96穴ポリスチレン製プレートに分注した．37℃で24時間培養後，プレートを蒸留水で洗い乾燥させた後，2%クリスタルバイオレットで45分間染色した．再度蒸留水で洗い乾燥させた後，付着したクリスタルバイオレットを5%酢酸エタノールに溶解させ，マイクロプレートリーダー（波長570nm）で吸光測定（OD_{570}値）を行った．

6. 臨床的背景

留置カテーテル，同時分離菌，37℃以上の発熱の有無について検討した．

7. 統計学的解析

Fisherの直接法およびMann-Whitneyの*U*検定を用い，P＜0.05の場合を有意差ありとした．

結　果

1. 各遺伝子の保有状況および菌体外酵素の産生性（Table 1）

E. faecalis 352株のうち*asa1*, *esp*, *cylA*, *gelE/sprE*を保有する株数（%）は，それぞれ291株（82.7%），254株（72.2%），164株（46.6%），306株（86.9%）であった．*cylA*保有株のうちHln産生株は63株（38.4%），*gelE-sprE*保有株のうちGel産生株は167株（54.6%）であった．

2. *asa1*および*esp*の保有状況と*cylA*, *gelE/sprE*保有との関連性

asa1, *esp*の両遺伝子，*asa1*のみ，*esp*のみ，両遺伝子のいずれも保有しない株は，それぞれ230株，61株，24株，37株であった．*asa1*もしくは*esp*を保有する株は315株であった．*cylA*保有株は，*asa1*, *esp*の両遺伝子を保有する群で147株（63.9%）と高くなっており，*asa1*, *esp*のいずれも保有しない群においては*cylA*保有株を認めなかった．一方*gelE/sprE*の保有の有無と*asa1*, *esp*両遺伝子の保有状況との間には関連性は認められなかった．Hln産生63株およびGel産生167株のうち，*asa1*および*esp*両遺伝子保有株はそれぞれ59株（93.7%），94株（56.3%）であった．

3. バイオフィルム形成能と*asa1*および*esp*の保有状況との関連性（Table 1, Fig. 1）

OD_{570}値により3群に分類すると，$OD_{570} \geqq 0.5$：64株(18.2%)，$0.5 > OD_{570} \geqq 0.2$：156株(44.3%)，

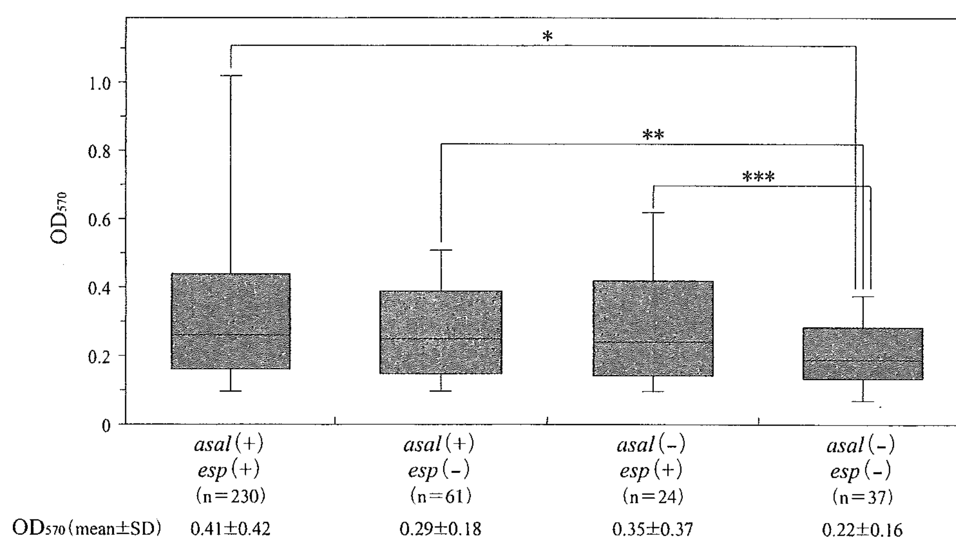

Fig. 1 Biofilm-forming capacities of *E. faecalis* isolates in four groups based on the presence/absence of *asa1* and *esp* genes

OD_{570} values of the isolates in the four groups are shown by the box and whiskers plot, which represents a five-number summary (upper extreme, upper quartile, median, lower quartile, and lower extreme). The mean OD_{570} values (mean ± SD) of the four groups are also shown. *P=0.038 **P=0.0449 ***P=0.1208 (Mann-Whitney's *U* test)

