Tyrosine protein phosphorylation in murine B lymphocytes by stimulation with lipopolysaccharide from Porphyromonas gingivalis

Shigenobu Kimura a,b, Toshiya Koga b, Taku Fujiwara a, Masanori Kontani a, Keiichiro Shintoku b, Hidehiro Kaya b, Shigeyuki Hamada a,*

*a Department of Oral Microbiology, Osaka University Faculty of Dentistry, 1-8 Yamanaka, Suita, Osaka 565, Japan
b Department of Endodontics and Periodontics, Fukuoka Dental College, 2-15-1 Tamara, Sawara-ku, Fukuoka, 814-01, Japan

Received 24 April 1995; accepted 25 April 1995

Abstract

The molecular effect of lipopolysaccharides (LPS) from Porphyromonas gingivalis as well as Escherichia coli on the tyrosine protein phosphorylation in the splenic B lymphocytes from LPS-responsive C3H/HeN and LPS-hyporesponsive C3H/HeJ mice was examined. P. gingivalis LPS induced tyrosine phosphorylation of selected membrane proteins that included the phosphoproteins with apparent molecular masses of 24.8 kDa and 26.0 kDa (p24.8 and p26.0) in the B lymphocytes from both strains of mice, while E. coli LPS induced p24.8 and p26.0 in C3H/HeN B lymphocytes only. These findings suggest that through the same tyrosine phosphorylation pathway as observed in C3H/HeN B lymphocytes, P. gingivalis LPS induced the activation of C3H/HeJ B lymphocytes in which a trigger signal by E. coli LPS could not be transduced to initiate tyrosine protein phosphorylation.

Keywords: Tyrosine protein phosphorylation; Murine B lymphocytes; Lipopolysaccharide; Porphyromonas gingivalis

1. Introduction

Selected species of Gram-negative anaerobic rods including Porphyromonas gingivalis have been implicated in the pathogenesis of periodontal diseases [1]. We have shown that lipopolysaccharides (LPS) from P. gingivalis are composed of unique constituents and exhibit characteristic immunobiological activities [2,3]. Splenic B lymphocytes from C3H/HeJ mice known to be hyporesponsive to the LPS from Enterobacteriaceae, including Escherichia coli, did proliferate after exposure to P. gingivalis LPS [4]. Since periodontitis lesions are characterized as infiltration with increased numbers of plasma cells, the LPS of periodontopathic Gram-negative rods have been considered to stimulate immunocompetent cells, especially B cells localized in the gingival tissue.

It is important to elucidate the mechanisms of signal transduction in terms of B cell activation upon stimulation with LPS and other B cell stimulants. However, the intracellular signals generated by LPS

* Corresponding author. Tel./Fax: +81 (6) 878-4755.
have not yet been well defined. Although activation of resting B cells by the cross-linking of membrane immunoglobulins induced phosphoinositide hydrolysis generating diacylglycerol, a potent activator of protein kinase [5], LPS did not initiate the phosphoinositide pathway [6]. Tyrosine protein phosphorylation could be important in the LPS-induced activation of B lymphocytes. In this regard, Dearden-Badet and Revillard [7] reported that the protein tyrosine kinase (PTK) inhibitors, genistein and/or herbimycin A, inhibited the proliferative response of the splenic B lymphocytes from BALB/c mice.

We demonstrate here that P. gingivalis LPS increases tyrosine protein phosphorylation in B lymphocytes not only from LPS-responsive C3H/HeN but also from LPS-hyporesponsive C3H/HeJ mice. We have also shown that pretreatment of B lymphocytes with herbimycin A abrogates the LPS-induced tyrosine protein phosphorylation pathway required for cellular proliferation.

