6-9-2012

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Journal of Molecular Pathophysiology

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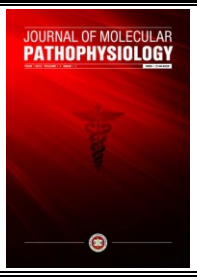

Original Research

Indoleamine 2,3-dioxygenase (IDO) and tryptophan dioxygenase (TDO) mRNA expression in oral squamous cell carcinoma cells is actively and differentially modulated

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Received: June 09, 2012

Accepted: June 09, 2012

Published Online: June 09, 2012

DOI: 10.5455/jmp.20120609040915

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Keywords: Indoleamine 2,3-dioxygenase (IDO), tryptophan dioxygenase (TDO), oral squamous cell carcinoma (OSCC)

Abstract
Objective: Oral cancers are slow developing tumors that affect thousands of individuals in the US annually. Evidence has shown that these cancers can create microenvironments to enhance their progression, survival and metastasis characteristics. Cancer cells will compete for extracellular nutrients with normal tissue cells, which can cause irregularities in immune regulation of progressive cancers. Recent evidence in other types of cancers have suggested that tumor cells may actually upregulate the consumption of extracellular L-tryptophan and increase the export of cytosolic kynurenine byproduct. These kynurenines have been linked to suppression of local immune response of T-cells. The primary goal of this study was to evaluate oral cancer cell lines for tryptophan dioxygenase (TDO) and Indolamine 2,3-dioxygenase (IDO) expression and elucidate whether or not other flavonoids inhibit enzyme expression and cancer growth. Methods: Oral cancer cell lines SCC25, SCC15, and CAL27 were screened for enzyme expression using mRNA specific primers and RT-PCR. The three cell lines were then subjected to proanthocyanidin (PAC) administration to determine potential growth and enzyme inhibition. Results: This study found that IDO and TDO are expressed in the SCC25, SCC15, and CAL27 oral cancer cell lines and enzyme expression is correlated with tumor growth. Additionally, it was observed that IDO and TDO expression, along with tumor growth, can be inhibited by flavonoid administration. Conclusions: With no real treatment advances in many years and survival rates remaining relatively unchanged, these results provide further knowledge in the understanding of oral cancer mechanisms and function that may lead to future treatment modalities. Our demonstration of enzyme down regulation and growth inhibition induced by flavonoids may also contribute to further research in future novel treatments.

INTRODUCTION

Oral cancers develop very slow over many years or decades and have recently been found to affect nearly 30,000 people annually in the US [1,2]. Oral health researchers and oncologists generally concur that most oral cancers are initiated due to the long-term use of tobacco and alcohol [3,4], although the mechanisms of oral carcinogenesis entail distinct and variable changes in the molecular systems, architecture, and genetics of each individual tumor [5,6]. Recent evidence suggests that aggressive tumors, including those of the oral cavity, create microenvironments with many common features that promote tumor progression, survival, angiogenesis, and metastasis [7].

One common cellular feature of oral cancers is accelerated growth and proliferation, which is dependent upon the uptake and metabolism of various micronutrients used in biosynthesis and energy generation [8,9]. For example, research has demonstrated that folic acid, which is crucial for DNA synthesis and repair, as well as specific metabolic processes, including DNA methylation via S-adenosylmethionine, is closely regulated in most tissues but may be dysregulated during oral

carcinogenesis [10-14]. More recent evidence now suggests that L-Tryptophan, an amino acid essential for growth and a variety of other cellular processes, including protein synthesis and both NADH / NADPH formation [15], may be preferentially imported and metabolized in many human cancers [16], such as those of the oral cavity [15,17].

Uptake of L-tryptophan into human cells may involve a variety of L-amino acid transporter (LAT) proteins, but mainly LAT1 counter-transport proteins that import extracellular L-tryptophan in exchange for the cytosolic by-product kynurenine following tryptophan catabolism within the cell by tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase enzymes (IDO) [18,19,20]. This exchange system functions not only to accelerate carcinogenesis by increasing L-tryptophan uptake from the local microenvironment, thus reducing availability to other cells, but also by up-regulating the export and extracellular concentration of kynurenines, which may play a significant role in suppressing the local adaptive immune response of T-cells [21-26].

