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Indoleamine 2,3-dioxygenase activity and L-tryptophan transport in human breast cancer cells

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Abstract

The activity and expression of indoleamine 2,3-dioxygenase together with L-tryptophan transport has been examined in cultured human breast cancer cells. MDA-MB-231 but not MCF-7 cells expressed mRNA for indoleamine 2,3-dioxygenase. Kynurenine production by MDA-MB-231 cells, which was taken as a measure of enzyme activity, was markedly stimulated by interferon-γ (1000 units/ml). Accordingly, L-tryptophan utilization by MDA-MB-231 cells was enhanced by interferon-γ. 1-Methyl-DL-tryptophan (1 mM) inhibited interferon-γ induced kynurenine production by MBA-MB-231 cells. Kynurenine production by MCF-7 cells remained at basal levels when cultured in the presence of interferon-γ. L-Tryptophan transport into MDA-MB-231 cells was via a Na^+-independent, BCH-sensitive pathway. It appears that system L (LAT1/CD98) may be the only pathway for L-tryptophan transport into these cells. 1-Methyl-D,L-tryptophan *trans*-stimulated L-tryptophan efflux from MDA-MB-231 cells and thus appears to be a transported substrate of system L. The results suggest that system L plays an important role in providing indoleamine-2,3-dioxygenase with its main substrate, L-tryptophan, and suggest a mechanism by which estrogen receptor-negative breast cancer cells may evade the attention of the immune system.

Keywords: Indoleamine 2,3-dioxygenase; Tryptophan transport; Breast cancer

1. Introduction

Indoleamine 2,3-dioxygenase is a cytoplasmic, heme-containing enzyme which catalyses the oxidative cleavage of the indole moiety of a number of substituted and unsubstituted indoleamines. In mammals, it catalyses the initial step in the metabolism of L-tryptophan along the kynurenine pathway [1]. Indoleamine 2,3-dioxygenase is expressed in a number of tissues including the lung, intestine, placenta and stomach [2]. Other features of the enzyme include stimulation of activity by interferon-γ [3] and inhibition by 1-methyl-tryptophan [4].

Although the properties of indoleamine 2,3-dioxygenase have been thoroughly studied its precise physiological roles are only just being elucidated. A major advance is the finding that indoleamine 2,3-dioxygenase is involved in protecting the immunologically foreign conceptus from the maternal immune system [5]. It is envisaged that placental indoleamine 2,3-dioxygenase prevents the proliferation of maternal T lymphocytes at the maternal–fetal interface by lowering the local L-tryptophan concentration. This has been termed as immunosuppression by starvation [6]. On the other hand, it has been suggested that tryptophan catabolites produced by indoleamine 2,3-dioxygenase may be involved in immunoregulation by inhibiting T lymphocyte proliferation [7–9].

Indoleamine 2,3-dioxygenase is also believed to be involved in regulating tumour growth [3]. A role for the enzyme as an anti-tumour agent has focussed primarily on the possibility that indoleamine 2,3-dioxygenase inhibits tumour growth as a consequence of depriving cells of the essential amino acid L-tryptophan. However, Mellor et al. [10] have recently proposed that tumour cells may utilize indoleamine 2,3-dioxygenase to protect themselves from the immune system in a manner analogous to that found in the fetal-placental unit (i.e. immunosuppression by starvation) as described above. In support of this notion is the report that 1-methyl-tryptophan, administered in vivo, resulted in the delay of Lewis lung carcinoma tumour growth in syngeneic mice [11].

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As a first step in examining the potential role of indoleamine 2,3-dioxygenase in breast cancer pathology, we have investigated the activity and expression of the enzyme in two human breast cancer cell lines. We have studied indoleamine 2,3-dioxygenase in MDA-MB-231 and MCF-7 cells which are, respectively, estrogen receptor-negative and -positive cells. We have also examined the pathways available for the transport of L-tryptophan in human breast cancer cells as the provision of substrate may be an important factor in the regulation of indoleamine 2,3-dioxygenase activity [12].

2. Methods

2.1. Culture of MDA-MB-231 and MCF-7 cells

MDA-MB-231 and MCF-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with L-glutamine (2 mM), heat-inactivated fetal bovine serum (10%), penicillin (50 IU/ml) and streptomycin (50 μg/ml). Both cell lines were cultured at 37 °C in a gas phase of air with 5% CO_2. Cells were cultured in 75 cm^2 flasks containing 12 ml of culture medium for 4–5 days until they had reached approximately 90% confluency. The cells were then cultured for a further 20 or 48 h in the absence or presence of interferon-γ. For transport studies, cells were seeded in 35 mm culture wells containing 2 ml of culture medium at a density of $0.3-1\times10^6$ cells per well and were used 24–48 h later when they had reached 60–90% confluency.