2. Materials and methods

2.1. Bacterial strains and preparation of LPS

P. gingivalis 381 was cultured anaerobically in GAM broth (Nissui Seiyaku, Tokyo, Japan) supplemented with 5 μg ml⁻¹ of hemin and 1 μg ml⁻¹ of menadione at 37°C in the BBL GasPak System (Becton Dickinson Microbiology Systems, Cockeysville, MD). E. coli K235 was grown aerobically in Trypticase soy broth. The LPS from P. gingivalis 381 and E. coli K235 were prepared as described previously [2].

2.2. Preparation of B cells and cellular stimulation with LPS

Spleens from inbred C3H/HeN and C3H/HeJ mice, 6 to 8 weeks of age, were removed aseptically and placed in sterile RPMI 1640 supplemented with 2-mercaptoethanol (5 × 10⁻⁵ M), penicillin (100 U ml⁻¹) and streptomycin (100 mg ml⁻¹) (incomplete RPMI 1640). Spleen cells were gently passed through sterile stainless screens and then resuspended in prewarmed incomplete RPMI 1640 containing 5% (v/v) heat-inactivated fetal calf serum (FCS). The depletion of adherent cells was performed by passage through Sephadex G-10 columns [8]. The eluted cells (G10-passed cells) were > 97% viable, and contained > 99% lymphocytes and < 0.1% esterase-positive cells. The G10-passed cells were enriched for B cells by panning with rabbit IgG anti-mouse F(ab')₂ as previously described [8]. Either G10-passed cells or panned B cells were resuspended in incomplete RPMI 1640 containing 10% FCS, and were then cultured with 10 μg ml⁻¹ of LPS at 37°C in a humidified 5% CO₂ atmosphere. In some experiments, cells were preincubated for 16 h with herbimycin A (0.5 μM) before LPS-stimulation.

2.3. Anti-phosphotyrosine immunoblotting

After incubation with LPS, cells were washed and sonicated for 1 min followed by centrifugation at 200 × g for 10 min at 4°C. The supernatant was ultracentrifuged at 100000 × g for 60 min at 4°C. The supernatant was then carefully removed and used as the cytosol fraction. The pellets were washed by ultracentrifugation to separate the residual cytosolic components, lysed in 0.25 ml of lysis buffer [1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM PMSF, 0.2 U/ml aprotinin, 4 mM EDTA, 2 mM Na₂VO₄], and used as the membrane fraction. SDS sample buffer was then added and boiled for 5 min. The samples were separated by electrophoresis on SDS-polyacrylamide gel (13%). The separated proteins were transferred to nitrocellulose (4 h at 7 V) and immunoblotted using monoclonal anti-phosphotyrosine antibody. Blots were then incubated with horseradish peroxidase-linked goat anti-mouse Ig antibody, and immunoreactive proteins were visualized by exposure to Hyperfilm-ECL using an enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Life Science, Buckinghamshire, UK). The molecular masses of the induced phosphoproteins were calculated using molecular size standards.

2.4. B cell proliferation by LPS

The G10-passed cells pretreated with or without herbimycin A (5 × 10⁵ cells per well in flat-bottom 96-well plates) were stimulated with 10 μg ml⁻¹ of
LPS for 48 h in incomplete RPMI 1640 containing 10% FCS at 37°C in a humidified 5% CO₂ atmosphere. Proliferation was measured by [³H]TdR incorporation during the last 20 h of a 48 h culture period [9].

3. Results

3.1. P. gingivalis LPS-induced tyrosine protein phosphorylation in B lymphocytes from C3H/HeN and C3H/HeJ mice

Both P. gingivalis LPS and E. coli LPS were found to increase tyrosine protein phosphorylation of the substrate B cell membrane proteins from C3H/HeN mice (Fig. 1). The molecular masses of phosphorylated proteins induced by stimulation of these LPS preparations in the G10-passed cells were identical to those in the panned B cells. Although tyrosine phosphoproteins were induced by stimulation with these LPS preparations, phosphorylated bands corresponding to molecular masses of 24.8 kDa and 26.0 kDa (p24.8 and p26.0, respectively) were most apparently and consistently induced.