OBJECTIVE

A few preliminary studies have observed the tryptophan influx/kynurenine efflux cycle in one or more oral cancer cell lines [15,17]. Several studies have demonstrated increased anti-tumor immunosuppression and tumor growth inhibition by down-regulation or interference with IDO [27-29]. However, only one study to date has successfully demonstrated IDO inhibition, transcriptional down-regulation, and growth inhibition in oral cancer cells (using the flavonoid epigallocatechin-3-gallate), as well as the elucidation of associated intracellular signaling pathways affected [17]. Therefore, the purpose of this study was to evaluate additional oral cancer cell lines, not previously examined, for IDO and TDO expression and to explore the inhibitory potential of other flavonoids on IDO and TDO expression. Based upon previous evidence, the working hypothesis was that cellular phenotypes that exhibit accelerated growth and proliferation may be correlated with expression of these enzymes. Furthermore, flavonoids may not only inhibit oral cancer growth but also IDO and TDO expression. These findings may be useful as they explore the potential effects of flavonoids as prevention and secondary treatment options for patients with oral cancer.

METHODS

Cell lines

The human oral scquamous cell carcinoma (OSCC) cell lines used in this study, CAL27 (CRL-2095), SCC15 (CRL-1623), and SCC25 (CRL-1628) were obtained from American Type Culture Collection (ATCC: Manassas, VA). CAL27 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4 mM L-glutamine, adjusted to contain 3.7 g/L sodium bicarbonate and 4.5 g/L glucose from Hyclone (Logan, UT). SCC15 and SCC25 cells were maintained in a 1:1 mixture of DMEM and Ham's F12 medium with 2.5 mM L-glutamine, modified to contain 15 mM HEPES, 0.5 mM sodium pyruvate, and 1.2 g/L sodium bicarbonate (ATCC), supplemented with 400 ng/ml hydrocortisone from Sigma-Aldrich (St. Louis, MO). The control oral cell line HGF-1 (CRL-2014) was maintained in DMEM with 4 mM L-glutamine, adjusted to contain 3.7 g/L sodium bicarbonate and 4.5 g/L glucose, from Hyclone (Logan, UT). Media for all cell lines were supplemented with 10% fetal bovine serum (FBS), and with 1% Penicillin (10,000 units/mL)-Streptomycin (10,000 µg/mL) solution (HyClone). Cell cultures were maintained in 75 cm2 Becton, Dickinson (BD) Falcon tissue-culture treated flasks (Bedford, MA) at 37°C and 5% CO_2 in humidified chambers.

RNA isolation, concentration, and yield

RNA was isolated from 1.5 x 107 cells of each of the experimental cell lines, using ABgene Total RNA Isolation Reagent (Epsom, Surrey, UK) in accordance with the procedure recommended by the manufacturer. RNA purity and concentration and purity were calculated using UV spectroscopy. The absorbance of diluted RNA samples (10 µL of RNA sample in 490 µL nuclease-free water, pH 7.0) was measured at 260 and 280 nm. RNA purity was determined by calculating the ratio of A260:A280, and comparing it to the expected ratio which should be > 1.80. Concentration of RNA samples was determined by the A260 reading of 1 = 40 µg/mL RNA, based on an extinction coefficient calculated for RNA in nuclease-free water. Concentration was then calculated as 40 x A260 absorbance measure x dilution factor (50). Total yield was determined by concentration x sample volume in mL.

Example:

RNA standard
A260 = 0.75
Concentration = 40 x 0.75 x 50 = 1,500 µg/mL
Yield = 1,500 µg/mL x 1.0 mL = 1,500 µg or 1.5 mg RNA

Reverse-transcription polymerase chain reaction (RT-PCR)

To quantify the expression of specific mRNA, RT-PCR was performed on total RNA using the ABgene Reverse-iT One-Step RT-PCR Kit (ReadyMix Version) and a Mastercycler gradient thermocycler (Eppendorf:

Hamburg, Germany) using the following primers for IDO [30], IDO2 [31], TDO [32], *c-myc [*33] , ornithine decarboxylase *ODC [*34] , *caspase*-2 [35], *caspase*-8 [36], and GAPDH [37], synthesized by SeqWright (Houston, TX):

