ORIGINAL CONTRIBUTION

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Nasal polyp fibroblasts produce MIP-3α in response to Toll-like Receptor ligands and cytokine stimulation*

Manabu Nonaka, Nozomu Ogihara, Akira Fukumoto, Atsuko Sakanushi, Kaoru Kusama, Ruby Pawankar, Toshiaki Yagi

Department of Otolaryngology, Nippon Medical School, Sendagi, Bunkyo-ku, Tokyo, Japan

SUMMARY

Objective: *Dendritic cells (DCs) play important roles in the development and perpetuation of immune responses. DCs are present in upper airway diseases such as chronic rhinosinusitis with nasal polyps. However, the mechanisms of how DCs migrate into the upper airway mucosa during upper airway inflammatory diseases remains unclear. Macrophage inflammatory protein-3α (MIP-3α) is known to be a migratory factor for immature DCs. There have been very few reports regarding cells producing this chemokine in the airways. To investigate this, we stimulated fibroblasts cultured from the nasal polyps with various toll-like receptor (TLR) ligands, which are derived from microorganisms, and IL-1β and TNF-α, which are proinflammatory cytokines, and analyzed their ability to produce MIP-3α.*

Methods: *Fibroblast lines were established from nasal polyps and stimulated with TLR2, 3, 4, 5, 7/8 and 9 ligands, IL-β1 and TNF-α. MIP-3α mRNA expression in nasal polyp fibroblasts (NPF) was evaluated by real-time RT-PCR and the protein levels of MIP-3α in the supernatants of stimulated NPF was measured by ELISA.*

Results: *Stimulation with TLR2, 3, 4 and 5 ligands, IL-1β and TNF-α, induced MIP-3α gene expression and protein production in the cultured NPF. This response was dose- and time-dependent.*

Conclusion: *NPF possibly play an important role in the recruitment of DCs in upper airway diseases such as chronic rhinosinusitis with nasal polyps through the production of MIP-3α.*

Key words: MIP3-α, fibroblasts, TLR ligands, IL-1β, TNF-α

INTRODUCTION

The nasal mucosa is the first site of the respiratory tract that environmental agents encounter. The osteomeatal complex (OMC) is the nasal site that is exposed to the greatest amount of airflow during respiration, especially during inspiration. The OMC is likely to be exposed to a great deal of potentially injurious agents, including microorganisms (e.g., viruses or bacteria). The nose is also susceptible to the development of nasal and paranasal inflammatory conditions such as rhinosinusitis with nasal polyps, rhinitis, etc., that are caused by various microorganisms [1]. It is thought that nasal and paranasal fibroblasts are at least partially involved in those inflammatory conditions based on their ability to produce a variety of cytokines/chemokines in response to proinflammatory cytokines such as IL-1β or TNF-α [2]. Furthermore, they also contribute to innate immunity via their responses through the Toll-like receptors (TLRs) [3-6].

Dendritic cells (DCs) are the only antigen-presenting cells (APCs) that are able to activate näive CD4+ and CD8+T cells [7,8]. DCs in the nasal mucosa may present antigens in such a way that Th1 or Th2 responses are induced [9]. Although a small number of DC is always present to sense invading pathogens, additional recruitment of immature DCs to the airways and sites of antigen exposure is needed and is dependent on the presence of select chemokines and expression of the corresponding specific chemokine receptors on DCs [10]. MIP-3α (also called CCL20) is a CC chemokine with an in vivo distribution that is rather restricted to mucosal and inflamed tissues. It is inducible in vitro by mediators of inflammation and is expressed by both hemopoietic cells (DCs, monocytes, granulocytes, T cells and B cells) and non-hemopoietic cells (epithelial cells, synoviocytes and endothelial cells) [11-13]. MIP-3α is a unique functional ligand for the chemokine receptor CCR6, and it was demonstrated to be the most powerful chemokine in inducing migration of immature DCs, even in

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comparison with RANTES and MIP-1α [11,14]. The CCR6 receptor is selectively expressed on immature DCs, such as Langerhans cell precursors, a subpopulation of DCs that reside on mucosal surfaces [15].

