Monitoring tryptophan metabolism in chronic immune activation

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Abstract

The essential amino acid tryptophan is a constituent of proteins and is also a substrate for two important biosynthetic pathways: the generation of neurotransmitter 5-hydroxytryptamine (serotonin) by tryptophan 5-hydroxylase, and the formation of kynurenine derivatives and nicotinamide adenine dinucleotides. The latter pathway is initiated by the enzymes tryptophan pyrrolase (tryptophan 2,3-dioxygenase, TDO) and indoleamine 2,3-dioxygenase (IDO). TDO is located in liver cells, whereas IDO is expressed in a variety of cells including monocyte-derived macrophages and dendritic cells and is preferentially induced by Th1-type cytokine interferon-γ. Tryptophan depletion via IDO is part of the cytostatic and antiproliferative activity mediated by interferon-γ in cells. In vivo tryptophan concentration can be measured by HPLC by monitoring its natural fluorescence (285 nm excitation and 365 nm emission wavelength). IDO activity is characterized best by the kynurenone to tryptophan ratio which correlates with concentrations of immune activation markers such as neopterin. Low serum/plasma tryptophan concentration is observed in infectious, autoimmune, and malignant diseases and disorders that involve cellular (Th1-type) immune activation as well as during pregnancy due to accelerated tryptophan conversion. Thus, in states of persistent immune activation, low tryptophan concentration may contribute to immunodeficiency. Decreased serum tryptophan can also effect serotonin biosynthesis and thus contribute to impaired quality of life and depressive mood. As such, monitoring tryptophan metabolism in chronic immunopathology provides a better understanding of the association between immune activation and IDO and its role in the development of immunodeficiency, anemia and mood disorders.

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Keywords: Tryptophan; Indoleamine (2,3)-dioxygenase; Depression

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1. Tryptophan metabolism

The essential amino acid L-tryptophan is required for the biosynthesis of proteins and is precursor for several biologically important compounds (Fig. 1): (a) 5-hydroxytryptamine (serotonin) which is formed by tryptophan (5)-hydroxylase (EC 1.14.16.4) following decarboxylation; (b) tryptophan (2,3)-dioxygenase (TDO, tryptophan pyrrolase, EC 1.13.1.2); and (c) indoleamine (2,3)-dioxygenase (IDO, EC 1.13.11.42). The latter two catabolize tryptophan via the so-called kynurenine-pathway synthesizing nicotinic acid, the vitamin niacin and nicotinamide adenine dinucleotides as end products [1]. Although TDO is localized to the liver and is up-regulated by corticosteroids, IDO is expressed by a variety of cells and is inducible preferentially by Th1-type cytokine interferon-γ (IFN-γ) [2–5]. Similar to TDO, IDO catalyzes the first step in tryptophan degradation, i.e., formation of N-formylkynurenine which subsequently deformylates to kynurenine. IFN-γ is a potent stimulus for IDO in vitro and also in vivo. Tryptophan degradation can be detected in various cells including antigen-presenting cells such as monocyte-derived macrophages, dendritic cells and fibroblasts [2–5] (Fig. 2) and in humans [6]. Other cytokines or lipopolysaccharides are also capable of inducing IDO, although less effectively [6–8]. In vivo, enhanced cytokine-induced degradation of tryptophan is observed whenever the cellular (Th1-type) immune response is induced. In this case, a decrease of serum tryptophan concentration and concomitant increase in kynurenine or other tryptophan catabolites can be detected [6,9–13].

Under normal conditions, kynurenine concentration is related to tryptophan level. Reduced dietary intake of tryptophan lowers endogenous tryptophan level. Under these circumstances, lower kynurenine concentration is also observed. The kynurenin (kyn) to tryptophan (trp) ratio (kyn/trp), i.e., the ratio of the concentration of the first product of TDO and IDO versus the concentration of their substrate is an appropriate indicator of tryptophan degradation. The kyn/trp ratio provides a better and normalized measurement than absolute tryptophan or kynurenine concentration. To substantiate that tryptophan degradation is due to activation of IDO rather than TDO it is necessary to demonstrate concomitant immune system activation [10,14] (Table 1). Thus, activated IDO is indicated when kyn/trp correlates with an immune activation parameter and endogenous IFN-γ formation. For example, determination of neopterin concentration is a sensitive laboratory diagnostic tool to detect and monitor Th1-type immune activation in humans and primates [34]. Neopterin concentration can be easily measured in body fluids or cell culture supernatants. Increased neopterin concentration is detected during viral infection, in autoimmune disease, allograft rejection and in malignant disease. In these clinical conditions a close association between neopterin concentration and accelerated tryptophan degradation is found.