IDO-forward
5′-TGTCCGTAAGGTCTTGCCAGG-3′;

IDO-reverse
5′-CGAAATGAGAACAAAACGTCC-3′;

IDO2-forward
5′-GACTACAAGAATGGCACACG-3’;

IDO2-reverse
5′ AATGTGCTCTTGTTGGGTTAC-3’;

TDO-forward
5’-GGG CATATG AAAAAACTCCCCGTAGAAGG-3’;

TDO-reverse
5’-GGGGATCCTTAATCTGATTCATCACTGCTG AAGTAGG-3’;

c-myc forward primer,
5’-TCCAGCTTGTACCTGCAGGATCTGA-3’;

c-myc reverse primer,
5’-CCTCCAGCAGAAGGTGATCCAGACT-3’;

ODC forward primer,
5’-AATCAACCCAGCGTTGGACAA-3’;

ODC reverse primer,
5’-ACATCACATAGTAGATCGTCG-3’;

caspase-2 forward primer,
5’-TGGCATATAGGTTGCAGTCTCGG-3’;

caspase-2 reverse primer,
5’-TGTTCTGTAGGCTTGGGCAGTTG-3’;

caspase-8 forward primer,
5’-GATATTGGGGAACAACTGGAC-3’;

caspase-8 reverse primer,
5’-CATGTCATCATCCAGTTTGCA-3’;

GAPDH forward primer,
5’-ATCTTCCAGGAGCGAGATCC-3’;

GAPDH reverse primer,
5’-ACCACTGACACGTTGGCAGT-3’;

One µg of template (total) RNA was used for each reaction. The reverse transcription step ran for 30 minutes at 47°C, followed by denaturation for 2 minutes at 94°C. Thirty-five amplification cycles were run, consisting of 20 second denaturation at 94°C, 30 seconds of annealing at 58°C, and 6.5 minutes of extension at 72°C. Final extension was run for 5 minutes at 72°C. Reaction products were separated by gel electrophoresis using Reliant 4% NuSieve® 3:1 Plus Agarose gels (Lonza: Rockland, ME). Bands were visualized by UV illumination of ethidium-bromide-stained gels and captured using a Kodak Gel Logic 100 Imaging System and 1D Image Analysis Software (Eastman Kodak: Rochester, NY). Quantitation of RT-PCR band densitometry and relative mRNA expression levels were performed using Adobe Photoshop (San Jose, CA) imaging software, Image Analysis tools.

Proliferation (growth) assays

Proliferation assays were performed in the appropriate complete media in Corning Costar 96-well assay plates (Corning, NY) at a concentration of 1.2 x 104 cells per well, and proliferation was measured over three days. Cultured cells were fixed at three time points, after 24 hrs (day 1 or d1), after 48 hrs (day 2 or d2), and after 72 hrs (day 3 or d3) using 50 µL of 10% buffered formalin, and were stained with crystal violet 1% aqueous solution (Fisher Scientific: Fair Lawn, NJ). The relative absorbance was measured at 630 nm using a Bio-Tek ELx808 microplate reader (Winooski, VT). Data were analyzed and graphed using Microsoft Excel (Redmond, WA). Three separate, independent replications of each experiment were performed.

Relative-fold increase (RFI)

Trypsinizing and plating cells may have proliferation-stimulating effects within laboratory cell culture-based assays, which have been observed between d0 and d1 in previously published work involving this specific method of proliferation assay in these cell lines [38-43]. To reduce the overall impact of these effects, the relative change in proliferation, measured as the change or relative-fold increase (RFI) in absorbance between d3 and d1, was calculated to more accurately assess the changes induced by these experimental treatments.

Viability

Prior to plating cells for proliferation assays, aliquots of trypsinized cells were stained using Trypan Blue (Sigma: St. Louis, MO), and live cells were enumerated by counting the number of Trypan-blue negative cells using a VWR Scientific Counting Chamber (Plainfield, NJ) and a Zeiss Axiovert 40 inverted microscope (Gottingen, Germany). At each time point (day 1-3), several wells were processed using the Trypan stain, and live (viable) cells were enumerated, and the overall percentage calculated, using this procedure.