2.2. RNA isolation and RT-PCR

Total cellular RNA was isolated from breast cancer cells and human placental tissue using Triazol reagent (InVitrogen). Maternal decidue was removed from the placental tissue prior to RNA extraction. RNA was suspended in water and the concentration determined by the A_{260}, where one unit of absorbance measured at 260 nm corresponds to 40 μg of RNA. The integrity of the RNA was checked by electrophoresis in a 1.2% agarose gel containing 2.2 M formaldehyde and 0.5 μg/ml of ethidium bromide [13].

PCR primers for indoleamine 2,3-dioxygenase and CD98 were designed from the sequences available in GenBank (accession numbers X17668 and AH001404 for indoleamine 2,3-dioxygenase and CD98 (4F2hc), respectively). Primer pairs were designed to different exons to prevent the amplification of any contaminating DNA. The primer pairs used in the subsequent PCRs were as follows: indoleamine 2,3-dioxygenase, forward 5′ AACAGCGCCTTTAGCAAAGTGTCCCGTTCTTG 3′, and reverse 5′ AGCGCCTTGCACGTCTAGTTCTGGGATGC 3′; CD98, forward 5′ CTGGCGGGTCTGAAGGGGCGTCTCGATTAC 3′ and reverse 5′ TCACCCCCGTAGTTGGGAGTAAGGTCCAGA 3′. The expected sizes of the PCR products were 376 bp for indoleamine 2,3-dioxygenase and 224 bp for CD98.

cDNA was prepared from 1 μg samples of total RNA using M-MLV reverse transcriptase (Promega) and random hexamers (Pharmacia). The cDNA (0.04 μg equivalent to RNA) was PCR amplified using Taq polymerase (ThermoStart Master Mix, ABgene) and 0.2 μM of each of the appropriate primer pair, in a total volume of 25 μl. Samples were denatured initially at 94 °C for 5 min, followed by 35 cycles of 92 °C for 30 s, 59.8 °C (for indoleamine 2,3-dioxygenase) or 60.3 °C (for CD98) for 30 s and 72 °C for 30 s with a final 5 min extension at 72 °C. Aliquots (5 μl) of the PCRs were then resolved on a 1% agarose/TBE gel and the products visualised by ethidium bromide staining. The authenticity of the PCR products were verified by digestion with specific restriction endonucleases.

2.3. Measurement of indoleamine 2,3-dioxygenase activity

Indoleamine 2,3-dioxygenase activity was measured using the method of Takikawa et al. [14] as modified by Kudo and Boyd [15]. MDA-MB-231 and MCF-7 cells were harvested by trypsin digestion. The cells were washed twice and resuspended in 1 ml of a buffer containing (mM) 130 NaCl and 50 Tris-MOPS, pH 7.4. The cells were disrupted by sonification for 30 s on ice at a power of 100 W. The homogenate was centrifuged at $12{,}000\times g$ for 5 min at room temperature. Samples of the supernatant were taken to determine the protein content by the Lowry assay using bovine serum albumin as standard. Following this, 0.4 ml of the supernatant was added to an equal volume of a solution containing 1 mM L-tryptophan, 20 μM methylene blue, 40 mM ascorbic acid, catalase (200 units/ml) and 100 mM potassium phosphate buffer, pH 6.5. Both the enzyme suspension and incubation buffer were pre-heated to 37 °C before mixing. The mixture was incubated for a further 30 min at 37 °C. The reaction was stopped by adding 0.2 ml of 30% (w/v) trichloroacetic acid. The mixture was incubated at 50 °C for 30 min to hydrolyse *N*-formylkynurenine produced by indoleamine 2,3-dioxygenase to kynurenine. The reaction mixture was then centrifuged at $12{,}000\times g$ at room temperature to remove sediment. Supernatant (0.8 ml) was added to 0.8 ml of 1% (w/v) *p*-dimethylaminobenzaldehyde in acetic acid. The absorbance at 480 nm was determined.