0.2 ＞ OD_{570} ＞ 0：132 株(37.5%)であった．OD_{570} 値の平均値は 0.36 ± 0.37(mean ± SD)であった．*asa1*, *esp*, *cylA* 保有株は非保有株に比し，それぞれの平均値が有意に高かった(P = 0.0176, P = 0.0276, P = 0.0116)．また，Hln 産生株は非産生株に比し，平均値が有意に高かった(P = 0.0384)．*asa1*, *esp* の両遺伝子を保有する株の平均値は 0.41 ± 0.42 で，いずれも保有しない株の 0.22 ± 0.16 に比べて有意に高かった(P = 0.038)．

4. 臨床的背景と *asa1* および *esp* の保有状況との関連性

352 症例中，カテーテル留置例は 107 例(30.4%)，複数菌分離症例は 202 例(57.4%)，発熱症例は 60 例(17.0%)であった(Table 1)．バイオフィルム形成能と臨床的背景との間に関連性は認められなかった．*asa1*, *esp* の両遺伝子保有株は，カテーテル留置単独菌分離症例，カテーテル留置複数菌分離症例，カテーテル非留置単独菌分離症例，カテーテル非留置複数菌分離症例から，それぞれ 20 株，46 株，79 株，85 株分離されていた．カテーテル留置かつ複数菌分離症例において，両遺伝子のいずれかを保有する株の分離率は 97.6%(80/82 株)であり，カテーテル非留置かつ単独菌分離症例における分離率 83.1%(103/124 株)と比較して有意差を認めた(P = 0.0020)．

考　察

腸球菌は院内感染の主要な原因菌である[3, 4]．尿路において *E. faecalis* が重篤な感染症を引き起こすことはまれであるが，尿路における *E. faecalis* の分離頻度は増加傾向にあり，そのバイオフィルム形成能および病原性を解明することは重要であると考えられる[13, 14]．本研究においては，尿路感染症由来 *E. faecalis* の Hln および Gel 産生率は緒家らの報告と差を認めなかったが，*asa1* および *esp* 保有率は明らかに高かった[15～17]．Agg は凝集素としての機能を持つとともに菌と菌との凝集に関与することにより，遺伝情報を交換する役割がある[10]．遺伝子伝達は微生物の進化および遺伝子の多様性において重要であり，自然環境において細菌はバイオフィルムを形成し，その内部で遺伝子を伝達していると考えられている[18]．我々が 2003 年に本誌に報告した成績においても，高頻度伝達(フェロモン反応性)プラスミド上に存在すると考えられる *asa1*, *cylA* の同時伝達を認めている[11]．これらの伝達性遺伝子は，*asa1*, *esp* 両遺伝子保有株に最も多く集積していた．

本研究においては，*asa1*, *esp* 両遺伝子保有株のバイオフィルム形成能は，いずれも保有しない株に比べて有意に高かった(P = 0.038)．Esp および Gel のバイオフィルム形成への関与については，必ずしも一定の見解が得られていない[19]．本研究では *esp* 保有株のバイオフィルム形成能は *esp* 非保有株に比べて有意に高くなっていた．一方で *gelE/sprE* 保有および Gel 産生の有無とバイオフィルム形成能との間には関連性を認めなかった．現在までにバイオフィルム形成能と Cyl との関連性についての報告はないが，我々の研究では *cylA* 保有株および Hln 産生株は，*cylA* 非保有株および Hln 非産生株に比べてバイオフィルム形成能が高くなっていた．*E. faecalis* の *fsr* が関与するクォーラムセンシングにより，Gel および serine protease の産生が制御されることが明らかにされている[1, 7～9]．最近，バイオフィルム形成は Gel の産生により制御されているとの報告がなされた[20]．本研究では Gel 非産生株においてもバイオフィルム形成能を認めており，この見解を裏付ける結果とはならなかった．

個々の病態における病原性を規定する因子は単一ではなく，宿主側の因子と細菌側の病原性因子との相対的な重要性は明らかにはされていない．今回の検討においても，臨床的背景とバイオフィルム形成能との間に明らかな相関性を認めなかった．しかしながら，本研究における実験成績に基づいて，病原性遺伝子を集積した *E. faecalis* はバイオフィルム形成能が高く尿路に定着するものと考えられた．

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Biofilm Formation by *Enterococcus faecalis* Isolated from Patients with Complicated Urinary Tract Infection