Materials

Proanthocyanidin (PAC) (Lot #3717HF7361), a dietary supplement intended to provide a concentrated source of commonly available fruit-derived flavonoids, was obtained from GNC Preventive Nutrition® (Pittsburgh, PA). This commercial source of PAC was extracted from U.S. grown wine grapes, *Vitus vinifera*. Such commercial sources of grape seed PAC extract are

demonstrated to contain 95% PAC and contain approximately 80-90% oligomeric PACs, including dimers, trimers, tetramers, and a small amount of other monomers [41,42,44]. The total concentrations of PAC used were between 10 and 100 µg/mL, added to the complete media prior to the start of each experimental assay and cell plating. The growth inhibitory maximum (GIMAX) previously determined for these cell lines was confirmed at 50 µg/mL [41,42]. Three independent replications of each experiment for each cell line (CAL27, SCC25, SCC15) were performed, each consisting of eight wells per experimental concentration.

Statistics

Comparisons of the effects of treatments were made using two-tailed *t* tests with $\alpha \leq 0.05$. All samples were analyzed using two-tailed *t* tests as departure from normality can make more of a difference in a one-tailed than in a two-tailed *t* test [45]. As long as the sample size is even moderate (>20) for each group, quite severe departures from normality make little practical difference in the conclusions reached from these analyses.

RNA was extracted from the OSCC cell lines CAL27, SCC25, and SCC15 to analyze IDO and TDO mRNA expression using RT-PCR (Figure 1). This analysis revealed expression of IDO and TDO mRNA in all three cell lines, although the levels in each of the cell lines varied significantly relative to their GAPDH levels (Fig 1A). In addition, CAL27 and SCC25 cells expressed comparatively greater levels of IDO mRNA than did SCC15 cells.

RESULTS

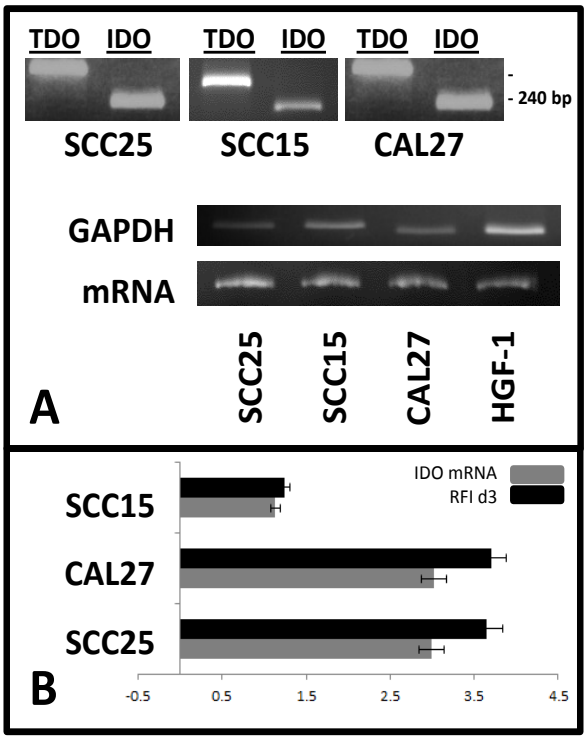

Figure 1.

To determine if proliferation or growth rates, measured by relative-fold increase (RFI) in growth after three days (3d), may be associated with these observations, an analysis was performed revealing IDO mRNA expression strongly correlated with RFI in CAL27, SCC25, and SCC15 cells, R=0.90, 0.91, and 0.76, respectively (Fig 1B). More specifically, the cell lines with the highest RFI (CAL27 and SCC25) were observed to express higher levels of IDO than SCC15 cells.