2. IDO to limit cellular growth via tryptophan deprivation

The pro-inflammatory cytokine IFN-γ induces the enzyme IDO in a variety of cells [15]. IDO activation limits availability of tryptophan (Fig. 2). Because tryptophan is required for protein synthesis, withdrawal of this essential amino acid from the micro-environment arrests protein biosynthesis and subsequent growth of pathogens and proliferating cells (Fig. 3). Consequently, tryptophan depletion is regarded as a defense mechanism induced by IFN-γ in immunocompetent cells during immune response. This phenomenon acts as an antimicrobial or antitumoral effector mechanism and limits the growth of intracellular pathogens or malignant cells [16–18]. Activation of IDO may also inhibit response of T-cells to mitogenic stimulation in vitro and in vivo [5,19–21]. This is especially true when the enzyme is induced by IFN-γ in macrophages and dendritic cells. Thus, in addition to tryptophan deprivation, the pro-
apoptotic effect of certain tryptophan catabolites such as kynurenine is also important [22,23]. These observations support the view that activation of IDO together with other biochemical pathways induced by IFN-γ represents an important antiproliferative mechanism of monocyte-derived macrophages and dendritic cells. This phenomenon, however, can also decrease the response of stimulated T-cells and thus contribute to development of immunodeficiency [24].

3. Measurement of tryptophan and kynurenine

Tryptophan measurement in serum, plasma and other body fluids is performed by HPLC using reversed phase C18 columns [14,25]. A pre-column is advantageous to protect the C18 separation column from apolar materials of biologic origin. Tryptophan is easily monitored by its natural fluorescence at an excitation wavelength of 285 nm and an emission wavelength of 365 nm. To accurately assess rate of tryptophan degradation, it is necessary to additionally analyze the concentration of the first product of IDO reaction (kynurenine) using a UV-detector set at a wavelength of 360 nm. Nitrotyrosine is useful as an internal standard since its concentration can be monitored at the same wavelength as kynurenine [14].

Following collection of blood specimens, serum/plasma is separated by centrifugation and can be stored at −20 °C for several weeks. For analysis, 100 µl serum/plasma are diluted with 100 µl potassium phosphate buffer (0.05 mol/L, pH 6.0). Protein is precipitated with 25 µl trichloroacetic acid (2 mol/L), and the capped tubes with the precipitate are immediately vortexed and centrifuged for 10 min at 13,000 ×g. An aliquot of the supernatant (150 µl) is transferred into micro-vials (Chromacol Ltd.) and placed into the autosampling device.

The external calibrator is prepared by the same method using freshly thawed stock solutions of tryptophan and kynurenine (1 mmol/L in double-distilled water, stored at −20 °C). The external calibrators tryptophan (50 µl) and kynurenine (10 µl) are then mixed with 940 µl albumin solution (70 g/L, corresponding to the average physiological protein content in human serum) for form a stock standard solution. Aliquots (200 µl) of stock standard solutions are then treated in the same way as serum specimens.

HPLC analysis was performed at a flow rate of 0.8 ml/min using a 55 mm column (3 µm grain size, LiChroCART, Merck, Darmstadt) at 25 °C. Potassium phosphate solution (0.015 mol/L, pH 6.4) containing 2.7% acetonitrile (v/v) is recommended as elution buffer. Retention times for kynurenine, tryptophan, and nitrotyrosine were 2.3, 4.3 and 3.3 min, respectively. Total analysis time is 7 min. After 30–40 measurements, the pre-analytical guard column is replaced and the HPLC column washed using a gradient from water to methanol and back (40 min).

In the serum healthy blood donors, concentration of metabolites were determined to be 73.0 ± 14.9 µmol/L tryptophan, 1.92 ± 0.58 µmol/L kynurenine, and 26.9 ± 8.10 kyn/trp (µmol/µmol) [14]. Tryptophan and kynurenine concentration was approximately 15% higher in men than women. This gender differences may be related to hormonal status since human chorionadotropin hormone (hCG) was found to induce IDO expression in monocytes obtained from healthy non-pregnant women in vitro [26].