Chang et al. [16] looked at genome-wide patterns of gene expression in cultured human fibroblasts derived from various anatomical sites by using cDNA microarrays, and found that fibroblasts from each site displayed distinct and characteristic transcriptional patterns. Those findings indicate that the use of in vitro fibroblast cell lines is an acceptable approach to studying the physiological characteristics of these cells. Thus, we carried out this study with primary cultures of nasal polyp fibroblasts (NPF).

Fibroblasts exist adjacent to and in close association with DCs in the stroma of the nasal and paranasal mucosae. Fibroblasts are known to respond to stimulation by TLR ligands and proinflammatory cytokines such as IL-1β and TNF-α. These stimuli may result in fibroblasts releasing selected cytokines and/or chemokines that influence the recruitment of local DC populations. We hypothesized that NPF would express and produce increased levels of MIP-3α in response to TLR ligands and cytokines.

MATERIALS AND METHODS

Reagents

Human recombinant TNF-α and IL-1β were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). TLR2 ligands [lipoteichoic acid (LTA), synthetic tripalmitoylated lipopeptide (Pam3CSK4), peptidoglycan (PGN)], TLR3 ligand [synthetic analog of double-stranded RNA (poly (I:C))], TLR4 ligand [lipopolysaccharide (LPS)], TLR5 ligand (flagellin), TLR7/8 ligand [lyophilized single-stranded poly(U) (sspoly(U))] and TLR9 ligand [bacterial DNA (DNA)] were purchased from InvivoGen Co. (San Diego, CA, USA).

Cell source

Primary fibroblast lines were established from human polyp biopsy tissues (n=5) removed at polypectomy and characterized as previously described [17]. Only fibroblast lines between the fourth and sixth passages were used in this study. All the nasal polyp specimens had been obtained from patients with chronic rhinosinusitis (four males and one female, aged 53.6 ± 14.3 yr (mean ± SD)). Three of the patients were atopic, diagnosed on the basis of elevation of at least one of the capsulated hydrophobic carrier polymer-radioallergosorbent tests (CAP-RASTs) against 8 common aeroallergens. Each had a cedar pollen score of 2 or more. One of them had a house dust CAP-RAST score of 2. Two of the atopic patients had asthma. All subjects had given written informed consent, and the study protocol was approved by the Ethics Committee of Nippon Medical School Hospital.

Culture

Before cytokine assay, fibroblasts (1×104 cells) were plated in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA). Before real-time PCR, fibroblasts (2×106 cells) were plated in 60-mm culture dishes (Corning, NY, USA). Plated fibroblasts were allowed to grow to confluence in regular growth medium (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum (FBS), penicillin at 100 U/ml, streptomycin at 100 g/ml and amphotericin B at 2.5 g/ml. The same culture medium was used when fibroblasts were exposed to the TLR ligands, IL-1β and TNF-α as stimuli. At specified time points, cultures were collected and centrifuged; supernatants were collected for cytokine protein assays, and the pelleted cells were collected for RNA extraction.

Analysis of MIP-3α mRNA by quantitative real-time RT-PCR

Total cellular RNA was extracted and purified using an RNeasy mini kit (Qiagen GmbH, Germany). Total RNA, 2 g, was reverse-transcribed at 37°C for 60 min using random primers (TAKARA, Otsu, Japan) and Omniscript reverse transcriptase (Qiagen, Tokyo, Japan) according to the manufacturers' protocols.

Quantitative real-time RT-PCR was carried out using the TaqMan assay and ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers and fluorogenic probes for MIP-3α and β-actin were purchased from Applied Biosystems. The amplification conditions were 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

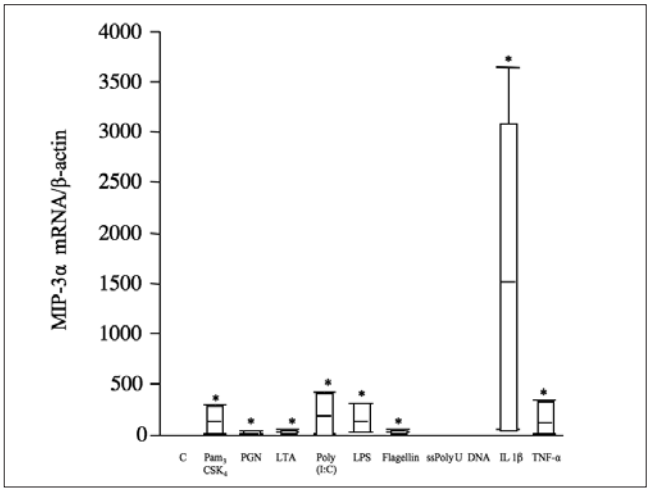


Figure 1. MIP-3α mRNA expression in NPF stimulated with TLR ligands and cytokines (IL-1β and TNF-α) for 6 hours.