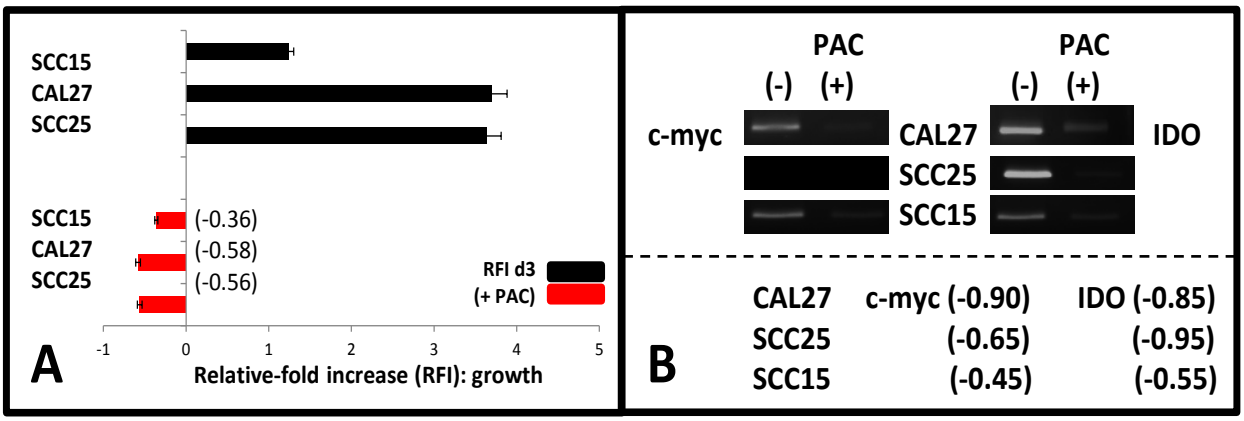

Figure 2.

To determine if these cell lines down-regulate IDO expression and are growth-inhibited by flavonoids, as previously observed with OSCC cell lines and epigallocatechin [17], a more diverse group of flavonoids (PACs) were administered at the GIMAX of 50 µg/mL (Figure 2). These experiments demonstrated that PACs were sufficient to significantly inhibit proliferation in a dose-dependent manner up to the growth inhibitory maximum (GIMAX) of 50 µg/mL (-36 to 58%, $p<0.05$), confirming previous observations in CAL27 and SCC25 cells [40-42], but also demonstrating these effects in SCC15 cells (Fig 2A). To determine if the growth inhibition induced by PAC administration also influenced IDO, RT-PCR was utilized to reveal a down-regulation in IDO mRNA in all cell lines (Fig. 2B). This decrease also corresponded with a down-regulation in the cell-cycle promoter *c-myc*, confirming previous observations [40,41].

To more accurately assess these effects and the possible mechanisms responsible, other key cell-cycle, survival and apoptosis-specific regulatory genes, including ODC, caspase-2, caspase-8, as well as TDO were assessed (Table 1). These analyses demonstrated that PAC administration is associated with down-regulation of both IDO and TDO mRNA between -0.3 and -0.95-fold. These analyses also confirmed the previously observed down-regulation of key growth-promoting regulators *c-myc* and *ODC*, as well as the up-regulation of apoptosis initiators and effectors, caspase-2 and caspase-8 [40]. Furthermore, these observations correlated with decreased cell viability (ranging from -0.42 and 0.82-fold) and growth (between 0.36 and -0.58).

Table 1. PAC induced effects on mRNA expression, growth and viability in oral cancer cells.

	CAL27	SCC25	SCC15
IDO	-0.85	-0.95	-0.30
TDO	-0.50	-0.55	-0.30
c-myc	-0.90	-0.65	-0.45
ODC	-0.45	-0.60	-0.35
Caspase-2	+0.70	+1.10	+0.65
Caspase-8	+0.80	+0.60	+0.55
RFI	-0.58	-0.56	-0.36
Viability	-0.71	-0.83	-0.42

These data provide evidence that administration of PACs may, directly or indirectly, down-regulate mRNA expression of both IDO and TDO in oral cancer cell lines (Figure 3). In addition, these data confirm previous observations suggesting that other key oral cancer cell-cycle signaling molecules, such as ODC and c-myc may also be down-regulated by PAC administration [40-42] – although these data may be the first to demonstrate that this process may occur in tandem. When combined with other previous reports of flavonoid-induced signaling interactions involving STAT [17] and FAK [46], a more comprehensive system involving the upstream and downstream effects of flavonoid-induced growth inhibition of oral cancer growth emerges.

