

Fig. 5. Effect of FTY720 or SEW2871 treatment on P0 autoreactivity and on Tregs in SAP. **A.** The proliferative response to P0 (180–199) was decreased in splenocytes from FTY720- or SEW2871-treated mice ($n=3-4$ each). * $p<0.01$ for FTY720 vs Ctrl; $p<0.02$ for SEW2871 vs Ctrl. **B.** Cytokine responses of splenocytes to the same Ags in replicate culture ($n=7$ for Ctrl; $n=3$ for FTY720; $n=4$ for SEW2871). IFN- γ and IL-2 secretion were decreased in splenocytes from FTY720- or SEW2871 gavaged mice. * $p<0.003$ for FTY720 or SEW2871 vs Ctrl for IFN- γ ; $p<0.05$ for FTY720 vs Ctrl or SEW2871 vs Ctrl for IL-2. **C.** There was an increase in the percentage of Tregs in spleens and LNs of FTY720- or SEW2871-gavaged mice compared to Ctrl ($n=3-5$ each). * $p<0.01$ for SEW2871 vs Ctrl in spleen; ** the rest: $p<0.001$ for FTY720 or SEW2871 vs Ctrl.

and related compounds could be extended to inflammatory diseases affecting the peripheral nervous system.

Preclinical studies are usually carried out in two or more animal models of the disease to demonstrate consistent response across species or models. Regarding autoimmune neuropathies, EAN can be induced by myelin proteins such as P0, P2 or by galactocerebroside in rodents and rabbits respectively, as reviewed previously (Hahn, 1996). More recently, we and other investigators found that spontaneous autoimmune neuropathy in B7-2 KO NOD mice is mediated by P0, although possible contributions from other antigens in disease progression cannot be excluded (Kim et al., 2008; Louvet et al., 2009). To our knowledge, there have been no studies of FTY720 or SEW2871 in a spontaneous model of inflammatory neuropathy or multiple sclerosis. We found that FTY720 treatment given at disease onset halted the progression of SAP in B7-2 KO NOD mice, which was confirmed by histological and electrophysiological findings. The rapid inhibition of the BNB by FTY720 and SEW2871 in SAP demonstrates the crucial role of S1P1 receptors on endothelial cells in autoimmune diseases. A two photon imaging study of living T cells also revealed that S1P1 agonist prevents lymphocytes from crossing into medullary sinuses, though it does not distinguish between actions at endothelial S1P1 vs lymphocyte S1P1 (Wei et al., 2005). SEW2871, like FTY720, halted the progression of SAP, though it is less potent as an agonist, has a shorter duration of action, and differs in S1P1 receptor fate after internalization. FTY720P and AFD-R induce S1P1 receptor degradation, while SEW2871 and the physiologic ligand S1P induce receptor

recycling, which correlates with the extent of ubiquitination (Gonzalez-Cabrera et al., 2007; Oo et al., 2007).

It is thought that effector mechanisms may not be affected by S1P receptor modulators (Brinkmann et al., 2002). The effector phase of SAP is mediated by Th1 cytokines, although there is an increase in IL-17 transcripts during the preclinical phase (Bour-Jordan et al., 2005; Kim et al., 2008). We found that FTY720P or SEW2871 had no direct effects on Th1 cytokine responses in vitro, yet splenocytes from FTY720-treated and SEW2871-treated SAP mice exhibited a decreased proliferative and/or Th1 cytokine response to myelin P0. Our observations may simply reflect FTY720-induced T cell depletion from the spleen and the circulation (Hofmann et al., 2006). Another possibility consists of indirect inhibition of pathogenic T cell expansion by S1P receptor modulators via increased number or function of Tregs. Indeed, we found increased number of Tregs in spleens and LNs of FTY720- or SEW2871-gavaged mice. Other investigators have reported that Tregs exhibit a reduced chemotaxis to S1P compared to other T cells, and that Tregs from FTY720-treated mice, but not those from vehicle-treated mice, could inhibit airway inflammation in C57BL/6 mice (Sawicka et al., 2005).

Aside from immune cells, neurons and glial cells also express S1P receptors, albeit differing in the predominating receptor subtypes. We found that Schwann cells express S1P3>S1P2>S1P1>S1P4=S1P5, in contrast to cells of oligodendroglial lineage where S1P1 and S1P5 predominate (Jaillard et al., 2005; Jung et al., 2007). Other investigators reported that S1P1 is expressed by terminal SCs but

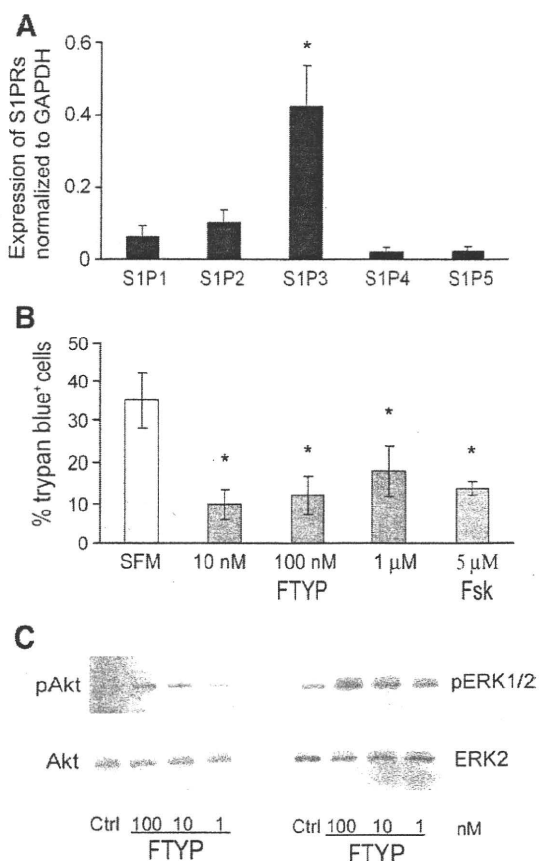


Fig. 6. Direct effects of FTY720P on cultured Schwann cells. **A.** S1P receptor mRNA levels relative to expression of GAPDH mRNA ($R = 2^{-\Delta CT}$) in SCs. * $p < 0.005$ for S1P3 vs other S1P receptors in rat SCs ($n = 3$). **B.** FTY720P rescues SCs from serum withdrawal-induced cell death. SCs were treated with 1 nM, 10 nM, 100 nM FTY720P (FTYP) or 5 μM forskolin (Fsk) ($n = 4-5$ each). * $p < 0.02$ for each treatment compared with serum-free medium (SFM). **C.** Representative examples of Western blot analysis demonstrating that exposure to FTY720P for 15 min stimulates the phosphorylation of ERK1/2 and Akt in rat SCs.

not by myelinating SCs, whereas S1P2, S1P3 and S1P4 receptors are expressed by both SC subtypes (Kobashi et al., 2006). S1P activates Rac1 and RhoA and increases SC migration (Barber et al., 2004). We found that FTY720P improves SC survival in vitro as well as activates both ERK2 and Akt pathways, which regulate SC survival, proliferation and differentiation (Hila et al., 2001; Ogata et al., 2004). FTY720P does not enhance SC differentiation as determined by P0 expression. Whether S1P receptor modulators modulate the function of SCs or axons in vivo is unclear and would require investigations in non-autoimmune neuropathies.

In summary, we found that both FTY720 and SEW2871 exert therapeutic actions in SAP, with acute effects mediated by functional agonism at S1P1 receptors of endothelial cells, followed by complex effects on the immune system characterized by T cell depletion with relative sparing of Tregs. Our data indicate that modulation of S1P1 receptors is sufficient to induce a protective effect on autoimmunity, but do not exclude possible contributions from other S1P receptors. One recognizes the limitations of SAP and EAN as animal models of autoimmune neuropathies given that previous attempts found only low frequency of humoral or cellular responses to myelin P0, P2 or glycolipids in human CIDP, perhaps reflecting epitope spreading or the heterogeneity of the disease. Nonetheless, our results suggest a potential therapeutic role for S1P receptor modulators in CIDP and other inflammatory neuropathies based on their actions on BNB permeability and differential sequestration of lymphocyte subsets.

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Proinflammatory cytokine interferon- γ increases induction of indoleamine 2,3-dioxygenase in monocytic cells primed with amyloid β peptide 1–42: implications for the pathogenesis of Alzheimer's disease

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Abstract

Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme of the kynurenine pathway of tryptophan metabolism, ultimately leading to production of the excitotoxin quinolinic acid (QUIN) by monocytic cells. In the Tg2576 mouse model of Alzheimer's disease, systemic inflammation induced by lipopolysaccharide leads to an increase in IDO expression and QUIN production in microglia surrounding amyloid plaques. We examined whether the IDO over-expression in microglia could be mediated by brain proinflammatory cytokines induced during the peripheral inflammation using THP-1 cells and peripheral blood mononuclear cells (PBMC) as models for microglia. THP-1 cells pre-treated with 5–25 μ M amyloid β peptide (A β) (1–42) but not with A β (1–40) or A β (25–35) became an activated state as indicated by their morphological

changes and enhanced adhesiveness. IDO expression was only slightly increased in the reactive cells but strongly enhanced following treatment with proinflammatory cytokine interferon- γ (IFN- γ) but not with interleukin-1 β , tumor necrosis factor- α , or interleukin-6 at 100 U/mL. The concomitant addition of A β (1–42) with IFN- γ was totally ineffective, indicating that A β pre-treatment is prerequisite for a high IDO expression. The priming effect of A β (1–42) for the IDO induction was also observed for PBMC. These findings suggest that IFN- γ induces IDO over-expression in the primed microglia surrounding amyloid plaques.

Keywords: Alzheimer's disease, amyloid β peptide, indoleamine 2,3 dioxygenase, microglia, proinflammatory cytokine, quinolinic acid.