Box plots represent the median values with 25% and 75% interquartiles from 3 donors, each studied in duplicate; the error bars represent the 10th and 90th percentiles. Results are normalized to β-actin expression. *$p < 0.05$ compared with control cultures stimulated under the same conditions but without TLR ligands and cytokines. C, control (no stimulation); Pam3CSK4, 1 g/ml; PGN, 10 g/ml; LTA, 10 g/ml; Poly (I:C), 50 g/ml; LPS, 1 ng/ml; flagellin, 1 g/ml; ssPoly U, 1 g/ml; DNA, 10 g/ml; IL-1β, 1 ng/ml; TNF-α, 10 ng/ml.

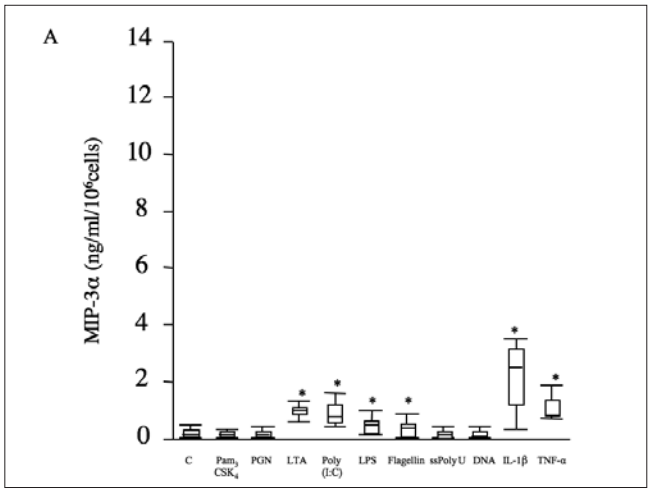


Figure 2. MIP-3α release by NPF stimulated with different concentrations of TLR ligands and cytokines (IL-1β and TNF-α) for 24 hours.
A) C, control (no stimulation); Pam3CSK4, 10 ng/ml; PGN, 100 ng/ml; LTA, 100 ng/ml; Poly (I:C), 500 ng/ml; LPS, 10 pg/ml; flagellin, 10 ng/ml; ssPoly U, 10 ng/ml, DNA, 100 pg/ml; IL-1β, 10 pg/ml; TNF-α, 100 pg/ml.

Data analysis was performed using a sequence detector system software (Applied Biosystems). Threshold cycles were used to calculate arbitrary mRNA concentrations by the relative standard curve method. The standard curve was constructed using serial dilutions of cDNA containing the message for MIP-3α. The level of MIP-3α mRNA was normalized to the level of β-actin mRNA.

Cytokine assay

The levels of MIP-3α in culture supernatants were measured by an enzyme-linked immunosorbent assay (ELISA) using a commercially available human MIP-3α ELISA kit (R&D Systems), which detects both recombinant and natural MIP-3α. The sensitivity of this assay system was more than 7.8 pg/ml. The data are presented as nanograms of MIP-3α per 1 $\times 10^6$ cells.

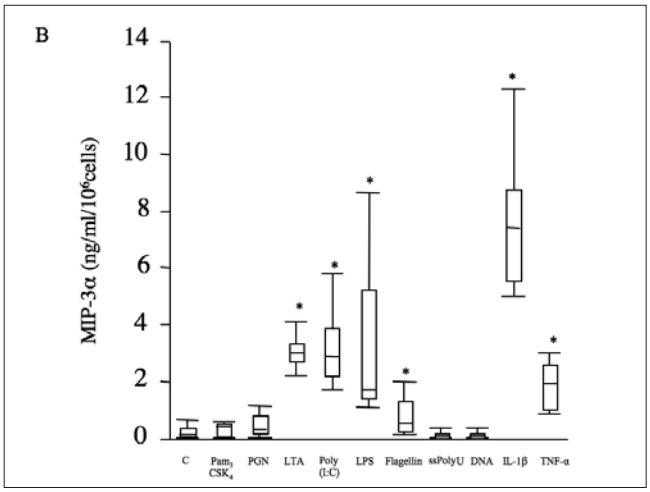
Statistical analysis

The paired Wilcoxon test was used for statistical analysis. A p-value < 0.05 was considered statistically significant.