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The potential relationships between biofilm formation and pathogenicity of *Enterococcus faecalis* in urinary tract infection (UTI) were investigated. Over a 12-year period from 1991 through 2002, a total of 352 *E. faecalis* isolates were collected from patients with complicated UTI (one isolate per patient) at the urology ward of Okayama University Hospital. We analyzed the prevalence of genes that may contribute to the pathogenicity of *E. faecalis* in the urinary tract. The presence of *asa1*, *esp*, *cylA* and *gelE/sprE*, which encode aggregation substance, enterococcal surface protein, cytolysin and gelatinase/serine protease, respectively, was confirmed by polymerase chain reaction (PCR) assay. Hemolysin and gelatinase production was detected using rabbit blood agar plates and agar plates containing gelatin, respectively. Biofilm formation was investigated using 96-well microtiter plates containing tryptic soy broth supplemented with 0.25% glucose and was quantified by staining the biofilms with crystal violet, and measuring the absorbance at 570 nm (A_{570}). Of 352 *E. faecalis* isolates, 315 (89.5%) possessed *asa1* and/or *esp* genes. Of the 63 hemolysin- and 167 gelatinase-producing isolates, 59 (93.7%) and 94 (56.3%) isolates, respectively, possessed both *asa1* and *esp* genes. *E. faecalis* isolates with both *asa1* and *esp* genes formed biofilms at significantly higher rates than those with neither gene (P=0.038). The genes encoding *asa1* and *cylA* on a pheromone-responsive plasmid were highly transferable and appeared to have accumulated in these isolates. In a review of the medical records, the *E. faecalis* isolates possessing *asa1* and/or *esp* genes were found from both catheter-related or -unrelated UTI. Our study indicates that the *E. faecalis* isolates that have accumulated virulence genes are apt to form persistent biofilms in the urinary tract.

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Transcriptional regulation of *β-defensin-2* by lipopolysaccharide in cultured human cervical carcinoma (HeLa) cells

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Abstract

Human β-defensin-2 (hBD-2) is an antimicrobial peptide with a broad spectrum of antimicrobial activity against bacteria, yeast and fungi. Here, we analyzed the transcriptional regulation of hBD-2 in cultured human cervical carcinoma (HeLa) cells with or without lipopolysaccharide (LPS). DNA from position −329 to −39 in the hBD-2 promoter region contained the consensus binding sites for transcription factors, one site for nuclear factor for IL-6 expression (NF-IL6) and two sites for nuclear factor-κB (NF-κB). Reporter gene assays for promoter activity revealed that the region had the highest level of responsiveness to LPS. Furthermore, mutations in both of the NF-κB binding sites caused a significant reduction of the responsiveness to LPS, whereas mutation in the NF-IL6 binding site resulted in an elevation of the basal promoter activity. Electrophoretic mobility shift assays demonstrated that LPS induced the binding of HeLa nuclear factors to 60-bp probe containing the two NF-κB binding sites, suggesting that the sites were essential for the binding. Our results suggest that the two NF-κB binding sites contribute to LPS-mediated hBD-2 transcription while the NF-IL6 binding site represses LPS-independent hBD-2 transcription in the HeLa cells.
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Keywords: *β-defensin-2*; Transcriptional regulation; Lipopolysaccharide; HeLa cells

1. Introduction

Epithelia not only serve as a physical barrier against infections, but also secrete substances that inhibit or neutralize invading microbes or their toxins. Antimicrobial peptides have recently been discovered in tissue, and one important subgroup of the peptides is the defensins, which are classified as α-, θ-, and β-defensins [1]. The human α-defensins are expressed in a few kinds of cells such as neutrophils, macrophages, and Paneth cells of the intestine [2], and θ-defensins have only been identified in leukocytes of rhesus macaques to date [3]. By contrast, 28 humans β-defensin (hBD) genes have been discovered by genomics-based approaches [1]; and in view of protein or mRNA level, hBD-1, -2, and -3 have been mainly detected in many tissues including secretory glands and epithelial cells [4], oral mucosa and salivary glands [5,6], and skin [7].

These hBD-2s vary in their distribution, induction, and antimicrobial properties. Among them, human β-defensin-2 (hBD-2) is induced in keratinocytes stimulated with Gram-negative or Gram-positive bacteria, *Candida albicans*, and is upregulated in inflamed epithelial tissues whereas it is poorly expressed in normal epidermal keratinocytes [8]. HBD-2 demonstrates in vitro

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antimicrobial activities against yeast and both Gram-negative and Gram-positive bacteria [9–11]. Therefore, the expression of hBD-2 might be tightly regulated in epithelial cells in response to microbial invasion.

