDO1 expression.
*$P < 0.05$ vs. RT4 or T24.

demonstrated in Figure 3, IDO1 expression was significantly reduced in IC cells compared to NIC cells. In a subsequent set of experiments, NIC and IC cells were incubated separately for 2 weeks to evaluate the expression of the same genes. No difference was found in the IDO1 expression between the 2 groups (Relative expression of 1.0 ± 0.2 in the NIC and 1.8 ± 0.8 in the IC).

Effect of the IDO inhibition on invasion

IDO1 expression was reduced using a small interfering RNA (Relative expression of 1.10 ± 0.35 in si-Control versus 0.04 ± 0.02 in si-IDO; $P < .05$). T24 cells were previously incubated with si-IDO1 and then submitted to Matrigel/Transwell system. The inhibition of IDO1 expression significantly reduced cell invasion (Figure 4).

Discussion

The severity of the BC disease is directly linked to the cell ability to invade locally and metastasize. In this context, EMT markers correlate positively to reduced overall survival and disease/progression-free survival in cancer patients.[11]

Immune cells-produced IDO1 has been the focus of many researchers interested in the context of tumor escape and tumor-promoting inflammation, however, IDO1 production by neoplastic cells cannot be disregarded. Although IDO1 is classically recognized as an immunomodulatory molecule, a body of evidence suggests other IDO1 effects in cancer, including cell invasiveness. In BC, IDO1 has been associated with markers of EMT and tumor progression.[8,12] IDO1 is constitutively expressed in some types of cancer and in BC it has been variable. While some tumors are negative for IDO1, others have subpopulation with variable IDO1 immunostaining. Why this occurs remains unknown. Inside the tumor, IDO1 expression could be induced by inflammatory cytokines, mainly by INF-γ and TNF-alpha.[13] In the current study, we demonstrated that T24 cells express naturally more IDO1 than RT4 (>500-fold) under normal condition and no difference was observed in INF-γ expression. The addition of INF-γ in the medium promoted a significant increase of IDO1 expression, showing that either RT4 or T24 respond to INF-γ by producing IDO1, but INF-γ is not responsible by the discrepant expression of IDO1 between the 2 BC cell lines in basal conditions.

Using 2 BC animal models characterized by intense invasiveness, we showed that IDO1 expression was variable accordingly to cellular localization. Immunohistochemistry analysis revealed IDO1-positive cells predominantly in the center of the tumor, while IDO1-negative cells were found in the tumor front, close to the musculature. Variation in IDO1 expression within the same cell line could be explained by the genomic instability and/or splice variant. For example, when mutations lead to the loss of the tumor suppressor gene Bin1, IDO1 is strongly induced.[14] Although this is a considerable hypothesis, we also hypothesized that IDO1 expression could be transient during cell invasion. In our experiments, we used the Matrigel/Transwell system to separate invasive from