2.4. Measurement of L-tryptophan by HPLC

Samples of culture medium either unconditioned or conditioned by culture with MDA-MB-231 and MCF-7 cells were deproteinised as follows: each sample (900 μl) was added to a mixture of 15% perchloric acid/2 mM cysteine (100 μl), vortexed and allowed to stand on ice for

at least 15 min. Following this, the mixture was centrifuged for 5 min at 20,000 × *g* at room temperature. The supernatants were removed and stored at 4 °C prior to assay. Standards containing 5 or 50 μM L-tryptophan and 1.5% perchloric acid/0.2 mM cysteine in double-distilled water were injected at the start, middle and end of each assay. The HPLC system was isocratic, using a mobile phase of 2.7% acetonitrile, 97.3% water v/v and 15 mM KH_2PO_4; delivered at a rate of 1 ml/min. For analysis, 20 μl of standard or sample were injected. Separation was achieved using an APEX 25 cm reverse-phase column (5 μm diameter packing; Jones Chromatography). The detector was a Kratos Spectroflow 98 fluorimeter, with excitation at 230 nm and emission >370 nm; tryptophan was measured at 0.2 fluorescence units full-scale. Chromatograms were recorded on a Shimadzu C-R6A Chromatopac integrator, with peak height being used to calculate concentrations. The mean coefficients of variation were 1.1% (intra-assay; $n=6$) and 10.4% (inter-assay; $n=7$).

2.5. Measurement of L-tryptophan uptake

The unidirectional uptake of L-tryptophan into MDA-MB-231 cells was measured according to the method of Shennan et al. [16] using L-[5-^{3}H]tryptophan (Amersham International, PLC; specific activity 33.0 Ci/mmol) as tracer. The culture medium was removed and the cells were washed three times with 3 ml of a buffer containing (mM) 0.01 L-tryptophan, 135 NaCl or choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4. The cells were then preincubated for 5 min at 37 °C in 3 ml of an appropriate buffer (see figure legends for details). L-Tryptophan influx experiments were initiated by aspirating the preincubation buffer and replacing it with 1 ml of a buffer containing (mM) 0.01 L-tryptophan, 135 NaCl or choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4 plus 1 μCi/ml of [^{3}H]L-tryptophan and 0.4 μCi/ml of [^{14}C]sucrose together with other additions such as unlabelled amino acids. The specific activity of the isotopes in the incubation buffer was determined by counting the radioactivity associated with 10 μl of buffer: the samples were prepared for counting by adding 0.5 ml of distilled water and 10 ml of UltimaGold liquid scintillation cocktail. After 30 s, the incubation buffer was removed and the cells were washed four times with 3 ml of an ice-cold buffer containing (in mM): 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4. The cells were lysed by incubating in 1 ml of distilled water for at least 3 h during which time the lysate was frequently agitated. A sample of the lysate (0.5 ml) was added to an equal volume of 10% TCA (w/v) and centrifuged at 12,000 × *g* for 10 min, 0.5 ml of the supernatant was prepared for counting by adding 10 ml of UltimaGold liquid scintillation fluid. Samples of the lysate (0.1 ml) were taken to determine the protein content by the Lowry assay using bovine serum albumin as a standard. L-Tryptophan uptake was calculated according to Eq. (1):

$$[\text{L-tryptophan}]_c = ([\text{L-tryptophan}]_t - (F[\text{L-tryptophan}]_m))/P \quad (1)$$

where $[\text{L-tryptophan}]_c$ is the amount of L-tryptophan in the cells (pmol/mg protein), $[\text{L-tryptophan}]_t$ is the total amount of L-tryptophan in the lysate (pmol), $[\text{L-trytophan}]_m$ is the amount of L-tryptophan in the medium (pmol), F is the ratio of radiolabelled sucrose in the lysate to that in the incubation medium and P is the amount of protein in the lysate (mg).

2.6. Measurement of tryptophan efflux

The efflux of L-tryptophan from MDA-MB-231 cells was assayed using L-[5-^{3}H]tryptophan as tracer. After removing the culture medium, the cells were washed three times with 2 ml of a solution containing (mM) 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4. The cells were loaded with radiolabelled L-tryptophan over a period of 10 min at 37 °C by incubating in 2 ml of a buffer similar in composition to that just described except that it also contained 1 μCi/ml of [^{3}H]L-tryptophan. After the loading period, the cells were washed four times in rapid succession with a radioactive-free buffer similar in composition to that described above. The efflux of [^{3}H]L-tryptophan from the cells was measured by the sequential addition and removal of 2 ml of buffer (see figure legends for precise details) at 1-min intervals. At the end of the wash-out period, cells were incubated in 2 ml of 10% TCA for at least 3 h. Following this, the samples were centrifuged at 12,000 × *g* for 5 min after which 1 ml of the supernatant was added to 10 ml of UltimaGold liquid scintillation fluid. The fractional release for each collection period was calculated according to Eq. (2):

$$\text{Fractional efflux} = \Delta X/(\Delta t X_t) \quad (2)$$

where ΔX is the amount of radiolabelled L-tryptophan released from the cells in the time interval Δt and X_t is the amount of isotope in the cells at the start of each collection period.