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The kynurenine pathway (KP) is the major route of tryptophan metabolism, leading to the production of NAD or the complete oxidation for energy production (Fig. 1). Indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase represent the first and rate-limiting enzyme in the KP (Takikawa *et al.* 1986; Takikawa 2005). IDO is known to be induced during various neuroinflammatory diseases and this induction causes the marked elevation of several neurotoxic metabolites including the free radical generator 3-hydroxykynurenine and the excitotoxic NMDA receptor agonist quinolinic acid (QUIN) in the brain (Heyes *et al.* 1992; Moroni 1999; Stone *et al.* 2001; Smith *et al.* 2007). The brain IDO induction also elevates the concentrations of NMDA receptor antagonist, kynurenine (Kyn), but generally to a lesser extent than the increases in QUIN (Heyes *et al.* 1992; Naritsin *et al.* 1995). Therefore, dysregulation of the KP, mainly associated with an over-expression of IDO, has been implicated in the pathogenesis

of neuroinflammatory and neurodegenerative disorders, such as Huntington's disease (Pearson and Reynolds 1992; Stoy *et al.* 2005), Parkinson's disease (Ogawa *et al.* 1992), amyotrophic lateral sclerosis (Guillemin *et al.* 2005a), and AIDS dementia complex (Heyes *et al.* 1992).

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Abbreviations used: AD, Alzheimer's disease; A β , amyloid β peptide; GM-CSF, granulocyte macrophage-colony stimulating factor; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL-18, interleukin-18; KP, kynurenine pathway; Kyn, kynurenine; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; qRT-PCR, quantitative RT-PCR; QUIN, quinolinic acid; TDO, tryptophan 2,3-dioxygenase; TNF- α , tumor necrosis factor- α .

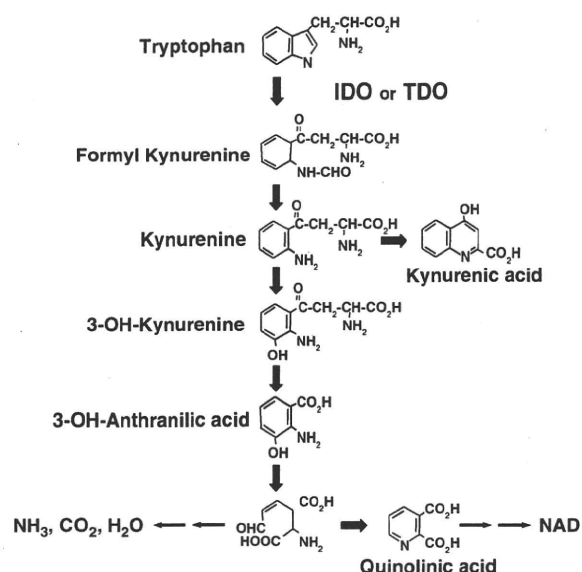


Fig. 1 The kynurenine pathway (KP) of tryptophan metabolism. IDO and tryptophan 2,3-dioxygenase (TDO) represent the first and rate-limiting enzymes in the KP.

Alzheimer's disease (AD) is the leading cause of dementia (Selkoe 2001). The pathological hallmarks of AD are extracellular amyloid plaques, intracellular neurofibrillary tangles, and dystrophic neurites (Selkoe 2001; Morgan *et al.* 2004). Amyloid β (A β) peptides consisting of 40 or 42 amino acid residues represent a primary component of amyloid plaques. A β (1–42) is considered to be the most neurotoxic form of the peptides (Drouet *et al.* 2000). A β (1–42) can activate primary cultured human microglia and induce IDO expression (Guillemin *et al.* 2003; Walker *et al.* 2006). Furthermore, IDO over-expression and increased production of QUIN have been observed in microglia associated with amyloid plaques in the brains of AD patients (Guillemin *et al.* 2005b). Thus, over-expression of IDO and over-activation of the KP in microglia are likely to be involved in the pathogenesis of AD.

We have previously studied the potential role of the KP in the pathogenesis of AD using a Tg2576 mouse model of AD. These mice express the Swedish mutant of amyloid precursor protein in the brains and have amyloid plaques consisting of A β increasing in an age-dependent manner (Hsiao *et al.* 1996; Kawarabayashi *et al.* 2001). We found that in the Tg2576 mouse brain the amyloid plaques could activate microglia but not sufficiently to induce IDO expression. We also found that an additional brain inflammation generated systemically, such as a peritoneal challenge with lipopolysaccharide, was required for IDO induction in the activated microglia infiltrating into amyloid plaques (Akimoto *et al.* 2007). In this sense, the microglial cells are 'primed' by A β which activates IDO and KP in response to a subsequent

brain inflammation. The secondary brain inflammation is likely to be mediated by proinflammatory cytokines including interleukin-1 β (IL-1 β), IL-6, tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ), all able to activate microglia (Basu *et al.* 2002). Increased levels of these cytokines have been found in the brain of Tg2576 mice during such systemic inflammation (Sly *et al.* 2001). We hypothesized here that proinflammatory cytokines might further stimulate the microglia primed by A β for the expression of IDO. In this study, we used THP-1 cells (a human monocytic cell line) and human peripheral blood mononuclear cells (PBMC), both of which are commonly used as model for the microglial response to A β and proinflammatory cytokines (Giri *et al.* 2003; Wilkinson *et al.* 2006).

Materials and methods

Materials

Synthetic A β peptides (1–40, 1–42, 42–1, and 25–35) were purchased from Peptide Institute Inc. (Osaka, Japan), and prepared as stock solution in dimethyl sulfoxide at a concentration of 4 mM. Human recombinant IL-1 β , IL-6, and TNF- α were purchased from Roche Diagnostics, Tokyo, Japan, and human recombinant IFN- γ was purchased from Sigma (St Louis, MO, USA). One unit of the biological activity of each cytokine was defined according to the manufacture's instruction. All cytokines were dissolved in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and stored at –80°C until use. Anti-human IDO monoclonal antibody (mAb) was purified from culture medium of a hybridoma clone established by Takikawa *et al.* (1988). Histofine® Simple Stain MAX PO, a horseradish peroxidase-conjugated secondary antibody for immunoblotting, was purchased from NICHIREI (Tokyo, Japan). Anti-TNF- α mAb (IgG₁) was obtained from R & D Systems (Tokyo, Japan). Control mouse IgG₁ was a product of Sigma. THP-1 cells, a human acute monocytic leukemia cell line (Tsuchiya *et al.* 1980), were obtained from Health Science Research Resources Bank (Osaka, Japan).

Cell culture and cellular assay

THP-1 cells were maintained in RPMI-1640 (Sigma, St Louis, MO, USA) containing 10% heat-inactivated fetal calf serum at 37°C in 5% CO₂ and 95% air. The cells used in this study were between passages 12 and 15. Human PBMC were isolated from blood of healthy volunteers with Ficoll-paque PLUS solution (GE Healthcare Life Sciences, Tokyo, Japan), and separated from lymphocytes on the basis of plastic adherence as described by Guillemin *et al.* (2003). PBMC were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 20% heat-inactivated fetal calf serum and 10% heat-inactivated human AB serum at 37°C in 5% CO₂ and 95% air. Before cellular assay, cells were washed once with a serum-free AIM-V medium (Invitrogen, Carlsbad, CA, USA), re-suspended in the AIM-V, and plated in culture plate. Kynurenine (Kyn) concentration in culture medium was determined after deproteinization with trichloroacetic acid at a final concentration of 5% with a Shimadzu Prominence® HPLC system (Kyoto, Japan) equipped with an Inertsil® reverse phased column (4.6 mm x 15 cm; GL Science,

Tokyo, Japan). The mobile phase was 10% methanol/10 mM ammonium acetate and Kyn was detected by absorbance at 360 nm. Phase contrast photomicrographs of THP-1 cells (Fig. 2) were taken using an Olympus IX70 inverted microscope (Tokyo, Japan) equipped with a Leica DFC290 digital camera (Wetzlar, Germany). The study with PBMC from blood of healthy volunteers was approved by the human ethical committee of the National Center for Geriatrics and Gerontology and all participants signed informed consent.

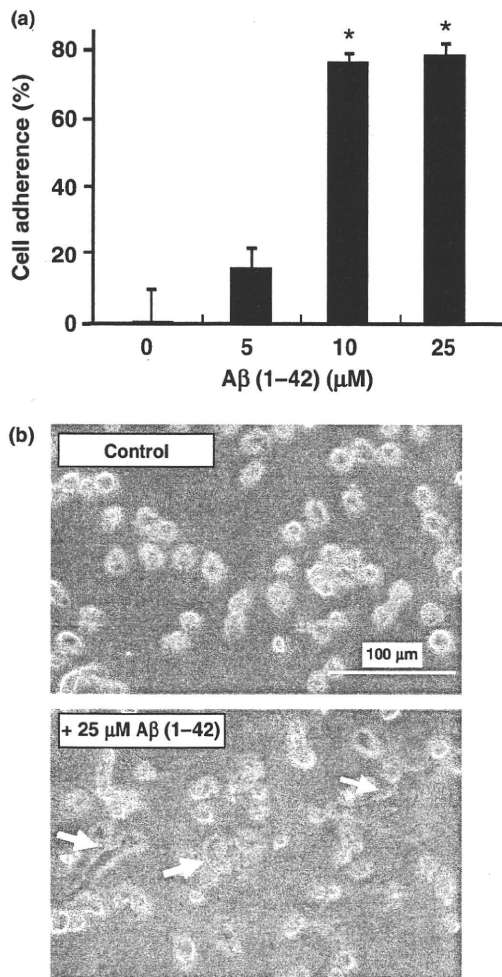


Fig. 2 Increase in adherent activity and change in morphology of THP-1 cells treated with A β (1-42). (a) Adherent activity of THP-1 cells treated with A β (1-42). THP-1 cells (1×10^5 cells) suspended in 1.0 mL of the AIM-V medium in a 24-well plate were treated with indicated concentrations of A β . After 24 h, non-adherent cells were gently collected from the culture plate by pipetting and counted with a hemocytometer under a microscope. * $p < 0.01$ compared with the control cells treated with the peptide vehicle (0.63% of dimethylsulfoxide at a final concentration). (b) Phase contrast microphotographs of THP-1 cells treated with the peptide vehicle (upper) or 25 μ M A β (1-42) (lower) for 24 h. Arrows indicate typical ameboid spread cells. Scale bar: 100 μ m. Magnification: 200 \times .