4. Accelerated tryptophan catabolism due to IDO activation in patients

In various diseases (Table 2), decreased tryptophan concentration and increased concentrations of kynurenine

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and other tryptophan catabolites such as quinolinic acid have been described [9–13,15,27]. The correlation found between neopterin concentration and kyn/trp ratio (Fig. 4) suggests that an increased endogenous production of IFN-γ takes place under these conditions. This finding supports the conclusion that activation of IDO is involved in enhanced rate of tryptophan catabolism.

4.1. Infection

Patients with HIV infection present with decreased tryptophan and increased kynurenine concentration thus indicating accelerated tryptophan degradation. Strong association between kyn/trp ratio and concentrations of kynurenine and neopterin, and IFN-γ [28] and other markers of immune activation like soluble cytokine receptor sTNF-R75 can be observed [29,30]. Antiretroviral therapy (ART) is able to significantly reduce tryptophan degradation: tryptophan concentration increases whereas kynurenine concentration decreases [29,31,32]. Change in kyn/trp ratio during ART correlates more strongly with the change of neopterin levels than with the change of HIV RNA level and only weakly with percentage change of CD4 cell count [32]. Activated IDO appears responsible for enhanced degradation of tryptophan. Thus, it is likely that ART is able to reduce effects of HIV infection on IDO activity via a reduction of immune activation, particularly IFN-γ production. The data confirm the premise that HIV production eliciting T-cell activation and release of IFN-γ may represent the main stimulus for activating IDO in monocyte-derived macrophages, dendritic cells and various other cells.

In trauma patients, degradation of tryptophan was found to be associated with neopterin concentration and development of sepsis. In tryptophan deficient states, a substantial decrease in lymphocyte count was also found in these patients [33]. An extremely high rate of tryptophan degradation was observed in the blood samples collected from humans infected by Streptococcus pyogenes [34]. Superantigenes appeared to be responsible for the high degree of immune activation in these patients.

Enhanced degradation of tryptophan is also found in patients with neuroborreliosis [35]. Changes observed are more dramatic in the brain. In some patients the cerebrospinal fluid concentration of tryptophan becomes undetectable (<0.1 μmol/L) whereas micromolar concentrations of kynurenine are produced. Upon successful antibiotic treatment, tryptophan and kynurenine concentrations rapidly return to normal ranges.

4.2. Malignancy

In several malignant diseases, including solid tumors and haematological neoplasias, accelerated tryptophan catabolism has been described [12,36–40]. Lower tryptophan concentration and increased kyn/trp ratio are associated with more advanced stages of disease. In patients with adult T-cell leukaemia [41] or colon carcinoma [42], lower tryptophan concentration is predictive of shorter survival. Enhanced IDO expression was demonstrated in patients with inflammatory bowel disease and colon carcinoma [36,37]. In tumor patients, it has been claimed that tryptophan degradation may represent an intrinsic immunoevasive mechanism of tumor cells [37]. However, correlations between kyn/trp ratio and neopterin concentration [38] support the view that IDO activation in monocyte-derived macrophages and/or dendritic cells due to an enhanced endogenous formation of IFN-γ during the host’s response against the tumor may suppress T-cell proliferation, thus acting as immunosuppressants (Fig. 3). Reduced immunoresponse may develop as a consequence of the antiproliferative mechanism induced by this particular cytokine [24].

4.3. Other diseases

In autoimmune diseases such as systemic lupus erythematosus (SLE) [43] and rheumatoid arthritis, tryptophan...
degradation correlates with disease activity and markers of immune activation [44,45]. The autoimmune process includes induction of various cytokine cascades involving also IFN-γ production. However, in autoimmune syndromes, accelerated tryptophan degradation is obviously insufficient to counteract the deleterious effects of the immune reaction against targeted structures.

Immune activation and degradation of tryptophan are also found in neurodegenerative disorders such as Alzheimer’s [46], Huntington’s [47] and Parkinson’s disease [48]. However, only minor changes of tryptophan degradation can be found in the early disease course. In the very late stages, significant acceleration of tryptophan degradation and immune activation is common. IDO activation appears to correlate with the disease stage. It is important to note that increased tryptophan degradation and signs of immune activation are common during normal ageing [49]. However, the degree of tryptophan degradation in patients with neurodegenerative disorders is substantially greater than in normal ageing. It is likely that lowered tryptophan concentration in the elderly as well as younger patients may be related to the increased risk of developing depression and/or memory loss (Fig. 5).