3. Results

3.1. Indoleamine 2,3-dioxygenase activity

Fig. 1 illustrates the activity of indoleamine 2,3-dioxygenase in MDA-MB-231 and MCF-7 cells which had been cultured in the absence and presence of interferon-γ (1000 units/ml) for 48 h. Kynurenine production was taken as a measure of enzyme activity. It is evident that indoleamine 2,3-dioxygenase activity was relatively low

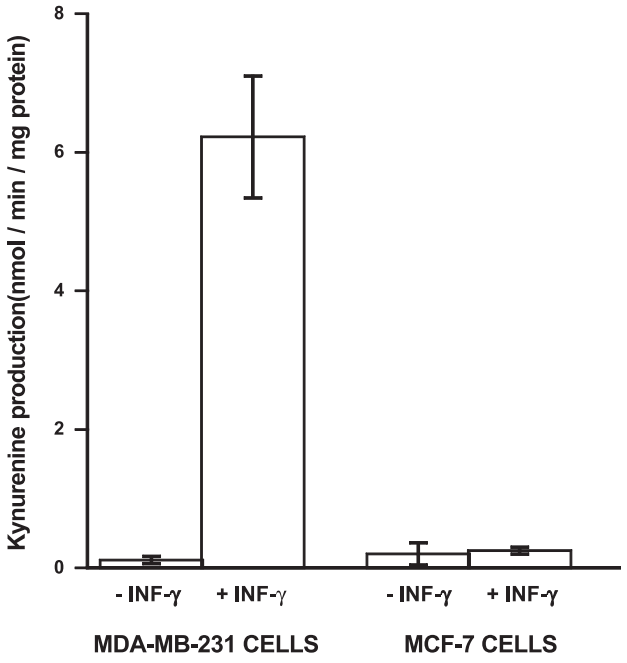

Fig 1. Indoleamine 2,3-dioxygenase activity in MDA-MB-231 and MCF-7 cells cultured in the presence and absence of interferon-γ (1000 units/ml) for 48 h (values shown are mean ± S.E., $n=9$ and 4, respectively, when MDA-MB-231 and MCF-7 cells were used).

in both MDA-MB-231 and MCF-7 cells when cultured in the absence of interferon-γ. However, interferon-γ markedly stimulated enzyme activity in MDA-MB-231 cells. Thus, kynurenine production by indoleamine 2,3-dioxygenase from cells which had been cultured in the absence and presence of the cytokine was, respectively, 0.12 ± 0.05 and 6.22 ± 0.88 nmol/min/mg protein (± S.E., $n=9$, $P<0.001$). In contrast, interferon-γ failed to significantly stimulate indoleamine 2,3-dioxygenase activity in MCF-7 cells: the activity of the enzyme from cells which had been cultured with and without interferon-γ was, respectively, 0.25 ± 0.04 and 0.20 ± 0.16 nmol/min/mg protein (S.E., $n=4$). The stimulation of indoleamine 2,3-dioxygenase activity in MDA-MB-231 cells by interferon-γ was time-dependent. Thus, the cytokine-induced production of kynurenine by MDA-MB-231 cells cultured for 20 h was significantly lower ($P<0.001$) than that found after a 48 h incubation (2.09 ± 0.22 *v* 6.11 ± 0.84 nmol/min/mg protein). We also found that 1-Methyl-DL-tryptophan (1 mM), a competitive inhibitor of indoleamine 2,3-dioxygenase [4], reduced the interferon-γ-induced production of kynurenine by MDA-MB-231 cells by 46.7%: kynurenine formation in the absence and presence of 1-methyl-DL-tryptophan was, respectively 7.83 ± 1.63 and 4.17 ± 1.01 nmol/min/mg protein (± S.E., $n=3$, $P<0.05$). 1-Methyl-DL-tryptophan had no effect on kynurenine production by cells which had been cultured in the absence of interferon-γ.