Assay of IDO activity

Indoleamine 2,3-dioxygenase activity in cells was assayed as described previously (Takikawa *et al.* 1988). Cultured cells were collected by trypsinization or pipetting, washed with twice with cold PBS, and disrupted by sonication for 30 s in cold PBS containing Complete proteinase inhibitor cocktail (Roche Diagnostics). The cellular homogenate was then centrifuged at 15 000 g for 10 min for 5 min at 4 $^{\circ}$ C and the supernatant (cellular extract) was used as the enzyme source. The reaction mixture (200 μ L) contained 50 mM potassium phosphate buffer (pH 6.5), 20 mM ascorbate, 10 mM methylene blue, 100 mg/mL catalase, 400 mM tryptophan, and the cellular extract. The reaction at 37 $^{\circ}$ C was started by the addition of tryptophan and terminated after 60 min with trichloroacetic acid at a final concentration of 5% and further incubated at 50 $^{\circ}$ C to hydrolyze *N*-formylkynurenine to Kyn. After centrifugation at 15 000 g for 5 min at 25 $^{\circ}$ C, Kyn in the supernatant was measured by HPLC as described above. Under this condition, the production of Kyn from tryptophan increased linearly for up to 90 min. Protein of the cellular extract was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as standard.

Immunoblotting

Cells were lysed in the lysis buffer (1% Triton X-100 in PBS containing Complete proteinase inhibitor cocktail) for 30 min on ice. After insolubles were removed from the lysate by centrifugation for 10 min at 10 000 g at 4 $^{\circ}$ C, protein concentration of the cell lysate was measured by bicinchoninic acid protein assay kit (Pierce). Aliquots containing 10 μ g of protein were resolved by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon-P transfer membranes (Millipore Corporation, Bedford, MA, USA). The membranes were sequentially reacted with blocking solution, primary antibody (1 : 10000), and the secondary antibody (1 : 500) for 1 h at 25 $^{\circ}$ C. Blots were detected by ECL plus (GE Healthcare). Band intensities were quantified using Scion image software (Scion Corp., Frederic, MD, USA).

RNA isolation and quantitative RT-PCR

Total RNA were isolated from THP-1 cells using a standard method with Trizol[®] reagent (Invitrogen), and reverse-transcribed by SuperScript[®]First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed using SYBER premix EX *taq* (Takara Bio Inc., Otsu, Japan) with a thermal cycler Dice RealTime System TP800 (Takara Bio Inc) according to the manufacturer's instruction. Comparison of IDO expression among the different experimental groups was performed with relative amount of IDO mRNA normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA as an internal standard. The sequences of primers were 5'-TCTTCTCA- TTTCGTGATGGAG-ACTG-3' and 5'-AAAGTGTCCCGTCTTGCATTG-3' for IDO, and 5'-GCACCGTCAAGGCTGAGAAC-3' and 5'-TGGTGAAGACGCCAGTGGA- 3' for glyceraldehyde-3-phosphate dehydrogenase.

Antibody neutralization assay

Anti-human TNF- α mAb or control isotype mouse IgG (IgG₁) was added into THP-1 cell culture at 1 μ g/mL with A β (1-42). After

culture for 24 h, IFN- γ was added to the culture and further incubated for 24 h. The concentration of Kyn in culture medium was determined by HPLC as described above.

Statistical analysis

Each value in all figures represents the mean \pm SD of the three separate experiments. All data were analyzed by ANOVA. If a significant difference was identified, multiple comparisons were adjusted using the Scheffe's test. $p < 0.01$ was regarded as statistically significant.

Results

Activation of THP-1 cells by A β (1–42)

To mimic the *in vivo* situation in which microglia surrounding amyloid plaques consisting of A β in Tg2576 mice are activated by continuous contact with A β , we added A β to culture of THP-1 cells and examined the cell adherence to plastic plate as cell adherence is a hallmark of monocyte/macrophage activation (Kamal and Harold 1998). After treatment with A β (1–42) for 24 h, THP-1 cells became adherent in a dose-dependent manner; at 5 μ M of A β (1–42), about 20% of the cells attached to the plate and at over 10 μ M, most of the cells were adherent (Fig. 2a). This effect was observed only for A β (1–42) but not A β (1–40) or A β 25–35 even at 25 μ M (data not shown). The negative control peptide A β (42–1) at 25 μ M was also totally inactive (data not shown). The activation of THP-1 cells by A β (1–42) was associated with marked changes in morphology from round shape to ameboid spread shape (Fig. 2b) which is a characteristic morphological feature of reactive monocytes/macrophages (Kamal and Harold 1998).

Effect of various proinflammatory cytokines on the Kyn production in THP-1 cells pre-treated with A β (1–42)

To examine if the adherence of the THP-1 cells generated by A β (1–42) induces IDO and activate the KP, we measured the level of tryptophan metabolite Kyn in the culture medium using HPLC. We found that the increase in the level of Kyn after stimulation with A β (1–42) for 24 h was very low (0.1–0.2 μ M), indicating that IDO was hardly activated. This was confirmed by immunoblotting of IDO protein and qRT-PCR for IDO mRNA as described below. Then we tested our hypothesis that a secondary stimulation by proinflammatory cytokines may induce the activation of KP in the reactive THP-1 cells. To this end, we first pre-treated the THP-1 cells with A β (1–42) at 25 μ M for 24 h, then added proinflammatory cytokines, IL-1 β , IL-6, TNF- α , or IFN- γ to the culture at 100 U/mL, and determined the levels of Kyn in the culture medium 24 h after the addition of cytokine. Of these cytokines, only IFN- γ enhanced markedly the production of Kyn from the reactive THP-1 cells (Fig. 3). This enhancement was more prominent at higher concentrations of IFN- γ and was also dependent on the concentration of A β (1–42)

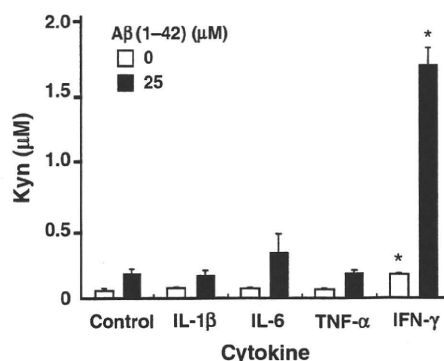


Fig. 3 Production of Kyn in THP-1 cells treated with A β (1–42) and proinflammatory cytokines. THP-1 cells (5×10^4 cells) suspended in 0.2 mL of the AIM-V medium in a 96-well plate were treated with the peptide vehicle or 25 μ M A β (1–42) for 24 h, and then further cultured for 24 h with proinflammatory cytokines at 100 U/mL or the cytokine vehicle (0.1% bovine serum albumin in PBS) (control). The levels of Kyn in the culture medium were measured after additional 24 h culture. □, the peptide vehicle; ■, 25 μ M A β (1–42). * $p < 0.01$ compared with each control treated with or without the A β .

(Fig. 4). It is worth noting that the dose-dependency of A β (1–42) for the Kyn production almost coincided with that for the conversion of THP-1 cells to the adherent reactive cells (Fig. 2). Pre-treatment with A β (1–40) or A β (25–35) at 5–50 μ M neither induced THP-1 cells to become adherent reactive cells nor activate the KP by the secondary stimulation with IFN- γ (10–1000 U/mL) (data not shown). Taken together these results indicated that changes in cell morphology induced by A β (1–42) were closely associated with the activation of the KP by IFN- γ .

Optimization of pre-treatment with A β (1–42) for the IFN- γ -mediated activation of KP in THP-1 cells

We determined the optimal duration of the pre-treatment with A β (1–42) required for the IFN- γ -mediated activation of KP in THP-1 cells. Lack of pre-treatment and pre-treatment for 12 h with the addition of A β did not induce any KP activation by IFN- γ . A minimum of 24 h pre-treatment was necessary to lead to a significant activation by IFN- γ (Fig. 5a). We also found that the pre-treatment with A β (1–42) for 24 h caused a long-term sustained activation of KP by IFN- γ for up to 72 h (Fig. 5b). These results demonstrated that pre-treatment of THP-1 cells with A β (1–42) for more than 24 h was required to the subsequent higher KP activation by IFN- γ .