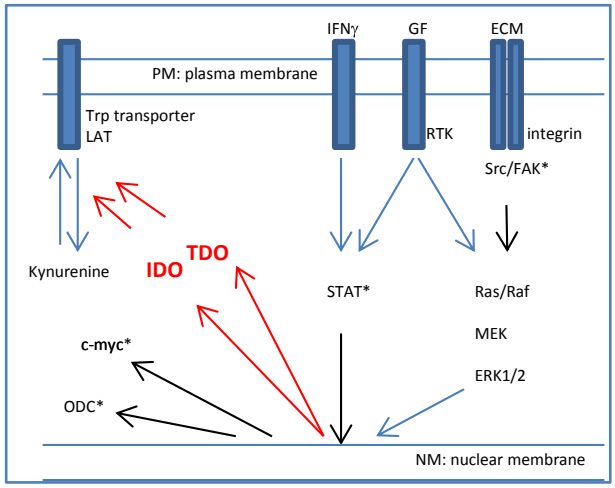

Figure 3.

DISCUSSION

The purpose of this study was to investigate whether previously unscreened oral cancer cell lines, expressed IDO and TDO; and additionally to explore whether this expression is correlated with any specific cellular phenotype. To test this hypothesis, a comprehensive series of integrated *in vitro* assays were performed that clearly demonstrate SCC25 and SCC15 cells expressed both IDO and TDO mRNA, as well as confirming this expression in CAL27 oral cancer cells. In addition, the differential expression of IDO, exhibited strong and positive correlations with cellular growth and proliferation rates. These experiments demonstrated that PACs were sufficient to significantly inhibit proliferation in a dose-dependent manner up to the growth inhibitory maximum. Finally, although these data confirm the growth-inhibitory properties of PACs on oral cancers [40-42,47,48], these data may be the first to demonstrate the potential for PACs and other fruit-derived flavonoids to inhibit IDO (and TDO) expression, similar to the effects previously observed with epigallocatechin-3-gallate [17], as well as an analysis of corresponding changes to key intracellular signaling molecules involved in cell cycle progression and the initiation of apoptosis.

Although several previous studies have evaluated the potential pro-growth and immune-evasion mechanisms

of IDO and TDO expression in other cancers [18-20], to date, only a few select studies have examined these processes in oral cancers [15,17]. The current study, therefore, provides novel data and further elucidation towards the understanding of how oral cancers function not only to increase survival and viability, but also to simultaneously down-regulate and evade local immunomodulatory responses [21,22,25,26]. In addition, these data provide novel evidence that IDO mRNA expression, but not TDO levels, may be correlated with oral cancer growth rates – a finding that may help to contextualize the understanding of the active, but differential, regulation of these enzymes in other oral cancer cell lines and tumors. In addition, this study provides novel data to suggest that PACs, and other flavonoids, may be sufficient to inhibit growth and down-regulate IDO expression in these cells.

A limitation of this study, similar to other preclinical studies, involves the use of oral cancer cell lines. These cells may harbor karyotypic alternations or other dissimilar genetic mutations that may potentially influence the experimental outcomes of this study. For example, CAL27 cells contain a nonsense mutation in the SMAD4 gene, which may influence the transforming growth factor (TGF) signaling pathway and cell growth potential [49]. In addition, SCC25 cells are known to harbor a deletion in the cell cycle promoter cdk1, resulting in a deletion of a critical transcriptional regulation sequence [50]. SCC15 cells however, harbor a single nucleotide polymorphism (SNP) that alters the expression of the tumor suppressor, S100A2 [51]. However, although oral cancers may harbor many genetic mutations and deletions, many lines of evidence now suggest that dysregulation and reduced expression of many tumor suppressors in oral cancers may, in fact, be the result of hypermethylation events rather than fixed genetic differences [52-54].

CONCLUSIONS

Although many studies have found that oral carcinogenesis may be strongly influenced by the ability of tumor cells to alter the local microenvironment, new evidence suggests that the ability to alter micronutrient uptake (L-tryptophan), while down-regulating adaptive immune responses to tumor growth may be critical to tumor growth and metastasis. This study may be the first to provide new evidence that IDO and TDO expression are found in SCC25 and SCC15 cells, as well as CAL27 cells, and to demonstrate that PAC administration is not only sufficient to inhibit oral cancer growth, but may also be associated with IDO and TDO mRNA down-regulation.

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