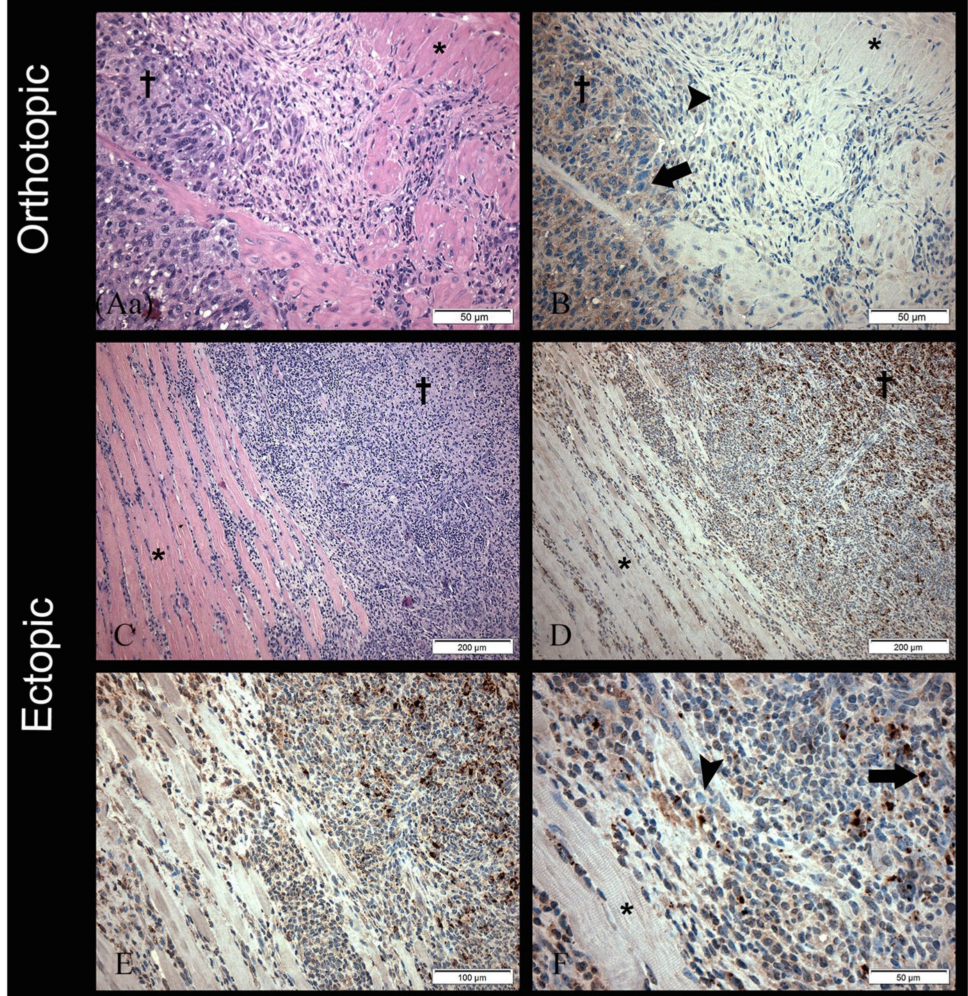

Figure 2. Orthotopic and ectopic BC models were performed to evaluate IDO1 immunostaining. C57Bl6 mice received MB49 cells, being euthanased two weeks after tumor diagnosis. (A) H&E staining revealed that in the orthotopic model MB49 cells formed an intravesical tumor (cross) and markedly infiltrated the detrusor muscle (asterisk). (B) In a serial section, Immunostaining for IDO1 was strong in the center of the tumor (arrow), while the tumor front presented weak IDO1 expression (arrowhead). In the ectopic model, (C) H&E staining revealed a solid tumor (cross), from which many MB49 cells infiltrated the skeletal thigh muscle (asterisk). (D) In a serial section of the same tumor, IDO1 immunostaining was strong in the central region of the tumor (arrow), while weak immunostaining was observed in the infiltrating cells (arrowhead). (E and F) High magnification of the field showed in (D) was performed.

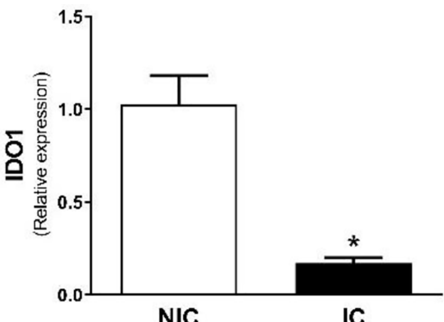

Figure 3. Expression analysis for IDO1 in non-invasive (NIC) and invasive (IC) T24 cells. The cells were seeded on Matrigel/Transwell system and after 24 h the IC were collected by trypsinization, while NIC were obtained directly from Matrigel surface for RNA extraction. IC expressed significantly less IDO1 compared to NIC. When the separated cells were cultured for two weeks under normal conditions, this difference was lost (data not represented in this figure), showing that IDO1 is transitorily expressed during the cell invasion process.
*$P < .05$ versus NIC .