Analysis of IDO expression in THP-1 cells stimulated by A β (1–42) and IFN- γ

The levels of Kyn in culture medium of various cells are closely related to those of IDO expressed by the cells (Takikawa *et al.* 1988). Therefore, we analyzed the changes in IDO expression in THP-1 cells stimulated with the

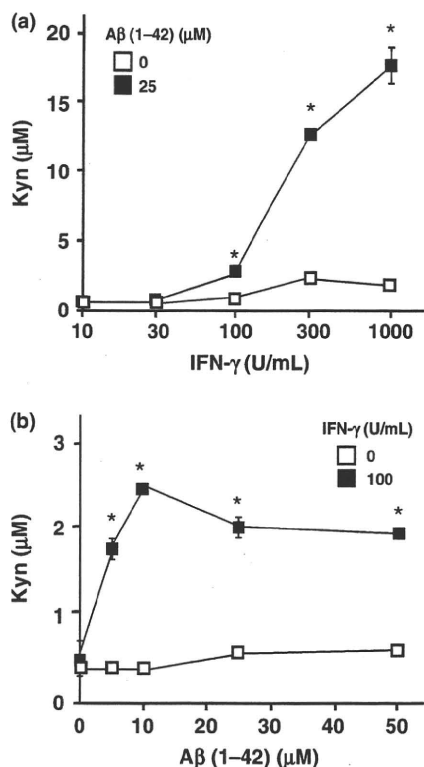


Fig. 4 Production of Kyn in THP-1 cells stimulated with the combination of A β and IFN- γ . (a) The dose-dependency of IFN- γ . THP-1 cells (5×10^4 cells) suspended in 0.2 mL of the AIM-V medium in a 96-well plate were first treated with (■) or without (□) 25 μ M A β (1-42) for 24 h and stimulated with indicated concentrations of IFN- γ for additional 24 h. The levels of Kyn in the culture medium were measured after the stimulation with IFN- γ . □, peptide vehicle; ■, +A β 1-42 (25 μ M). * p < 0.01 compared with the values obtained with the peptide vehicle. (b) The dose-dependency of A β (1-42). THP-1 cells (5×10^4 cells) suspended in 0.2 mL of the AIM-V medium in a 96-well plate were first treated with indicated concentrations of A β (1-42) for 24 h and further stimulated with 100 U/mL of IFN- γ (■) or the cytokine vehicle (0.1% BSA in PBS) (□) for additional 24 h. The levels of Kyn in the culture medium were measured after the secondary stimulation. □, cytokine vehicle; ■, IFN- γ (100 U/mL). * p < 0.01 compared with the values obtained with the cytokine vehicle.

combination of A β (1-42) and IFN- γ . IDO mRNA was very weakly expressed in THP-1 cells when cultured without any stimulation. This expression increased by 7.2 ± 1.7 -fold upon stimulation with 25 μ M A β (1-42) alone and was strongly elevated by 3156 ± 488.8 -fold in combination with IFN- γ at 100 U/mL (Fig. 6a). Stimulation with IFN- γ alone without pre-treatment with A β (1-42) resulted in small increase of 65 ± 5.2 -fold in mRNA levels; (Fig. 6a). In accordance with these qRT-PCR results, both IDO enzyme activity (Fig. 6b) and the level of IDO protein (Fig. 6c) were markedly elevated by the sequential stimulation with A β and IFN- γ . Taken together, these results showed that KP

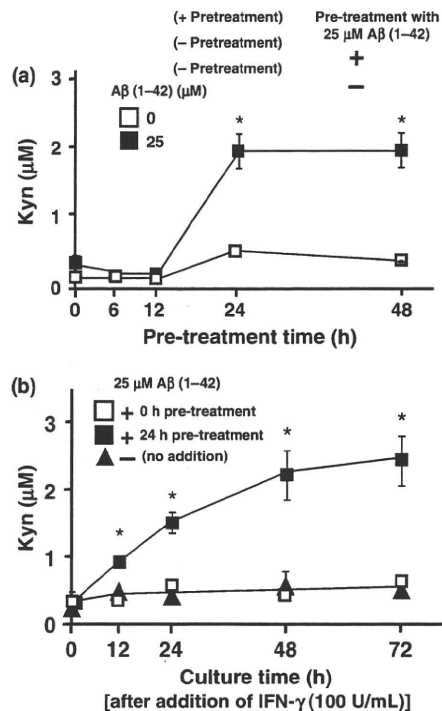


Fig. 5 Optimum duration of the A β (1-42) pre-treatment for the activation of KP by IFN- γ in THP-1 cells and the time course of the production of Kyn in the reactive THP-1 cells stimulated with IFN- γ . (a) Effect of pre-treatment time with A β (1-42) for the production of Kyn by the subsequent stimulation with IFN- γ in THP-1 cells. THP-1 cells (5×10^4 cells) suspended in 0.2 mL of the AIM-V medium in a 96-well plate were cultured with 25 μ M A β (1-42) (■) or without the peptide (i.e., with the peptide vehicle) (□). At indicated culture periods (6, 12, 24, and 48 h), IFN- γ (100 U/mL) was added to the cultures and the levels of Kyn in culture medium were measured after additional 24 h culture. □, peptide vehicle; ■, +25 μ M A β (1-42). * p < 0.01 compared with the values obtained without the pre-treatment with the A β . (b) The time course of the production of Kyn in the THP-1 cells stimulated with the combination of A β (1-42) and IFN- γ . THP-1 cells (5×10^4 cells) suspended in 0.2 mL of the AIM-V medium in a 96-well plate were pre-treated with 25 μ M A β (1-42) for 24 h and further cultured with 100 U/mL IFN- γ for up to 72 h. The time course of Kyn production in the cultures after the addition of IFN- γ was depicted with ■. Those of the cultures stimulated simultaneously with 25 μ M A β (1-42) and 100 U/mL IFN- γ or stimulated with 100 U/mL IFN- γ alone after pre-treatment of the peptide vehicle for 24 h were indicated with □ and ▲, respectively. * p < 0.01 compared with the values obtained without culture with IFN- γ .

activation in THP-1 cells by A β (1-42) and IFN- γ was associated with an increase in IDO transcript and functional IDO protein.

Analysis of IDO expression in PBMC stimulated with A β (1-42) and IFN- γ

It could be possible that the above-mentioned KP activation may be the unique property of THP-1 cells acquired during immortalization. Therefore, we tested if the similar

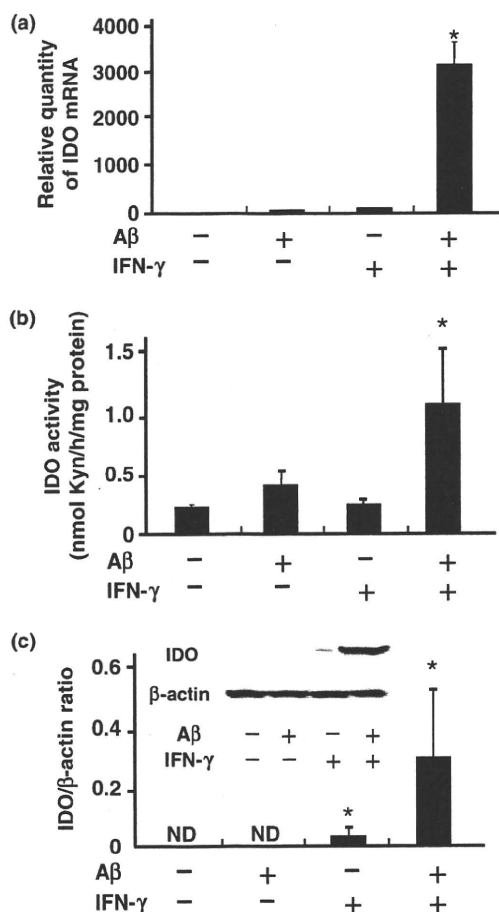


Fig. 6 Analysis of the expression of IDO in THP-1 treated with the combination of Aβ (1–42) and IFN-γ. THP-1 cells (1×10^5 cells) suspended in 1.0 mL of the AIM-V medium in a 24-well plate were analyzed after pre-treatment with or without 25 μM Aβ (1–42) for 24 h and further stimulation with or without 100 U/mL IFN-γ for 24 h. (a) qRT-PCR of IDO mRNA. The levels of IDO mRNA were normalized with those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and expressed as relative quantity to that of the control (the control value: 1) without stimulation with 25 μM Aβ (1–42) nor 100 U/mL IFN-γ. (b) IDO enzyme activity. (c) Immunoblot analysis of IDO protein. The band intensities of IDO were normalized with those of β-actin. * $p < 0.01$ compared with others. ND, not detectable.

combinational effects of Aβ (1–42) and IFN-γ could also induce IDO in primary cultures of human peripheral blood mononuclear cells (PBMC). PBMC were first pre-treated with 25 μM Aβ (1–42) for 24 h, then stimulated with different concentrations of IFN-γ for another 24 h, and analyzed by immunoblotting. Similar to THP-1 cells, IDO expression in PBMC was undetectable without any stimulation but weakly induced by IFN-γ alone (Fig. 7a). However, with the Aβ (1–42) pre-treatment, IDO expression was greatly enhanced by IFN-γ in a dose-dependent manner (Fig. 7a). In fact, the pre-treatment increased IDO protein

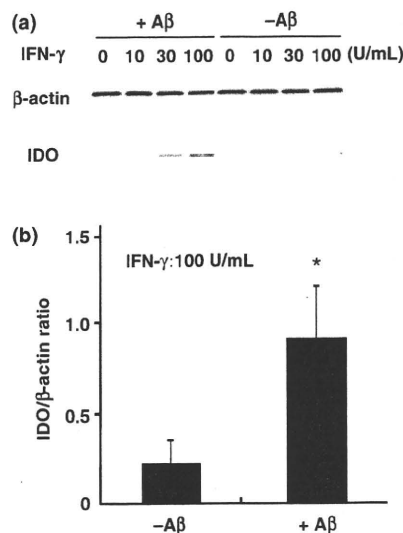


Fig. 7 Analysis of IDO expression in PBMC treated with Aβ (1–42) and IFN-γ. (a) Immunoblotting of IDO in PBMC. PBMC (1×10^5 cells) suspended in 1.0 mL of AIM-V medium and cultured in a 24-well plate with the peptide vehicle or 25 μM Aβ (1–42) for 24 h and further stimulated with indicated concentrations of IFN-γ for 24 h. IDO expression in PBMC was analyzed by immunoblotting after stimulation with IFN-γ. (b) Quantitative analysis of the immunoblots. The band intensities obtained with 100 U/mL IFN-γ were normalized with those of β-actin. * $p < 0.01$ compared with the value obtained with the peptide vehicle (-Aβ).

level by about fourfold with 100 U/mL IFN-γ (Fig. 7b), which was comparable with results with THP-1 cells (Fig. 6c). Thus, the high IDO induction by the sequential stimulation with Aβ (1–42) and IFN-γ was well similar between human monocytic cell line THP-1 cells and human primary PBMC.