The concentration of L-tryptophan in the conditioned medium following culture of MDA-MB-231 and MCF-7 cells was measured (Table 1). In this set of experiments, cells were cultured with or without interferon-γ (1000 units/ml) for 48 h. It is evident that inclusion of interferon-γ in the culture medium markedly stimulated the degradation of L-tryptophan by MDA-MB-231 cells and to a lesser extent by MCF-7 cells.

3.2. Expression of indoleamine 2,3-dioxygenase mRNA

We subjected total RNA isolated from MDA-MB-231 and MCF-7 cells (which had been cultured in the absence of interferon-γ) to RT-PCR to test for the expression of indoleamine 2,3-dioxygenase mRNA. Human placental tissue was used as a positive control since the presence of indoleamine 2,3-dioxygenase mRNA in human placental trophoblast has been reported [17]. CD98 expression was also used as a positive control given that it is expressed in MDA-MB-231 cells, MCF-7 cells and human placental tissue [16]. In accordance with previous results, we found a product for the expected size for CD98 in all three cell/tissue types. A product for the expected size for indoleamine 2,3-dioxygenase mRNA was found in placental tissue and MDA-MB-231 cells but not MCF-7 cells (Fig. 2).

3.3. L-Tryptophan transport by MDA-MB-231 cells

Indoleamine 2,3-dioxygenase is a tryptophan catabolizing enzyme, therefore, the provision of the essential amino acid could be an important point in the regulation of enzyme activity [12]. In view of this, we investigated the transport (i.e. influx and efflux) of L-tryptophan by MDA-MB-231 cells. Fig. 3 shows that L-tryptophan uptake into MDA-MB-231 cells was not affected by replacing extracellular Na^+ with choline. Tryptophan uptake in the presence and absence of extracellular Na^+ was, respectively, 1.17 ± 0.05 and 1.29 ± 0.05 nmol/30 s/mg protein. Fig. 3 also shows that L-tryptophan influx was inhibited by BCH (2-amino-2-norbornane-carboxylic acid), a compound regarded to be a relatively specific inhibitor of system L. Replacing Na^+ with choline did not affect the inhibition of L-tryptophan uptake by BCH (Fig. 3). Thus, BCH inhibited L-tryptophan uptake by 91.3 ± 3.6% and 94.2 ± 2.0%,

Table 1
Effect of interferon-γ on L-tryptophan concentrations in medium conditioned following culture of MDA-MB-231 and MCF-7 cells

	L-Tryptophan concentration (μM)		
	Non-conditioned	− Interferon-γ	+ Interferon-γ
MDA-MB-231	66.2 ± 2.7	53.1 ± 1.7*	8.0 ± 6.8**††
MCF-7	72.2 ± 2.7	56.8 ± 2.5*	41.2 ± 1.8*†

Culture conditions were the same as those described in Fig. 1. Each value is the mean ± S.E. of three experiments.
† $P<0.05$ vs. − Interferon-γ
†† $P<0.02$ vs. − Interferon-γ

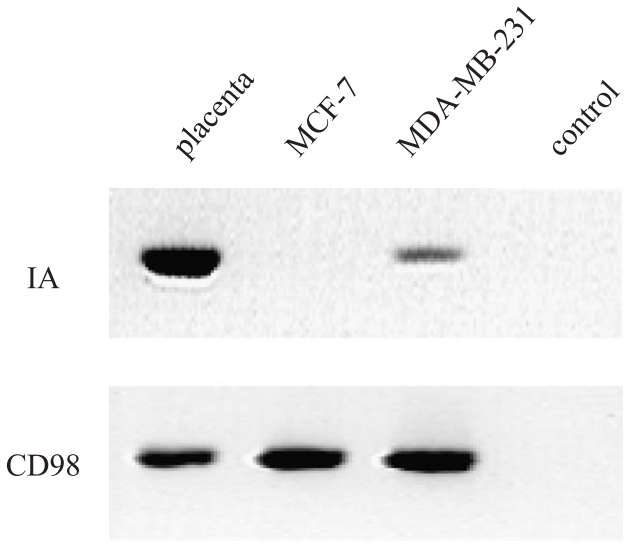

Fig. 2. Indoleamine 2,3-dioxygenase and CD98 RT-PCR products from human placenta, MCF-7 cells and MDA-MB-231 cells. The control track represents PCR in the absence of cDNA. Samples are representative of three independent RNA preparations for each cell/tissue type.

respectively, in the presence and absence of extracellular Na^+.