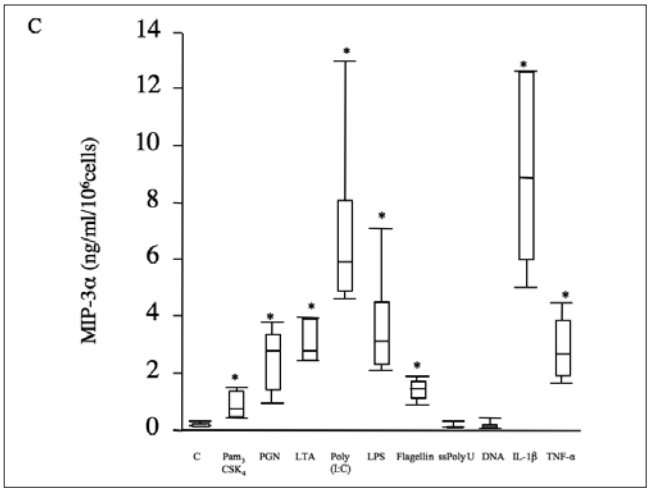
RESULTS

Expression of MIP-3α mRNA in NPF

After stimulation of NPF with TLR ligands and cytokines (1 g/ml IL-1β and 10 ng/ml TNF-α) for 6 hours, real-time RT-PCR was performed. The dose of each TLR ligand used for the experiment was decided on the basis of the manufacturer's recommendation. TLR2, 3, 4 and 5 ligands, but not TLR7/8 or 9 ligands, and the cytokines (IL-1β and TNF-α) upregulated MIP-3α mRNA expression in the fibroblasts (Figure 1).



B) C, control (no stimulation); Pam3CSK4, 100 ng/ml; PGN, 1 g/ml; LTA, 1 g/ml; Poly (I:C), 5 g/ml; LPS, 100 pg/ml; flagellin, 100 ng/ml; ssPoly U, 100 ng/ml, DNA, 1 g/ml; IL-1β, 100 pg/ml; TNF-α, 1 ng/ml.



C) C, control (no stimulation); Pam3CSK4, 1 g/ml; PGN, 10 g/ml; LTA, 10 g/ml; Poly (I:C), 50 g/ml; LPS, 1 ng/ml; flagellin, 1 g/ml; ssPoly U, 1 g/ml, DNA, 10 g/ml; IL-1β, 1 ng/ml; TNF-α, 10 ng/ml. Box plots represent the median values with 25% and 75% interquartiles; the error bars represent the 10th and 90th percentiles ($n = 5$). *$p < 0.05$ compared with control cultures stimulated under the same conditions but without TLR ligands or cytokines.

MIP-3α production by NPF

To determine whether NPF can produce the MIP-3α protein, supernatants from cultured NPF stimulated with TLR ligands and cytokines (IL-1β and TNF-α) were assayed for the level of MIP-3α. The dose of each TLR ligand used for the experiment was decided on the basis of the manufacturer's recommendation. In agreement with the findings regarding enhanced mRNA expression, TLR2, 3, 4 and 5 ligands, but not TLR7/8 or 9 ligands, and the cytokines (IL-1β and TNF-α) stimulated NPF to produce significantly greater amounts of MIP-3α compared with unstimulated NPF (Figures 2A-C). Dose-dependent production of MIP-3α was also demonstrated by stimulation with TLR2, 3, 4 and 5 ligands and the cytokines (IL-1β and