Involvement of TNF-α in KP activation by Aβ (1–42) and IFN-γ

We previously demonstrated that IDO is induced by IFN-γ in many different cell types (Takikawa *et al.* 1988), and other showed that this induction could be enhanced by TNF-α in macrophages and epithelial cells (Currier *et al.* 2000; Robinson *et al.* 2003). Other studies also reported that TNF-α secretion by microglial cells was induced by a combination of Aβ (1–42) and IFN-γ (Meda *et al.* 1995; Klegeris *et al.* 1997). We therefore hypothesized that an autocrine stimulation by TNF-α may be involved in KP activation in THP-1 cells by the sequential treatment with Aβ (1–42) and IFN-γ. To test this hypothesis, we stimulated THP-1 cells with Aβ (1–42) and IFN-γ in the presence of anti-TNF-α mAb at 1 μg/mL, which was able to neutralize as much as 100 U/mL of TNF-α. The anti-TNF-α mAb inhibited the Kyn production by more than 60% ($39.1 \pm 8.7\%$) whereas the control isotype IgG₁ at 1 μg/mL

Table 1 Effect of anti-TNF- α mAb on the production of Kyn in THP-1 cells treated with the combination of A β (1–42) and IFN- γ

	Kyn (μ M)	Percent of control
Control	2.3 \pm 0.2	100.0
+Anti-TNF- α (1 μ g/mL)	0.9 \pm 0.2	39.1 \pm 8.7*
+IgG1 (1 μ g/mL)	2.2 \pm 0.1	95.6 \pm 4.3

TNF- α , tumor necrosis factor α ; Kyn, kynurenine.

THP-1 cells (5×10^4 cells) suspended in 0.2 mL of AIM-V medium were cultured in a 96-well plate with 25 μ M A β (1–42) in the presence of anti-human TNF- α mAb (IgG₁) or control isotype IgG₁ at 1 μ g/mL for 24 h. Control cells were cultured with 25 μ M A β (1–42) alone. Then IFN- γ was added to the cultures at 100 U/mL and cultured for another 24 h. Kyn was measured in culture supernatants after the additional 24 h culture.

* $p < 0.01$ compared with others.

was totally ineffective (Table 1). These results indicated that an autocrine TNF- α was significantly involved in the KP activation in THP-1 cells.

Discussion

In this study, we tested our hypothesis that activated microglia surrounding amyloid plaques consisting of A β are 'primed' for the activation of KP in response to the additional stimulation by proinflammatory cytokines. We used THP-1 cells and human primary PBMC as models for microglia, and found that both monocytic cell types pre-treated with A β (1–42), one of the main components of amyloid plaques, become highly responsive to a secondary stimulation with the proinflammatory cytokine IFN- γ and markedly activate the KP through induction of IDO. Only the combination of A β (1–42) and IFN- γ was able to activate the KP whereas A β (1–40) or A β (25–35) and other major proinflammatory cytokines including IL-1 β , TNF- α , or IL-6 were not (Fig. 3), although an autocrine TNF- α was partly involved in the induction of IDO (Table 1). However, the combined concomitant stimulation of THP-1 cells with A β (1–42) and IFN- γ did not activate the KP. We showed that a pre-treatment with A β (1–42) for at least 24 h was prerequisite for the secondary stimulation with IFN- γ to be effective on the KP activation (Fig. 5). This pre-treatment with A β lead to a cellular activation as shown by the morphological change and enhanced adhesiveness (Fig. 2). This activation by A β (1–42) alone, however, was not sufficient to switch on the cellular machinery involved in IDO induction (Fig. 4). Thus, our *in vitro* model further supported our hypothesis that the microglia receiving a chronic stimulation by amyloid plaques consisting of A β (1–42) are 'primed' for the activation of KP by the induction of IDO in a response to the proinflammatory cytokine IFN- γ .

Both THP-1 cells primed with A β (1–42) and PBMC differentiated by granulocyte macrophage-colony stimulating

factor (GM-CSF) have a similar response in term of KP activation by IFN- γ (Jansen and Reinhard 1999). However, PBMC required longer time of culture (3 to 7 days) with GM-CSF to adopt a reactive state in response to the IFN. These differentiated cells did not exhibit such strong morphological changes and adhesiveness as we found with THP-1 cells after a short (24 h) pre-treatment with A β (1–42) (Fig. 2). Therefore, the cellular changes induced by GM-CSF appear to be different from those with A β 'priming'.

What is the relevance of these present findings to the pathogenesis of AD? It is known that systemic inflammatory infection is the risk factor for AD progression (Holmes *et al.* 2003) and that similar systemic inflammation caused by an intraperitoneal injection of lipopolysaccharide increased proinflammatory cytokine production including IFN- γ in the mouse brain (Pitossi *et al.* 1997). Level of IFN- γ in the mouse brain increased with aging (Frank *et al.* 2006; Kumagai *et al.* 2007), which represented the major risk factor for AD (Evans *et al.* 1989; Hebert *et al.* 2003). On the other hand, it has been recently reported that IFN- γ can be produced by mouse microglia when stimulated with IL-18 (Kawanokuchi *et al.* 2006) and that expression of IL-18 is elevated in microglia, astrocytes, and neurons within the AD brains (Ojala *et al.* 2009). Under stress conditions, IL-18 was also involved in the activation of murine microglia (Sugama *et al.* 2007), which accelerated learning and memory impairment and worsened the amyloid pathology in the mouse models of AD (Dong *et al.* 2004; Jeong *et al.* 2006). Therefore, under neuroinflammatory conditions (infection, aging, and stress) associated with the accelerating progression in both human AD or the AD-like pathology of the mouse models, 'primed' microglia surrounding amyloid plaques appear to be activated by IFN- γ to induce IDO and to produce neurotoxic QUIN, thereby promoting the neurodegeneration. In the rat brain QUIN neurotoxicity was greatly enhanced by proinflammatory cytokine IL-1 β (Stone and Behan 2007). This neurotoxic combination is likely to reach neurons around amyloid plaques in human AD brains, as production of both QUIN and IL-1 β was increased in activated microglia attacking senile plaques (Griffin *et al.* 1989; Guillemin *et al.* 2005b). Thus, our findings may explain some part of the molecular mechanisms underlying the accelerated neurodegeneration by risk factors known to enhance inflammation in the AD brain.

A β (1–40) and A β (1–42) are the major components of amyloid plaques (Gravina *et al.* 1995; Kawarabayashi *et al.* 2001). However, considerable circumstantial evidence suggests that A β (1–42) rather than A β (1–40) is the critical molecule involved in the pathogenesis of AD (Jarrett *et al.* 1993; Iwatsubo *et al.* 1994; Gravina *et al.* 1995; McGowan *et al.* 2005). In fact, even at lower concentrations A β (1–42) was significantly more toxic to cultured neurons compared with A β (1–40) (Drouet *et al.* 2000; Dahlgren *et al.* 2002). A β (1–42) can form soluble oligomeric structures, insoluble

fibrils, and highly aggregated form of the fibrils at lower concentrations and higher rates compared with A β (1–40) or any other A β variants (Burdick *et al.* 1992; Jarrett *et al.* 1993). These unique properties of A β (1–42) may be responsible for the specificity for the priming effect on the induction of IDO in THP-1 cells.

Several surface receptors expressed in monocytic cells including microglia have been reported to interact with A β fibrils; among them the scavenger receptor complex consisting of class A scavenger receptor, CD36, α 6/ β 1-integrin, and CD47 (El Khoury *et al.* 1996; Wilkinson *et al.* 2006), the receptor for advanced glycation end products (Yan *et al.* 1996), and Toll-like receptors 2 and 4 (Chen *et al.* 2006; Richard *et al.* 2008; Udan *et al.* 2008). Our preliminary data (not shown) suggested that the scavenger receptor complex was not involved in the priming effect of A β (1–42) because the antagonist for the receptor complex, 4N1K peptide at up to 300 μ M (Wilkinson *et al.* 2006) did not suppress the induction of IDO. Moreover, the phagocytosis of A β fibrils was not required for the effect of A β (1–42) as treatment with an inhibitor of phagocytosis, cytochalasin D (3 μ M) (Sulahian *et al.* 2008) did not inhibit the enzyme induction (data not shown). The possible involvement of receptor for advanced glycation or Toll-like receptor 2/4 in the priming effect of A β (1–42) is currently under investigation.

It is generally accepted that inflammation-mediated neurotoxicity in neurodegenerative disease including AD can occur as a consequence of microglial overactivation (Perry *et al.* 2003; Block *et al.* 2007). This concept is based on the fact that such over-activated microglia can generate neurotoxic products including reactive oxygen species and proinflammatory cytokines (Cunningham *et al.* 2005; Block *et al.* 2007). In addition to these neurotoxic compounds, we previously demonstrated that activated microglia associated with amyloid plaques in human AD brains produced neurotoxic amounts of QUIN (Guillemin *et al.* 2005a, 2007). Based on our previous *in vivo* findings in Tg2576 mice and our present *in vitro* data with models of microglia, we concluded that the microglia producing QUIN in AD brains were over-activated by the combination of the chronic exposure to amyloid peptides, and more particularly A β (1–42), and the secondary inflammatory cytokine, IFN- γ . Several drugs that block the KP are currently under therapeutic investigation by our laboratory and others. Targeting IDO or other KP enzymes with specific inhibitors would lead to a decrease in QUIN production and may therefore bring new therapeutic strategies for AD.

Acknowledgment

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Indoleamine 2,3-Dioxygenase Expression and Regulation in Chronic Periodontitis

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Background: Indoleamine 2,3-dioxygenase (IDO) is an intracellular tryptophan-oxidizing enzyme with immunosuppressive characteristics. Its expression and regulation in periodontal tissues are unknown. The aim of this study was to determine IDO expression in healthy gingiva and chronic periodontitis lesions. In addition, the effect of inflammatory cytokines and bacterial products on the expression and activity of IDO in human gingival fibroblasts (HGFs) was assessed.