We examined the properties of L-tryptophan efflux from MDA-MB-231 cells incubated under Na^+-free conditions. BCH stimulated L-tryptophan efflux from MDA-MB-231 cells (Fig. 4A). Thus, BCH at a concentration of 1 mM increased L-tryptophan efflux (basal-to-peak) from 0.032 ± 0.005 min^{-1} to 0.523 ± 0.050 min^{-1} (S.E., $n=3$, $P<0.01$). We also found that 1-methyl-D,L-tryptophan stimulated L-tryptophan efflux from MDA-MB-231 cells in a dose-dependent manner (Fig. 4B). 1-Methyl-D,L-tryptophan at a concentration of 0.01, 0.1 and 1.0 mM, respectively, stimulated L-tryptophan efflux (basal-to-peak) by $327.8 \pm 19.2\%$ ($P<0.01$), $624.4 \pm 38.6\%$ ($P<0.01$) and $958.9 \pm 71.4\%$ ($P<0.001$). Notably, BCH (1 mM) did not

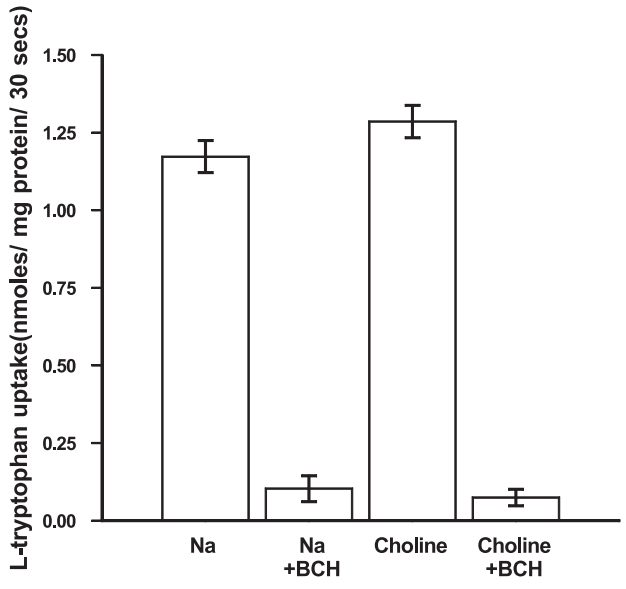

Fig. 3. The effect of BCH on L-tryptophan uptake by MDA-MB-231 cells in the presence and absence of extracellular Na^+. The incubation medium contained (in mM): 0.01 L-tryptophan, 135 NaCl or choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4, 1 μCi/ml [^{3}H]L-tryptophan and 0.4 μCi/ml [^{14}C]sucrose ± 10 BCH. Uptake was measured at 37 °C after 30 s of incubation. The results are the mean ± S.E. of three experiments.