4.4. Pregnancy

Increased rate of tryptophan degradation is observed during pregnancy [50]. Tryptophan degradation correlates to week of pregnancy with highest kyn/tryptophan ratio and lowest tryptophan concentration observed at term. Significant associations between kyn/tryptophan ratio, tryptophan and neopterin as well as soluble TNF receptor concentrations confirm that immune-mediated IDO activation is responsible for the decline of tryptophan in pregnancy. Recently, human choriogonadotropic hormone (hCG) was found to induce IDO expression in monocytes of healthy non-pregnant women in vitro [26].

After birth, immune activation ceases and tryptophan concentration returns to normal. Kynurenine concentration, however, remains increased contributing to increased kyn/tryptophan ratio [51]. Correlation between kyn/tryptophan ratio and immune activation markers no longer exists. This finding suggests that sustained increase of kynurenine (up to 6 weeks after pregnancy) occurs independently of IDO activation. As such, liver metabolism influenced by hormonal changes may play a role in this process [51].

An immunopathogenetic involvement of IDO activation during pregnancy was established recently [52]. Tryptophan degradation by IDO was found to be necessary for induction of tolerance to the fetus [51]. When the specific IDO inhibitor 1-methyl tryptophan was applied, successful pregnancy was no longer possible.

4.5. Immunostimulatory treatment

Treatment of patients with cytokines results in enhanced tryptophan degradation [4,6,53,54]. In addition to interferon, other cytokines possess this capacity, e.g., interleukin-2, which by itself has no effect on monocyte-derived macrophages to activate IDO [49]. Although all interferons directly stimulate IDO in target cells such as dendritic cells [5,55], interleukin-2 action appears to be indirect stimulating release of IFN-γ by T-cells which then activates IDO.

In patients with malignant melanoma under treatment with IFN-α, an association between decreased tryptophan concentration and development of depressive mood was observed [54].

5. Pathogenetic effects related to accelerated tryptophan degradation

IFN-γ is probably the most important antiproliferative cytokine released during Th1-type immune response. It induces several biochemical pathways and mechanisms in order to stop growth of microbes and tumor cells. Because of its outstanding potency it is not surprising to find side effects especially during clinical conditions of continuous immune system stimulation and IFN-γ production which may exert strong negative impact on cells of the host [56].

5.1. Immunodeficiency, anemia, cachexia

Enhanced degradation of tryptophan has been demonstrated in several diseases correlated to or characterized by acquired immunodeficiency. This is especially true in patients with HIV infection, but also in various mostly chronic diseases like autoimmune disorders or malignancy in which immunodeficiency develops with progressive disease, a sign of poor prognosis. Acquired immunodeficiency is the hallmark of progressing HIV infection. Activation of several immune compartments has been observed including B-cells, T-cells and macrophages. Several parameters of immune activation were found to predict disease development in patients with HIV infection [57,58], and data show that immune activation coexists with
immune deficiency [10,59]. Activation of IDO by IFN-γ could diminish T-cell responsiveness in patients with HIV infection (Fig. 3). In patients with HIV infection, reduced T-cell proliferative response to soluble antigens in vitro has been associated with immune activation status [60]. Similar relationships also exist in cancer patients.

Accelerated catabolism of tryptophan appears to play a role in the pathogenesis of the anemia of inflammation as well as in weight loss and the development of cachexia. It is well established that pro-inflammatory cytokines IFN-γ and TNF-α suppress growth and differentiation of erythroid progenitor cells [60]. These cytokines are related to cachexia. Interestingly, TNF-α was known as cachectin before its molecular characterization. Recently an association between lowered tryptophan concentration and drop in hemoglobin was reported in patients with anemia of inflammation [61]. Thus, IFN-γ induced tryptophan deprivation appears to be involved in the hematopoietic suppression in the patients and limitation of tryptophan availability may represent a key mechanism in cytokine-mediated inhibition of erythroid progenitor cells (Fig. 3). Likewise, in patients with hematologic neoplasias, low tryptophan concentration was associated with low serum albumin concentration and weight loss [12,40]. This association was apparent at patient study entry and during follow-up. IFN-γ-mediated tryptophan deprivation may slow down protein biosynthesis and in turn accelerate breakdown of muscle proteins.