Methods: Human gingival tissue samples were obtained from patients who underwent periodontal surgery. IDO expression in healthy gingiva and periodontitis lesions was determined by immunohistochemistry. HGF cells were treated with interferon-gamma (IFN- γ), interleukin (IL)-1 β , tumor necrosis factor-alpha (TNF- α), and lipopolysaccharides from *Porphyromonas gingivalis* (PgLPS). IDO mRNA expression was determined by reverse transcription-polymerase chain reaction. The IDO enzymatic activity was determined by measuring the kynurenine level using a colorimetric method.

Results: In gingival tissues, IDO expression was detected in epithelial cells, fibroblasts, endothelial cells, and inflammatory mononuclear cells. IDO expression was higher in periodontitis lesions than in healthy gingiva. HGFs did not constitutively express IDO. IFN- γ strongly induced IDO expression and activity in HGFs, in a dose-dependent manner. IL-1 β , TNF- α , and PgLPS were also able to induce IDO expression in HGF cells. IFN- γ in combination with IL-1 β , TNF- α , or PgLPS showed enhanced IDO expression.

Conclusions: IDO was expressed in human gingiva, and the expression was upregulated in chronic periodontitis. The increased IDO expression in periodontitis lesions may be due, in part, to the activation of HGFs by inflammatory cytokines and bacterial products. *J Periodontol* 2009;80:114-121.

KEY WORDS

Fibroblasts; indoleamine 2,3-dioxygenase; inflammation; periodontitis.

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Indoleamine 2,3-dioxygenase (IDO) is an enzyme that metabolizes the amino acid tryptophan, the least abundant essential amino acid for mammals. The majority of tryptophan is metabolized along the kynurenine pathway, leading to the synthesis of nicotinamide adenine dinucleotide or the complete oxidation of the amino acid.¹ IDO is expressed in many cell types, including monocytes, macrophages, dendritic cells, fibroblasts, epithelial cells, astrocytes, and several cancer cell lines.¹ Early studies showed that IDO was involved in the interferon-gamma (IFN- γ)-mediated host defense to many intracellular pathogens, including *Toxoplasma*,² *Chlamydia*,³ *Mycobacterium*,⁴ *Staphylococcus aureus*,⁵ cytomegalovirus,⁶ and herpes simplex virus.⁷ This antimicrobial effect was mainly mediated through IFN- γ -induced IDO expression and activity.

IDO also plays a role in immunoregulation and tolerance induction. Macrophages and dendritic cells expressing IDO can suppress T-cell responses and promote tolerance.⁸ IDO-dependent T-cell suppression seems to be mediated by depletion of tryptophan in the microenvironment. Excess tryptophan was able to reverse the inhibition of T cells.^{9,10} Toxic metabolites of tryptophan, such as quinolinic acid and 3-hydroxy-anthranilic acid, may also mediate the immunosuppressive effects of IDO.¹¹⁻¹³

IDO expression is induced by several inflammatory cytokines and immunomodulating agents. IFN- γ is a potent inducer of IDO expression. Interferon-alpha (IFN- α) and -beta (IFN- β) are also able to induce IDO expression, but to a lesser extent.¹⁴ Tumor necrosis factor-alpha (TNF- α), interleukin (IL)-1, and lipopolysaccharide (LPS) also induce IDO expression alone or in combination with IFN- γ .^{15,16} Because IDO expression may lead to suppression of T-cell proliferation and function, induction of IDO expression by inflammatory cytokines and immunomodulating agents may limit excessive T-cell activation at local sites of inflammation, thus, serving an anti-inflammatory role.

Various cytokines that regulate IDO expression and activity were detected in periodontal tissues. High levels of IFN- γ , a strong IDO inducer, were detectable in inflamed gingival tissues.^{17,18} IL-1 and TNF- α levels were shown to be elevated in the gingiva of chronic periodontitis and from active periodontitis sites.¹⁹ To the best of our knowledge, the expression and function of IDO in periodontal tissues have not been explored. The aim of this study was to determine IDO expression in healthy and periodontitis gingiva. In addition, we studied IDO expression and the regulation of human gingival fibroblasts (HGFs), one of the most abundant cell types in gingival tissues.

MATERIALS AND METHODS

Gingival Tissue Sample Collection

The study protocol was approved by the ethics committee of the Faculty of Medicine, Chulalongkorn University. Tissue samples were obtained from marginal gingiva excised during tooth extraction or periodontal surgery at the Graduate Periodontology Clinic between April 2006 and December 2007. Written informed consent was obtained prior to tissue collection. For immunohistochemistry, we obtained gingival samples from 12 individuals. Six samples were from healthy gingiva, and six samples were from periodontitis tissues. Healthy gingival samples were collected from sites with clinically healthy gingiva, no bleeding on probing, no radiographic bone loss, and probing depth <4 mm. Periodontitis tissue samples were collected from sites with gingival inflammation, bleeding on probing, radiographic bone loss, and probing depth >5 mm. Tissue samples were washed briefly in normal saline solution, placed in the optimum cutting temperature (OCT) embedding compound, snap-frozen in liquid nitrogen, and stored at -80°C. For fibroblast cell culture, healthy gingival samples were collected from four subjects. Tissues were washed briefly in normal saline solution and placed immediately into tissue culture media. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, gentamicin (50 μ g/ml), penicillin G (50 U/ml), streptomycin (50

μ g/ml), and fungizone (2.5 μ g/ml)[§] was used for tissue collection and cell culture. The samples were kept on ice and processed within a few hours.

Immunohistochemistry

Gingival tissue samples were cut to 5 μ m in thickness. Cryosections were fixed with ice-cold acetone for 10 minutes, air-dried, and washed with phosphate buffered saline (pH 7.4). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes. Immunoperoxidase staining was performed using a commercially available kit.^{||} Non-specific binding was reduced by applying blocking serum for 20 minutes. Sections were incubated overnight at 4°C with purified IDO-specific mouse monoclonal antibody (immunoglobulin G₁; 1 mg/ml diluted at 1:100 in blocking serum) or non-specific mouse antibody.[¶] Antibody binding was detected using an avidin-biotin complex detection technique, following the manufacturer's protocol. Immunostaining was visualized using 3,3'-diaminobenzidine chromogen. The slides were counterstained with hematoxylin. For quantitative analysis, the tissue sections were magnified at $\times 400$. Three sample areas within the epithelium were taken from each section. The number of epithelial cells stained positive for IDO was counted, and the percentage of IDO⁺ cells was calculated.

Primary Culture of HGFs

The method used to obtain HGF cells from the gingival tissue was described by Murakami et al.²⁰ Briefly, the biopsy was washed twice with DMEM to remove blood clots and adherent erythrocytes. Then it was cut into fragments of 1 to 3 mm³ with a sterile scalpel. These tissues were transferred to a 35-mm tissue culture dish containing 2 ml culture medium and incubated at 37°C in a humidified 5% CO₂-air atmosphere. Culture medium was changed twice weekly. When the fibroblast cells surrounding the tissue explants were confluent, they were subcultured into a larger tissue culture dish. HGFs at passages 3 to 6 were used in this study.

Detection of IDO mRNA Expression by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

HGFs (2.5 $\times 10^5$ cells/well) were seeded into a six-well tissue culture plate overnight. The cells were stimulated with the following agents: IFN- γ [#] (10 to 1,000 U/ml), TNF- α ^{**} (0.5 to 50 ng/ml), IL-1 β ^{††} (0.5 to 50 ng/ml), and LPS from *Porphyromonas gingivalis* strain 381²¹ (PgLPS; 0.1 to 10 μ g/ml). In addition,

§ Gibco, Grand Island, NY.

|| VECTASTAIN ABC kit, Vector Laboratories, Burlingame, CA.

¶ Universal negative control mouse antibody, DakoCytomation, Carpinteria, CA.

R&D Systems, Minneapolis, MN.

** R&D Systems.

†† R&D Systems.

the combination of IFN- γ and each reagent (TNF- α , IL-1 β , or *Pg*LPS) was used. Unstimulated HGFs served as a control. After 24 hours, HGFs were harvested to determine IDO expression. Total RNA was extracted, according to the manufacturer's protocol.^{††} One microgram of total RNA was used for reverse transcription with random hexamer and reverse transcriptase,^{§§} following the manufacturer's instruction. The cDNA was used for detection of IDO mRNA by PCR. PCR was carried out with PCR mixture containing 15 mM MgCl₂, 20 μ M IDO primer, 10 mM deoxynucleotide triphosphate, 5 unit/ μ l Taq polymerase, and the DNA template. The PCR conditions consisted of a first heating step (95°C for 5 minutes), 30 amplification cycles (95°C for 15 seconds, 60°C for 30 seconds, and 70°C for 30 seconds), and one final extension step (72°C for 7 minutes). Specific primer sequences for the gene were as follows: human GAPDH: forward 5'-TCATCTCTGCCCCCTCTGCTG-3' and reverse 5'-GCCTGCTTCACCACTTCTTG-3' (approximate size 400 base pairs [bp]); human IDO: forward 5'-CTT-CCTGGTCTCTCTATTGG-3' and reverse 5'-GAA-GTTCCTGTGAGCTGGT-3' (approximate size 430 bp).²² PCR products were separated by electrophoresis in a 1.2% agarose gel containing ethidium bromide. GAPDH was used as an internal control. The target bands were visualized with an ultraviolet illuminator and analyzed with image-analysis software.^{|||}

Detection of IDO Enzymatic Activity

HGFs (3×10^4 cells/well) were seeded into a 96-well microtiter plate overnight. The cells were stimulated with the following agents: IFN- γ , TNF- α , IL-1 β , *Pg*LPS, or their combinations. Unstimulated HGFs served as a control. After 24 and 48 hours, culture supernatants were harvested and assayed for the presence of kynurenine, the first stable catabolite of tryptophan in the kynurenine pathway. Kynurenine was detected by a modified spectrophotometric assay.²³ Briefly, 50 μ l 30% trichloroacetic acid was added to 100 μ l culture supernatant, vortexed, and centrifuged at $8,000 \times g$ for 5 minutes. Seventy-five microliters of the supernatant was added to an equal volume of Ehrlich reagent (100 mg *p*-dimethylbenzaldehyde and 5 ml glacial acetic acid) in a 96-well microtiter plate. Optical density was measured at 492 nm. A standard curve of defined kynurenine concentration (0 to 100 μ M) permitted analysis of unknown samples.