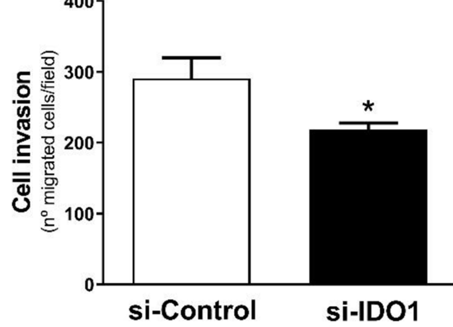

Figure 4. Effect of IDO1 inhibition by siRNA on cell invasiveness. IDO1 inhibition diminished cell invasion in Matrigel/Transwell system.
*$P < .05$ versus si-Control.

non-invasive cells of the same cell line. Invasive cells expressed less IDO1 than noninvasive cells during the invasion. In order to verify if they were different subclones, we cultivated the invasive and noninvasive cells separately for 2 weeks.

Interestingly, no difference was found in the expression of IDO1 between invasive and noninvasive cells after a long time subculture. These results suggest that IDO1 expression is transient during invasion. The loss of IDO1 expression does not mean it is not important in the process of cell invasion, as its inhibition with siRNA decreased the cell the invasion in Matrigel/Transwell system. IDO1 seems to modulate this process. Clearly further studies will be needed to reveal the precise mechanisms by which IDO1 influences cellular invasiveness.

Through an analogy with the female reproductive apparatus, additionally to its immunomodulatory effects, IDO1 has been involved in non-immune processes during pregnancy. IDO1 expression was described in trophoblasts and it appears to be involved in the invasion of the endometrial tissue.[15] In the preeclampsia disease, there is a deficient invasion of the trophoblast into the uterine wall and this effect has been associated with decreased IDO1 expression and activity.[16,17] The importance of IDO1 was also demonstrated mediating proliferation and migration of trophoblasts under STAT3 signaling.[18] A decrease in STAT3 phosphorylation with consequent downregulation of IDO1 expression has been associated with recurrent spontaneous abortion by affecting trophoblast cell proliferation and migration.[18] Intense trophoblast differentiation occurs for the embedding of the blastocyst into the endometrium and this is accompanied by transient IDO1 production. Ban et al[15] demonstrated that there is the expression of IDO1 in human cytotrophoblast, syncytiotrophoblast, and invasive extravillous cytotrophoblasts, however, in almost half of the cases, weak staining was observed in syncytiotrophoblasts when compared to cytotrophoblast and extravillous cytotrophoblats. In addition, Hönig et al[19] demonstrated that IDO1 immunostaining was absent in syncytiotrophoblats when compared to cytotrophoblasts and invasive extravillous cytotrophoblasts.

One limitation of this study was the impossibility to evaluate IDO1 expression in real-time during the cell invasion. We have tried to construct a vector carrying a fluorescent gene reporter under the IDO1 promoter, but we are not yet successful.

The current study reinforces the hypothesis that IDO1 has a role in the BC that goes beyond its immunomodulatory action. The transient IDO1 expression may explain the variability of the IDO1 immunostaining found in BC human specimens and should be taken into consideration in terms of diagnosis and in the proposal of new therapeutic modalities.

In summary, IDO1 is differentially expressed among BC and its expression is changed during the cell invasion, in which IDO1 exerts effect. This variation should be considered in the IDO1-based prognosis of the BC.

Author Contributions

HJSPS: Conceptualization, methodology, formal analysis, and writing
LHGM: Methodology and follow-up
AS: Methodology and data curation
SVD: Methodology and data curation
AAS: Methodology and writing
LRARS: Methodology and statisctic analysis
DMS: Methodology and statisctic analysis
STR: Conceptualization and writing (review & editing)
IPN: Conceptualization and writing (review & editing)
HD: Conceptualization, formal analysis, supervision and project administration, writing (original draft preparation and review & editing), and funding acquisition

ORCID iDs

Ivan Pereira Nascimento https://orcid.org/0000-0001-9843-1594

Humberto Dellê https://orcid.org/0000-0001-6072-3671

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