In the membrane fractions of B lymphocytes from C3H/HeJ mice, however, only P. gingivalis LPS induced tyrosine protein phosphorylation of the substrate proteins including p24.8 and p26.0 (Fig. 1). The disability of E. coli LPS in activating signals to stimulate tyrosine protein phosphorylation in C3H/HeJ B lymphocytes was not ascribed to the concentration of E. coli LPS used for stimulation. In this regard, our preliminary experiments indicated that the tyrosine protein phosphorylation response occurred following stimulation with either P. gingivalis LPS in C3H/HeJ and C3H/HeN B lymphocytes or E. coli LPS in C3H/HeN B lymphocytes at a concentration of 0.1 μg ml⁻¹ (data not shown).

In the cytosol fractions, on the other hand, no apparent tyrosine protein phosphorylation was observed with the substrate proteins following LPS stimulation in B lymphocytes from C3H/HeN or C3H/HeJ mice (Fig. 1). Although LPS-stimulated B lymphocytes were sonicated when ice-cold, followed by separation of the cytosol fractions by ultracentrifugation at 4°C, it might be possible that proteolytic enzymes released from the cells prevented tyrosine phosphorylation in the cytosol fractions.

We also examined the kinetics of LPS-induced tyrosine protein phosphorylation in B lymphocytes

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Fig. 1. LPS-induced tyrosine protein phosphorylation in the membrane and cytosol fractions of the G10-passed spleen cells and panned B cells from C3H/HeN and C3H/HeJ mice. Molecular masses (in kDa) are indicated on the left.
from C3H/HeN (Fig. 2) and C3H/HeJ mice (Fig. 3). *P. gingivalis* LPS-induced tyrosine protein phosphorylation in B lymphocytes was detectable after 24 h incubation, and reached a peak after 48 h incubation. Significant phosphorylation continued with another 48 h incubation (data not shown). These phosphorylation profiles were basically similar to B lymphocytes from C3H/HeN (Fig. 2) and C3H/HeJ mice (Fig. 3). *E. coli* LPS also induced tyrosine protein phosphorylation in C3H/HeN B lymphocytes in the same manner that was observed in *P. gingivalis* LPS stimulation.

**C3H/HeN**

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Molecular masses (in kDa) are indicated on the left.

**C3H/HeJ**

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Molecular masses (in kDa) are indicated on the left.

Fig. 2. Kinetics of LPS-induced tyrosine protein phosphorylation in the membrane fractions of the cells from C3H/HeN mice. G10-passed spleen cells were stimulated with 10 μg ml⁻¹ of *P. gingivalis* LPS and/or *E. coli* LPS for the indicated time. Molecular masses (in kDa) are indicated on the left.

Fig. 3. Kinetics of LPS-induced tyrosine protein phosphorylation in the membrane fractions of the cells from C3H/HeJ mouse. G10-passed spleen cells were stimulated with 10 μg ml⁻¹ of *P. gingivalis* LPS and/or *E. coli* LPS for the indicated time. Molecular masses (in kDa) are indicated on the left.
3.2. Effect of herbimycin A on LPS-induced tyrosine protein phosphorylation and cellular proliferation of B lymphocytes

We next examined whether a PTK inhibitor, herbimycin A, inhibited *P. gingivalis* LPS-induced tyrosine protein phosphorylation in *B* lymphocytes from C3H/HeN and/or C3H/HeJ mice. Herbimycin A was found to inhibit *P. gingivalis* LPS-induced tyrosine protein phosphorylation in both C3H/HeN and C3H/HeJ B lymphocytes, and *E. coli* LPS-induced tyrosine protein phosphorylation in C3H/HeN B lymphocytes as well (Fig. 4). The inhibition of LPS-induced tyrosine protein phosphorylation in B lymphocytes was not due to cellular toxicity, since herbimycin A of the concentration used exhibited little effect on cell viability as revealed by trypan blue exclusion (data not shown).