Statistical Analysis

The independent sample *t* test was used to compare the mean percentage of IDO-expressing cells in healthy and periodontitis gingiva. The one-sample *t* test was used to compare the level of IDO activity between samples. Results were expressed as mean \pm SE. Data were analyzed using statistical software.^{¶¶} Sta-

tistical differences with a *P* value <0.05 were considered significant.

RESULTS

IDO Expression in Gingival Tissues

The gingival tissue samples were obtained from 12 subjects (six males and six females) with a mean age of 40 ± 12 years (range, 24 to 66 years). We observed IDO expression in healthy gingiva and periodontitis gingiva. Figure 1 shows representative findings. Within epithelium, the number of epithelial cells expressing IDO was lower in healthy tissues (Fig. 1A) than in periodontitis tissues (Fig. 1B). Gingival epithelial cells showed distinct nuclear staining of IDO (Fig. 1C). Within connective tissues, IDO expression was observed in fibroblast cells, endothelial cells, and inflammatory mononuclear cells (Fig. 1D). The number of cells stained positive for IDO was also lower in healthy tissues (Fig. 1E) than in periodontitis tissues (Fig. 1F). The control sections showed no immunoreactivity (Figs. 1G and 1H). Quantitative analysis showed that the percentage of epithelial cells stained positive for IDO was $40.6\% \pm 8.4\%$ in healthy gingiva compared to $77.7\% \pm 8.4\%$ in periodontitis gingiva. This difference was statistically significant ($P < 0.001$). The percentage of IDO-expressing cells within connective tissue was not determined; this was due to the close proximity and clustering of cells, which did not allow accurate cell counting.

IDO mRNA Expression by HGFs

RT-PCR analysis was performed to investigate whether inflammatory cytokines and bacterial products induce IDO mRNA expression in HGF cells. Figure 2 depicts a representative RT-PCR result. HGF cells normally did not express detectable levels of IDO mRNA. However, IDO expression was induced upon treatment with IFN- γ , IL-1 β , TNF- α , and *Pg*LPS. IFN- γ was the strongest inducer of IDO expression. Relative IDO mRNA expression, as determined by the ratio of IDO mRNA to GAPDH mRNA, is shown in Figure 3. IFN- γ , TNF- α , and *Pg*LPS seemed to induce IDO mRNA expression in a dose-dependent manner. However, stimulation with IL-1 β did not show the dose-dependent effect. We also evaluated whether combinations of these cytokines showed an additive effect on IDO induction. HGF cells treated with combinations of IFN- γ and IL-1 β , TNF- α , or *Pg*LPS showed increased IDO expression compared to IFN- γ alone (Fig. 4).

†† TRIzol reagent, Invitrogen, Carlsbad, CA.

§§ Improm-II, Promega, Madison, WI.

||| Gene Genius Bio Imaging System, Syngene, Cambridge, U.K.

¶¶ SPSS version 12.0, SPSS, Chicago, IL.

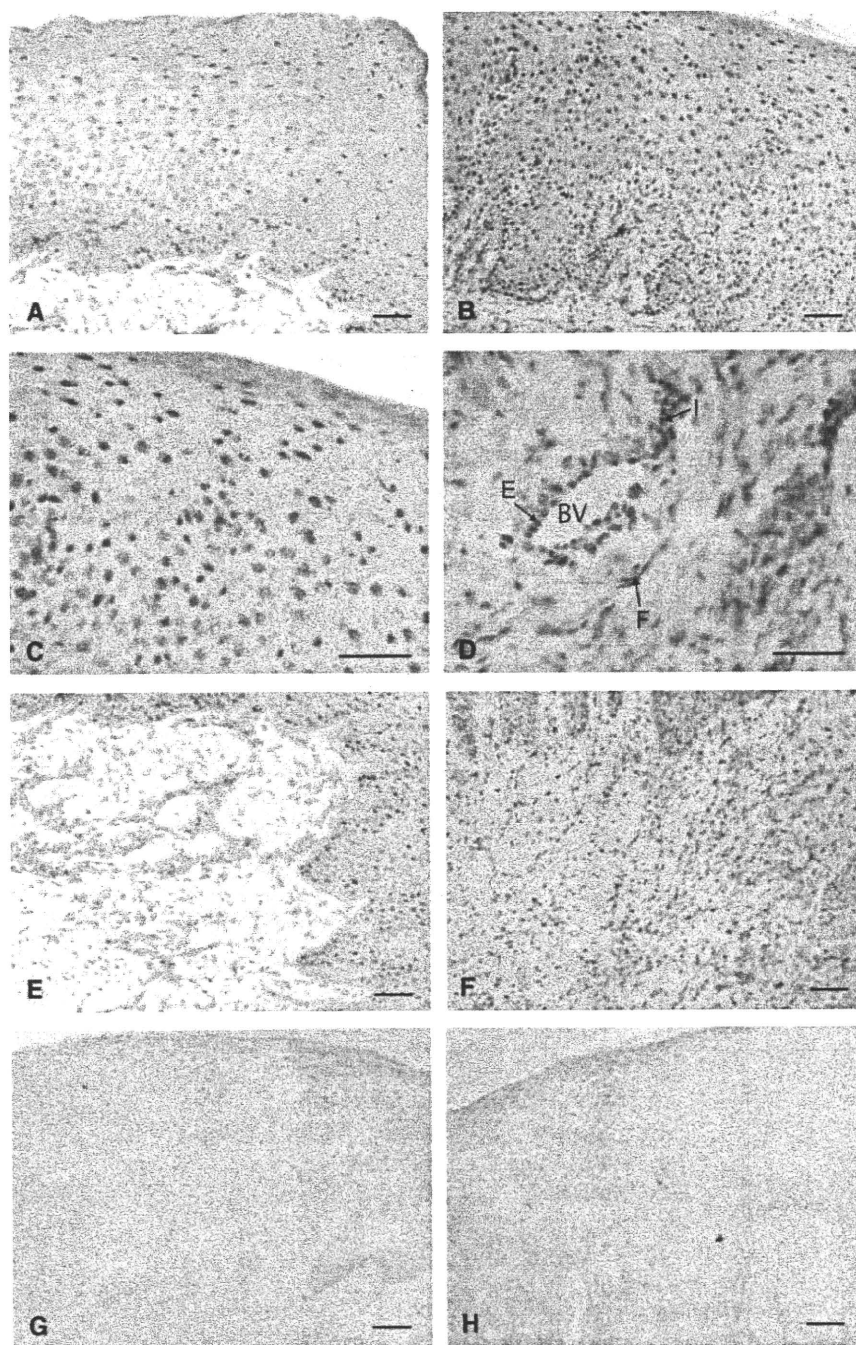


Figure 1.

Immunolocalization of IDO expression in human gingiva. **A)** The epithelium of healthy gingiva. **B)** The epithelium of periodontitis gingiva. IDO expression was stained with diaminobenzidine and is shown in brown. Cell nuclei were counterstained with hematoxylin and were shown in blue-purple. There were fewer epithelial cells expressing IDO in healthy gingiva than in periodontitis gingiva. **C)** The nuclear localization of IDO in gingival epithelial cells. **D)** IDO expression by gingival fibroblasts (F), endothelial cells (E), and inflammatory mononuclear cells (I) (arrows). BV = blood vessel. **E)** The connective tissue of healthy gingiva. **F)** The connective tissue of periodontitis gingiva. Cells stained positive for IDO were fewer in healthy tissues than in periodontitis tissues. **G)** Negative control section of healthy gingiva stained with non-specific immunoglobulin G (IgG). **H)** Negative control section of periodontitis gingiva stained with non-specific IgG. Scale bars = 50 μ m.

Expression of IDO Activity by HGFs

To evaluate whether HGF cells produced functional IDO, we detected IDO enzymatic activity by measuring the level of kynurenine, which is the first stable catabolite in the metabolic pathway of tryptophan. IDO activity seemed to be increased over time. Significantly increased IDO activity was detected in HGF cells treated with IFN- γ . Increased IDO activity was also observed in HGF cells treated with IL-1 β and TNF- α , although it did not reach statistical significance (Fig. 5). The combination of IFN- γ and IL-1 β as well as IFN- γ and TNF- α showed significantly higher IDO activity than that of IFN- γ alone (Fig. 6).

DISCUSSION

IDO is an enzyme that metabolizes the amino acid tryptophan. This enzyme has a complex role in immunoregulation in infection, pregnancy, autoimmunity, transplantation, and cancer.²⁴ In this study we showed that IDO was expressed in human gingiva and may play a role in the pathogenesis of periodontal disease. IDO expression was detected in many cell types within gingival tissues, including epithelial cells, fibroblasts, endothelial cells, and inflammatory mononuclear cells. Within epithelium, the number of epithelial cells expressing IDO was lower in healthy tissues than in periodontitis tissues. Cultured foreskin keratinocytes did not express IDO mRNA unless they were induced by IFN- γ .²⁵ Tissue samples from inflammatory skin diseases, including psoriasis and atopic dermatitis, showed increased IDO mRNA expression compared to the uninvolved skin.²⁶ Therefore, the IDO expression in epithelial cells seemed to be upregulated in the presence of inflammation. This was consistent with our findings that IDO expression in epithelium of periodontitis tissues was higher

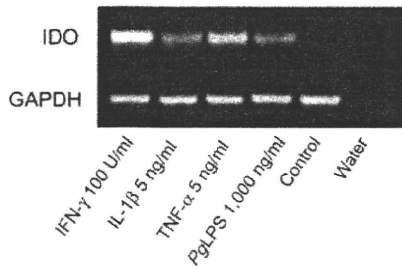


Figure 2. IDO mRNA expression of HGF cells upon stimulation with IFN- γ , IL-1 β , TNF- α , and PgLPS. Unstimulated HGFs served as a control.