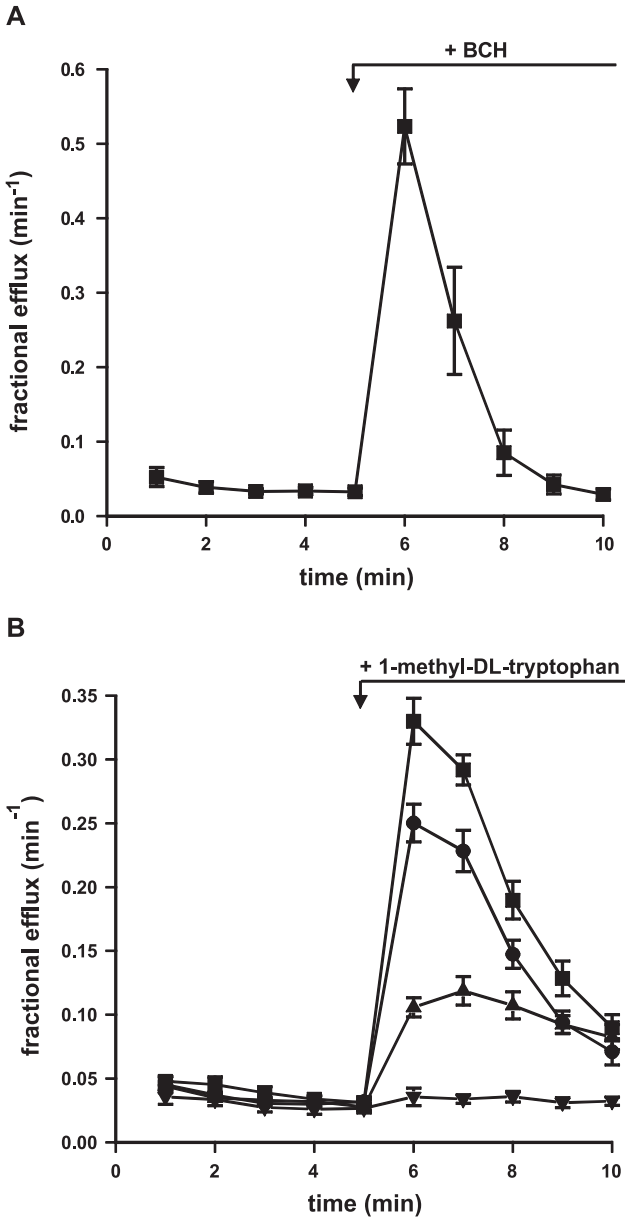

Fig. 4. The effect of (A) BCH and (B) 1-methyl-DL-tryptophan (B) on L-tryptophan efflux from MDA-MB-231 cells. The incubation medium contained (in mM): 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose and 10 Tris-MOPS, pH 7.4. BCH when required was used at a concentration of 1 mM. 1-Methyl-DL-tryptophan, when required was used at a concentration of 0.001 mM (▼), 0.01 mM (▲), 0.1 mM (●) and 1 mM (■). Each point is the mean ± S.E. of three experiments.

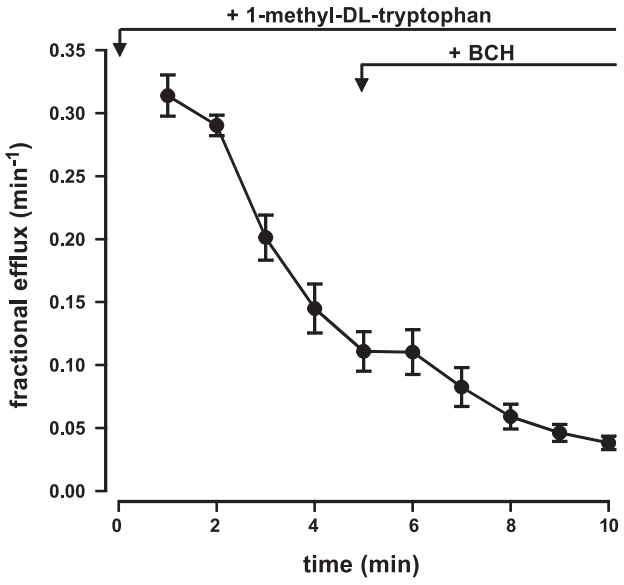

Fig. 5. The effect of BCH in the presence of 1-methyl-DL-tryptophan on L-tryptophan efflux from MDA-MB-231 cells. The incubation medium contained (in mM): 135 choline-Cl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 10 glucose, 1 1-methyl-DL-tryptophan and 10 Tris-MOPS, pH 7.4 ± 1 BCH. Each point is the mean ± S.E. of six experiments.

trans-stimulate L-tryptophan efflux in the presence of 1 mM 1-methyl-DL-tryptophan (Fig. 5).

4. Discussion

4.1. Activity and expression of indoleamine 2,3-dioxygenase

On the basis that indoleamine 2,3-dioxygenase appears to play a central role in protecting the fetus from a maternal immune response, Mellor et al. [10] suggested that the catabolism of L-tryptophan by indoleamine 2,3-dioxygenase may also help tumour cells escape the attention of the immune system. This prompted us to investigate the potential role of indoleamine 2,3-dioxygenase in breast cancer pathology. Thus, we have investigated the expression and activity of indoleamine 2,3-dioxygenase in two human breast cancer cell lines, namely MDA-MB-231 (estrogen receptor-negative) and MCF-7 (estrogen receptor-positive). The results show that MDA-MB-231 cells express indoleamine 2,3-dioxygenase at the functional and molecular level. Thus, MDA-MB-231 cells express mRNA for the enzyme and are capable of metabolizing L-tryptophan via the kynurenine pathway in a time-dependent fashion when cultured in the presence of interferon-γ. Accordingly, we found that interferon-γ increased L-tryptophan utilization by MDA-MB-231 cells. Another important feature is that interferon-γ-induced kynurenine production by MDA-MB-231 was sensitive to 1-methyl-DL-tryptophan. Indoleamine 2,3-dioxygenase from the rabbit intestine and human placenta is competitively inhibited by 1-methyl-DL-tryptophan [4,12]. Indeed, this compound was used by Munn et al. [5] to inhibit indoleamine 2,3-dioxygenase in pregnant mice which, in turn, resulted in fetal rejection.