Pretreatment with herbimycin A abrogated the *P. gingivalis* LPS-induced proliferative response of both C3H/HeN and C3H/HeJ B lymphocytes (Fig. 5). Similar results were obtained with *E. coli* LPS in C3H/HeN B lymphocytes, while no apparent mitogenic responses were observed in C3H/HeJ B lymphocytes irrespective of the pretreatment with herbimycin A. The inhibition of [3H]TdR incorporation was not due to a shift in kinetics of the peak responses of cellular proliferation (data not shown).

4. Discussion

We demonstrated here that *P. gingivalis* LPS increased tyrosine phosphorylation in the cell membrane proteins including p24.8 and p26.0 in B lymphocytes from C3H/HeN as well as C3H/HeJ mice, while *E. coli* LPS induced phosphorylation only in C3H/HeN B lymphocytes (Fig. 1). This was confirmed by the fact that preincubation of the cells with herbimycin A inhibited the ability of *P. gingivalis* LPS to induce increased tyrosine protein phosphorylation in B lymphocytes (Fig. 4). The treatment with herbimycin A also abrogated the *P. gingivalis* LPS-induced proliferative response of both C3H/HeN and C3H/HeJ B lymphocytes and *E. coli* LPS-induced proliferative response of C3H/HeN B lymphocytes (Fig. 5). These findings strongly suggested that increased tyrosine protein phosphorylation in B lymphocytes following LPS stimulation could be an important signaling event that might lead to cellular responses, which is in accordance with the data of Dearden-Badet and Revillard [7].

Moreover, the phosphoproteins p24.8 and p26.0 were clearly observed in both C3H/HeN and C3H/HeJ B lymphocytes following *P. gingivalis* LPS stimulation (Fig. 1). This finding suggests that the basic signaling pathways that involve tyrosine protein phosphorylation appear to be intact in C3H/HeJ B lymphocytes. The functional 'defect' of
C3H/HeJ B lymphocytes, as revealed by the stimulation with *E. coli* LPS, is most likely ascribable to the putative LPS receptor(s), which may be either missing or altered in some way. This hypothesis is supported by the observation of partial reconstitution of the LPS mitogenic response in splenic C3H/HeJ B lymphocytes by fusing plasma membranes from LPS-responsive C3H/eb B lymphocyte with C3H/HeJ B lymphocytes [10].

We also noticed that the kinetics of LPS-induced tyrosine protein phosphorylation in B lymphocytes was markedly delayed, showing a peak response after 48 h incubation (Fig. 2 and Fig. 3). We initially employed a relatively shorter incubation time with LPS, since the tyrosine protein phosphorylation in macrophages or macrophage cell lines has been demonstrated with incubation times ranging from 15 min to 4 h [11-13]. Under these experimental conditions, however, no apparent LPS-induced tyrosine protein phosphorylation in B lymphocytes was observed (Fig. 2 and Fig. 3). One possible explanation for the delayed response to the stimulation of LPS is that increased expression of the putative LPS receptor(s) on murine B lymphocytes stimulated with LPS requires significant duration, i.e., 24 to 48 h incubation. Indeed, Ernst et al. [14] reported that increased expression of transferrin receptor on murine B lymphocytes stimulated with LPS was detectable only with 12 to 24 h of culture, which coincided with entry into later phases of G1 and initial entry into S phase. Furthermore, it was reported that about 20% of the cells could express the specific LPS receptor after 24 h of incubation with LPS, while constitutive LPS-binding sites were not detectable before incubation using bone marrow cells including pre-B cells [15]. Although specific LPS receptor(s) on B lymphocytes has not been identified yet, onset of the surface LPS receptor hyperexpression may occur after 24 h or more of incubation with LPS. The signal triggered by LPS could then be transduced to initiate tyrosine protein phosphorylation in B lymphocytes.

References