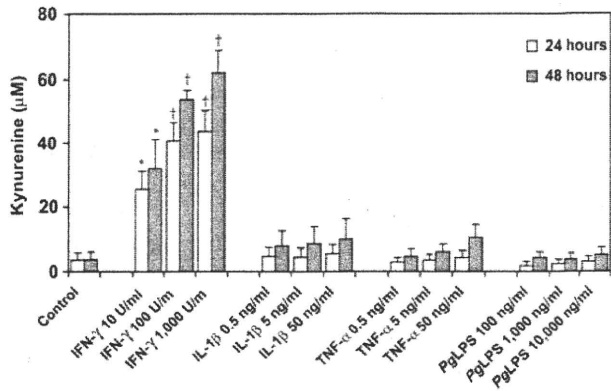


Figure 5. IDO activity in HGF cells treated with different concentrations of IFN- γ , IL-1 β , TNF- α , or PgLPS. Data are shown as mean \pm SE from four separate experiments. Significantly different from the untreated control: * $P < 0.05$; $^{\dagger}P < 0.01$.

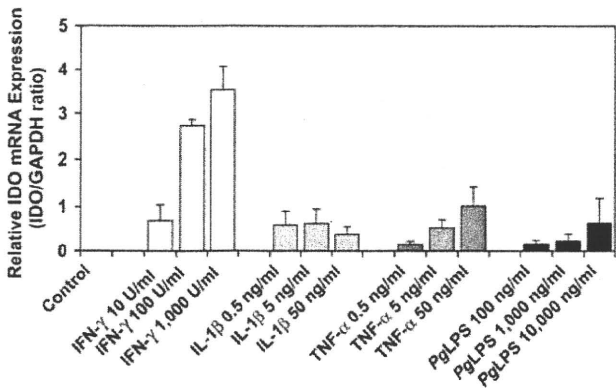


Figure 3. Relative IDO mRNA expression in HGF cells treated with different concentrations of IFN- γ , IL-1 β , TNF- α , or PgLPS. Data are shown as mean \pm SE from four separate experiments.

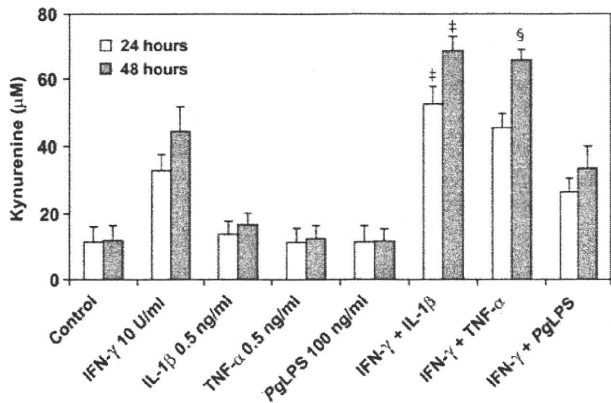


Figure 6. IDO activity in HGF cells treated with combinations of IFN- γ and IL-1 β , TNF- α , or PgLPS. Data are shown as mean \pm SE from four separate experiments. Significantly different from IFN- γ alone: $^{\#}P < 0.05$; $^{\S}P < 0.01$.

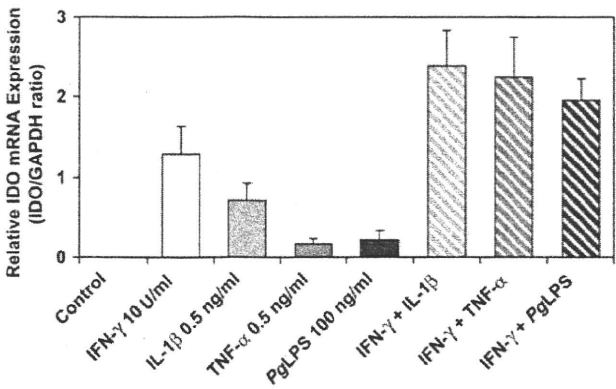


Figure 4. Relative IDO mRNA expression in HGF cells treated with combinations of IFN- γ and IL-1 β , TNF- α , or PgLPS. Data are shown as mean \pm SE from four separate experiments.

mononuclear cells,²⁷ stromal cells, luminal and glandular epithelial cells of human placenta,²⁸ and ovarian cancer cells.²⁹ However, there was a report³⁰ of IDO immunostaining localized primarily to the nuclei of placental endothelial cells. How IDO translocates to the nucleus and whether it also has a nuclear function are not known.

In gingival connective tissue, the level and extent of IDO expression were also higher in periodontitis tissues than that of healthy tissues. Periodontitis lesions had high levels of several inflammatory cytokines and bacterial products that were inducers of IDO.¹⁷⁻¹⁹ Therefore, upregulation of IDO expression in periodontitis lesions may be due to the presence of these agents. In this study, we assessed IDO expression of

cultured HGFs upon stimulation with IFN- γ , IL-1 β , TNF- α , and *Pg*LPS. Gingival fibroblasts are the major cell type within periodontal tissues and may participate directly with several inflammatory mediators in periodontitis. We found that HGFs did not constitutively express IDO; however, IDO expression was inducible in these cells. IFN- γ is a potent inducer for IDO expression in HGFs. Lower levels of IDO expression were detected upon stimulation with IL-1 β , TNF- α , and *Pg*LPS. IFN- γ was shown to be a strong inducer of IDO expression in many cell types, including dendritic cells, macrophages, epithelial cells, skin fibroblasts, and many cancer cell lines.^{14,31,32} It was shown that the IDO promoter contains the interferon-stimulated response element (ISRE) and gamma-activated sequence (GAS). These sequences were the binding site for the transcription factor IFN-regulatory factor-1 (IRF-1) and signal transducer and activator of transcription 1 (STAT1), which allowed activation of the IDO gene in response to IFN- γ .³³

We showed that IL-1 β , TNF- α , and *Pg*LPS, when used as a single agent, induced low levels of IDO expression in HGFs. TNF- α alone was ineffective in IDO induction of peripheral blood mononuclear cells, macrophages, epithelial cell line, and an astrocytoma cell line,^{5,15,34} but it showed weak IDO induction in a fibroblast cell line.³⁵ IL-1 alone also was unable to induce IDO expression in macrophages³⁶ or epithelial cells.¹⁵ Bacterial LPS induced IDO expression in dendritic cells³⁷ and monocyte-derived macrophages¹⁶ but not in epithelial cells from the cervix¹⁵ or lung.³⁴ Therefore, IDO expression in response to inflammatory cytokines and mediators seems to be cell-type specific.

IFN- γ in combination with IL-1 β , TNF- α , or *Pg*LPS augmented the level of IDO expression in HGFs compared to IFN- γ alone. Combinations of IFN- γ and IL-1 β as well as IFN- γ and TNF- α also increased IDO expression in human monocyte-derived macrophages and human cervical epithelial cells.^{15,36} The synergistic effect of TNF- α and IFN- γ on IDO induction was shown to be mediated at the level of transcription through an increase in IFN- γ receptor expression that enhanced the binding of STAT1 to GAS and IRF-1 to ISRE sites.³³ The combination effect of inflammatory cytokines and bacterial products on IDO expression may be important for regulating IDO function in vivo.

The control of IDO activity seems to be complex and cell-type specific. The presence of IDO mRNA and protein may not correlate with its functional activity. Human dendritic cells constitutively express IDO protein, but the protein does not have functional enzymatic activity until these cells are activated by IFN- γ and cluster of differentiation (CD) 80/CD86 ligation.³⁸ In this study, we showed that increased IDO mRNA expression in HGFs upon treatment with IFN- γ was

positively correlated with increased IDO activity. In addition, the IDO activity was increased over time. However, IDO activity in HGFs treated with IL-1 β , TNF- α , or *Pg*LPS was not significantly different from that of untreated controls. This may be due to the low level of IDO mRNA expression in these cells and the wide variation in the level of response between primary cell lines. We also showed that IL-1 β and TNF- α augmented the IDO activity in IFN- γ -treated HGF cells. IL-1 β , TNF- α , and LPS were shown to enhance the IDO activity induced by IFN- γ in human monocyte-derived macrophages.^{16,36} In contrast, the presence of IL-1 β and TNF- α decreased the IDO activity induced by IFN- γ in a uroepithelial cell line and showed no effect on the IDO activity induced by IFN- γ in an astrocytoma cell line.⁵ It seemed that HGFs were able to produce functional IDO in response to several inflammatory cytokines and immunomodulating agents. These agents may work together or regulate each other to control IDO expression and activity in periodontal tissues.

The role of IDO in periodontal disease pathogenesis is not known. A previous study³⁹ from our laboratory showed that coculturing of peripheral blood mononuclear cells with HGF cells treated with IFN- γ and *Pg*LPS resulted in the suppression of T-cell proliferation. This effect could be reversed by the addition of 1-methyl-tryptophan, an inhibitor of IDO. Therefore, IDO expression may be one of several mechanisms to downregulate the inflammatory process in periodontitis. This effect may be beneficial to the host and prevent excessive inflammation and the destruction of periodontal tissues. Further studies are needed to explore this hypothesis.

CONCLUSIONS

IDO was expressed in human gingiva, and the expression was upregulated in chronic periodontitis. The increased IDO expression in periodontitis lesions may be due, in part, to the activation of HGFs by inflammatory cytokines and bacterial products.

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