In contrast to MDA-MB-231 cells, we found that MCF-7 cells did not express indoleamine 2,3-dioxygenase mRNA. Moreover, in accordance with previous results [18], we found that basal kynurenine production in MCF-7 cells was low and could not be induced by interferon-γ. In view of this, it is tempting to suggest that there may be a difference between estrogen receptor-positive and -negative breast cancer cells with respect to the expression of indoleamine 2,3-dioxygenase. We did find, however, that interferon-γ stimulated L-tryptophan utilization by MCF-7 cells, albeit to a smaller extent. It is apparent that the interferon-γ-induced catabolism of L-tryptophan by MCF-7 cells must occur via another, as yet unidentified, pathway.

The finding that MDA-MB-231 cells express indoleamine 2,3-dioxygenase suggest that estrogen receptor-negative breast cancer cells may express the enzyme in vivo. Therefore, this study forms the basis for future work on the potential role of indoleamine 2,3-dioxygenase in human breast cancer pathology. A major focus should be identifying the presence of the enzyme in primary breast cancer cells and examining the relationship between its expression and hormone-receptor status. Based on our findings with cell lines, there is the distinct possibility that Munn et al.'s hypothesis of immunosuppression by starvation could be applicable to estrogen receptor-negative breast cancer cells. It is envisaged that breast cancer cells could utilize indoleamine 2,3-dioxygenase to reduce L-tryptophan in their immediate environment and consequently inhibit T cell proliferation. Alternatively, production of L-tryptophan metabolites by indoleamine 2,3-dioxygenase could possibly result in T cell apoptosis [9]. Whilst this report was being prepared, Uyttenhove et al. [19] reported that indoleamine 2,3-dioxygenase protein could be detected in a variety of human tumours including breast carcinomas. However, whilst indoleamine 2,3-dioxygenase was present in all prostate, colorectal, pancreatic and cervical tumours analysed, only 30% of breast tumours exhibited the enzyme. It is possible, as suggested above, that the expression of the enzyme in vivo may be related to the hormone-receptor status of the tumour which was not reported by Uyttenhove et al. [19].

4.2. Transport of L-tryptophan by MDA-MB-231 cells

L-Tryptophan transport, via system L, in human placental tissue has been shown to play an important role in the regulation of indoleamine 2,3-dioxygenase [12]. In view of this, we examined L-tryptophan transport in MDA-MB-231 cells because an understanding of the transport mechanism could be important in relation to developing potential therapeutic strategies aimed at inhibiting indoleamine 2,3-dioxygenase activity in breast cancer cells. The results show that L-tryptophan, the major substrate of indoleamine 2,3-dioxygenase, gains access to MDA-MB-231 cells via a BCH-sensitive, Na^+-independent pathway. Therefore, the

results are consistent with system L, a Na^+-independent neutral amino acid transport mechanism, being the major conduit for L-tryptophan entry into MDA-MB-231 cells. System L is not a single transporter and recent studies have shown that there are at least two molecular correlates of system L [20–24]. Two proteins termed LAT1 and LAT2 induce system L-like activity when co-expressed with CD98. Thus, the transport protein is a heterodimer consisting of a light chain (LAT1 or LAT2) and a heavy chain (CD98). In this connection, we have recently shown that MDA-MB-231 cells express LAT1 (but not LAT2) and CD98 [16]. Indeed, from the present results, it appears that system L (LAT1/CD98) may be the only pathway available for L-tryptophan transport into MDA-MB-231 cells under these conditions. This places system L in a potentially important position as a therapeutic target. We have also shown that 1-methyl-D,L-tryptophan, a competitive inhibitor of indoleamine 2,3-dioxygenase, was able to *trans*-stimulate L-tryptophan efflux from MDA-MB-231 cells. Importantly, BCH failed to *trans*-accelerate L-tryptophan efflux in the presence of extracellular 1-methyl-D,L-tryptophan suggesting that both compounds are acting at a single locus. This suggests that 1-methyl-D,L-tryptophan is acting to stimulate L-tryptophan efflux via system L (LAT1/CD98). Moreover, the finding that 1-methyl-D,L-tryptophan *trans*-stimulates L-tryptophan efflux from MDA-MB-231 cells suggest that 1-methyl-D,L-tryptophan is a transported substrate of system L. This finding may assume more importance in light of the suggestion that 1-methyl-tryptophan could possibly be used to enhance anti-tumour immunity in cancer patients [10,19].

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