5.2. Mood changes

Decreased tryptophan availability reduces the biosynthesis of neurotransmitter 5-hydroxytryptamine (serotonin) which can increase susceptibility for development of mood disturbances and depression and also impair cognitive function [62]. Immune-mediated tryptophan degradation by IDO may thus elicit neuropsychiatric symptoms when the availability of tryptophan is insufficient for normal serotonin biosynthesis [10,62–64] (Fig. 5). In fact, decreased tryptophan and decreased serotonin neoplasias are found in blood of HIV infected patients [65]. The concentration of kynurenine and other catabolites such as quinolinic acid accumulate as a consequence of tryptophan degradation [13]. The latter is a potent neurotoxin which interferes with the NMDA receptor and may therefore additionally influence the neuroendocrine system.

Major depression is closely related to disturbed tryptophan metabolism [66]. Reduced concentration of 5-hydroxyindoleacetic acid, the main catabolite of serotonin, confirms insufficient availability of serotonin. Therapeutic treatment with selective serotonin-reuptake inhibitors (SSRIs) can be very effective in patients with depression [67]. Similarly, enhanced degradation of tryptophan due to immune stimulation could underlie the increased risk for development of depression in patients with chronic inflammatory diseases in general and specifically during normal ageing [63,64]. Decreased serum tryptophan concentration is found in patients with major depression and correlates with increased concentration of immune activation markers [66].

In patients with HIV infection, an association exists between decreased tryptophan concentration and progressed cognitive inability [10]. Antiretroviral therapy improves cognitive impairment [68,69] and also reduces tryptophan degradation [30,32]. Such data support a role of tryptophan deficiency in contributing to cognitive loss. Interestingly, an association between tryptophan degradation and cognitive ability appears to exist in patients with late phase Alzheimer’s and Huntington’s disease [46,47].

In patients with advanced colorectal cancer enhanced degradation of tryptophan was found to coincide with impaired quality of life [42]. Recently, a relationship between lower tryptophan concentration and increased susceptibility to depression was reported in malignant melanoma patients during treatment with interferon-α [50]. Finally, rise in tryptophan concentration during ART could be related to the observation that such treatment also improves depressive symptoms in patients with HIV infection [70].

In the studies available thus far, a huge overlap of tryptophan concentration between diagnostic categories was found. However, it has to be noted that most of these studies relied on serum/plasma measurements only. Investigations involving the brain, i.e., cerebrospinal fluid, would allow more precise conclusions to be drawn with respect to involvement of tryptophan metabolism in neuropsychiatric disorders.

6. Treatment options to counteract accelerated tryptophan degradation

Until the late 1980’s, supplementation of tryptophan was used to correct sleep disturbances. Supplementation in patients with accelerated tryptophan catabolism is, however, hampered by increased production of potentially harmful products such as quinolinic acid. Tryptophan supplementation also circumvents the antiproliferative strategy of the immune system and it is unclear whether tryptophan supplementation would up-regulate malignant cell growth in cancer patients. On the other hand, immunoresponse could be improved. An alternative strategy is to increase the tryptophan pool via supplementation with nicotinamide to suppress IDO activity [71]. In patients with HIV infection, treatment with nicotinamide was found to increase plasma tryptophan concentration by 40% with no major side effects [71]. As such, administration of nicotinamide might provide a valuable strategy to counteract tryptophan depletion by IFN-γ-stimulated IDO in cells.

To improve mood of patients due to tryptophan deficiency, the application of SSRIs certainly remains the therapeutic option of choice. Controlling the immunopatho-
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6.1. In vitro assay to monitor effects of drugs and compounds on cytokine-induced tryptophan degradation

Cellular immune activation can be monitored by determination of tryptophan degradation in vivo and in vitro. To accomplish this goal, peripheral blood mononuclear cells (PBMC) stimulated with mitogens like phytohaemagglutinin or concanavalin A and tryptophan degradation is monitored to detect immunomodulatory properties of cytokines, drugs and plant extracts (see also Refs. [73–75]); IFN-γ-interferon-γ.

Fig. 6. Cytokine-induced tryptophan degradation applied in an in vitro assay using peripheral blood mononuclear cells from healthy donors. Cells are stimulated with mitogens like phytohaemagglutinin or concanavalin A and tryptophan degradation is monitored to detect immunomodulatory properties of cytokines, drugs and plant extracts (see also Refs. [73–75]); IFN-γ-interferon-γ.

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