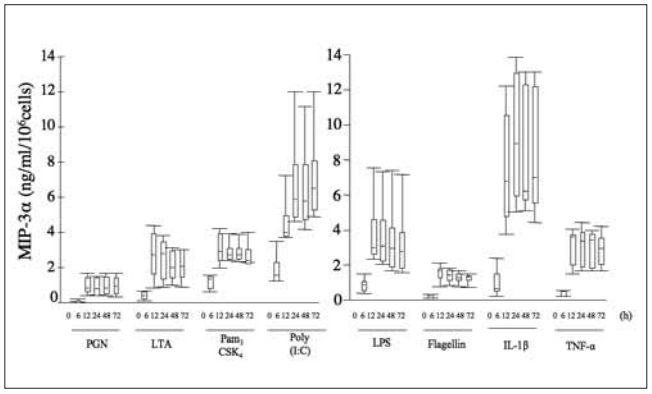


Figure 3. Kinetics of MIP-3α release from NPF.
Cells were cultured in the presence of TLR ligands and cytokines (IL-1β and TNF-α) for 0, 6, 12, 24, 48 and 72 h. Box plots represent the median values with 25% and 75% interquartiles; the error bars represent the 10th and 90th percentiles (n = 5). Pam3CSK4, 1 g/ml; PGN, 10 g/ml; LTA, 10 g/ml; Poly (I:C), 50 g/ml; LPS, 1 ng/ml; flagellin, 1 g/ml; IL-1β, 1 ng/ml; TNF-α, 10 ng/ml.

TNF-α) (Figures 2A-C). TLR2, 4 and 5 ligands and TNF-α induced production of MIP-3α in a time-dependent manner from 0 to 12 h, and a plateau was reached at 12-72 h. In the case of TLR3 ligand and IL-1β, production of MIP-3α increased from 0 to 24 h and reached a plateau at 24-72 h (Figure 3).

DISCUSSION

Chronic inflammatory diseases of the airways such as chronic rhinosinusitis is characterized by cell-cell interactions between blood-derived cells such as monocytes, T cells, B cells, eosinophils and DCs, and structural cells such as epithelial cells and fibroblasts. Such cell-cell interaction and activation of these cells can perpetuate chronic inflammation leading to narrowing of the sinus ostia and further obstruction of the sinus drainage and culminating in a secondary bacterial infection [18]. The sinuses that are most susceptible to chronic inflammation are the frontal, anterior ethmoid and maxillary sinuses. These sinuses all open into the OMC [19]. The OMC is a site that is exposed to various injurious environmental agents, including microorganisms (e.g., viruses or bacteria) and endotoxins, in sinus effusions from infected sinuses and in the inspired air [20]. Bacteria that are isolated from the OMC of patients with chronic rhinosinusitis include not only such Gram-positive bacteria as *Staphylococcus aureus* and *S. pneumonia*, but also many Gram-negative bacteria such as *Haemophilus influenzae*. In addition, ciliated bacteria such as *Pseudomonas aeruginosa* are sometimes detected [21]. Nasal polyps associated with chronic rhinosinusitis often form in the OMC, and it can be surmised that the various microorganisms, their products (e.g., TLR ligands) and proinflammatory cytokines such as IL-1β and TNF-α which have been shown to be expressed in nasal polyps [22] are involved in the pathogenesis of those polyps. The findings [23] that fibroblasts comprised 47% of the total cell population in nasal polyps, that the proportion of activated fibroblasts was significantly higher in nasal polyps than in normal nasal mucosa and that fibroblasts are an important source of inflammatory mediators all support the concept that fibroblasts also may play an important role in the pathogenesis of nasal polyps. In fact, it has been shown that NPF produced MCP-4 in response to TLR2, 3, 4 and 5 ligands, but not TLR7/8 ligands, and that NPF derived from eosinophil-rich nasal polyps were more sensitive than those from non-eosinophilic nasal polyps regarding eotaxin production induced by IL-4 and TNF-α [2,6].

TLRs are transmembrane receptors having an extracellular domain composed of leucine-rich repeats, an intracellular domain structure that is shared with that of IL-1 receptor and a Toll/IL-1 receptor homologous domain. TLRs activate the transcription factor NF (nuclear factor)-κB, a key regulator of immune and inflammatory genes [24]. To date, 10 TLR family members have been identified in humans, and ligands have been identified for all but TLR10 [25]. Except for mycoplasmas, almost all bacteria possess peptidoglycan (PGN) and lipopeptides (lipoteichoic acid (LTA) and Pam3CSK4), which are found in the cell membrane and are recognized mainly by TLR2. LPS, a component of the cell wall of Gram-negative bacteria, is recognized by TLR4, while flagellin, a protein that constitutes cilia and is produced by ciliated bacteria such as *P. aeruginosa*, is recognized by TLR5. In addition, the DNA and RNA sequences that are characteristic of bacteria and viruses are recognized by TLR molecules that are distributed in intracellular endosomes. That is, viral double-stranded RNA is recognized by TLR3 and single-stranded RNA by TLR7/8, while bacterial CpG DNA is recognized by TLR9 [26]. It has been reported that nasal fibroblasts express various TLRs and have the ability to produce chemokines such as MCP-4 in response to TLR2, 3, 4 and 5 ligands and IL-4 [6]. In the present study, we have demonstrated that TLR2, 3, 4 and 5 ligands and proinflammatory cytokines (IL-1β and TNF-α) induce both mRNA expression and release of MIP-3α in NPF. This induction occurred in a dose-dependent manner. Additionally, maxillary sinus-derived fibroblasts also produced MIP-3α in response to the same stimuli (data not shown). Considering that nasal polyp and paranasal fibroblasts respond not only to Gram-negative and Gram-positive bacterial substances, which are often found in the OMC of patients with chronic rhinosinusitis, but also to viral substances, which predispose patients to the development of chronic rhinosinusitis, these fibroblasts may contribute to DC recruitment in nasal polyps via the TLR/ proinflammatory cytokine-induced production of MIP-3α. Athough in vitro experiments do not always reflect in entirety the in vivo conditions, these findings are suggestive of a role of NPF in DC recruitment.

The ability of DCs to present antigens in a context that determines a specific T-cell response makes them central in the

development and perpetuation of airway immune responses. They are abundantly present in allergic nasal polyps, and may play essential roles in the activation of effector Th2 functions leading to eosinophilic inflammation in chronic rhinosinusitis [27]. In the airways, DCs are located not only in the epithelium but also in stromal lesions where fibroblasts are the most abundant cell type. The close proximity of fibroblasts to DCs in the stroma and their ability to secrete various cytokines/chemokines, including RANTES and GM-CSF, in response to TLR ligands and local stimuli such as IL-1β and TNF-α makes them likely candidates to modify DC responses [5].

MIP-3α is a recently described CC-chemokine most powerful in inducing migration of immature DCs, even compared with MIP-1α and RANTES [11,14]. It binds to the CCR6 receptor that has a distinct pattern of expression. Because of the restricted expression of CCR6 and the selective interaction between MIP-3α and CCR6, the pattern of expression of MIP-3α is critical for DC recruitment [27]. MIP-3α has recently been described in mucosal and inflamed tissues [28], and it is an inducible in vitro mediator of inflammation that is expressed by both hemopoietic cells (DCs, monocytes, granulocytes, T and B cells) and non-hemopoietic cells (epithelial cells, endothelial cells and synoviocytes). Our data suggest that fibroblasts in nasal polyps may be involved in the recruitment of DCs via their responses to certain TLR-ligands and proinflammatory cytokines. In a separate study, we have demonstrated that NPF is a source of TSLP [29], which is crucial for the maturation of DCs. Therefore, NPF can regulate DC migration to the tissue as well as maturation. Further studies are needed to clarify the in vivo levels of MIP-3α expression by fibroblasts in nasal polyps.

Taken together, our present findings suggest that NPF may play additional roles in the pathogenesis of nasal polyps by recruitming DCs via the TLR/pro-inflammatory cytokine-induced production of MIP-3α. Specific inhibitors targeting such cell-cell interaction or transcription factors like NF-κB that commonly regulate the induction of several effector genes [24,30] deserve further investigation as potential therapeutic modalities for chronic rhinosinusitis with nasal polyps.

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Manabu Nonaka
Department of Otolaryngology
Nippon Medical School
Sendagi
Bunkyo-ku
Tokyo
Japan

Tel: +81-3-3827-9126
Fax +81-3-3827-9126
E-mail: nonaka@nms.ac.jp