HBD-2 mRNA expression was upregulated strongly in airway epithelial cells by lipopolysaccharide (LPS) derived from *Escherichia coli* [12]. In contrast to airway epithelial cells, hBD-2 mRNA was expressed weakly in human gingival epithelial cells exposed to LPS from *E. coli*, *Fusobacterium nucleatum*, and *Porphynomonas gingivalis* [8]. Weak expression was detected in human cervical carcinoma (HeLa) cells stimulated with *E. coli* LPS (our unpublished data). The findings suggest that the regulation of hBD-2 gene activation may differ in the types of epithelial cells. Recently, the hBD-2 promoter has been cloned [13], and found to contain several consensus transcription factor binding sites [14]. The hBD-2 promoter region between −324 and −180, which contains motifs that resemble nuclear factor-κB (NF-κB) and nuclear factor for IL-6 expression (NF-IL6) binding sites, has both basal and LPS-induced reporter gene activities in bovine tracheal epithelial cells (TECs) [12]. Moreover, the NF-IL6 binding site is necessary for the response to LPS in TECs [12]. However, the regulation of the hBD-2 gene in stratified epithelial cells may be different from that in simple epithelial cells such as TECs, because stratified epithelia of skin, gingiva, and cervix are continuously exposed with commensal bacteria, fungi, or viruses whereas simple epithelia of trachea and bronchus are sometimes invaded by pathogenic microbes.

In this study, we investigated the promoter region of hBD-2 in HeLa cells responsive to *E. coli* LPS. Furthermore, the specific binding of nuclear factors to hBD-2 promoter sequences in HeLa cells was examined by electrophoretic mobility shift assay (EMSA).

2. Materials and methods

2.1. Cloning of hBD-2 promoter

Human genomic DNA was amplified by nested polymerase chain reaction (PCR) using Genome Walker Kits (Clontech Laboratory Inc., Palo Alto, CA, USA). The gene-specific primers used were designed based on 5′ coding region of hBD-2 cDNA (GenBank Accession No. AF040153); first primer, 5′-TCAGGAATATGAAGAGGAACGAGAAGA-3′, and second primer, 5′-AAGAGGAACGAGAAGAGGAGATACAAG-3′. The PCR-based DNA fragment was cloned into the pCR-Blunt Vector (Invitrogen, Carlsbad, CA, USA), and the plasmid DNA was prepared using Plasmid Miniprep Kits (Qiagen, Hilden, Germany). A clone containing the longest insert was sequenced by the dideoxy sequencing procedure [15] using the Automatic 377 sequencer (Perkin–Elmer, Foster City, CA, USA). The sequence was analyzed for the presence of consensus transcription factor binding sites using the TFSEARCH program (http://www.cbrc.jp/research/db/TFSEARCHJ.html) and SIGNAL SCAN search program (http://www-bimas.cit.nih.gov/molbio/signal/). The genomic DNA obtained was subcloned into a reporter plasmid ligated with *secreted alkaline phosphatase* (*SEAP*) (pSEAP2-Basic; Clontech), and it was used for experiments.

2.2. Construction of deletant of hBD-2 promoter-SEAP reporter

The deletants used were named according to the number of remaining hBD-2 promoter base pairs from 5′ to the transcription start position. To obtain a series of deletants, the pSEAP2-Basic vectors containing the genomic DNA were digested using exonuclease III/Mung Bean Deletion Kit (Promega, Madison, WI, USA), and cloned again. The sequences of all constructs were confirmed by sequencing.

2.3. Cell culture, transfection, and reporter assay

HeLa cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 μg ml^{-1} streptomycin, 60 μg ml^{-1} kanamycin, and 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA). The cells at 5×10^6 per well in 35-mm diameter plates (Corning, Corning, NY, USA) were co-transfected with 0.67 μg of hBD-2 promoter-*SEAP* reporter and 0.33 μg of pSV-β-galactosidase control vector (Promega) using Lipofect AMINE Plus (Invitrogen) according to the manufacturer's instructions. The plasmid DNA of each deletant was prepared using the EndFree Plasmid Maxi Kit (Qiagen). After transfection for 4 h, the cells were stimulated with 100 ng ml^{-1} *E. coli* LPS (055:B5; Sigma–Aldrich, St. Louis, MO, USA) for 24 h. The culture medium was collected and treated using Great EscAPe SEAP Chemiluminescence Detection Kit (Clontech), and then the SEAP activity was assayed quantitatively with a fluorometer (Millipore, Billerica, MA, USA). For monitoring transfection efficiency, the β-galactosidase (β-gal) activity was assayed using a luminometer (Wallac, Gaithersburg, MD, USA). The experiment was carried out three times for each construct, and the SEAP activity was normalized to the β-gal activity. The result of the reporter assay was indicated as a fold increase in the SEAP activity relative to that in the plasmid containing the minimum length of promoter region. A deletant exhibiting the strongest promoter activity was used for the mutation analysis and EMSA.

2.4. Site-directed mutagenesis and reporter assay

Point mutations were generated into the deletant for the hBD-2 promoter using QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Briefly, two synthetic oligonucleotide primers, each complementary to opposite strands of the vector, were designed for the desired mutation. Incorporation of the primers into the deletant was performed by PCR using *PfuTurbo* DNA polymerase (Stratagene) according to the following conditions: denaturing at 95 °C for 30 s, and 18 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C for 1 min, and elongation at 68 °C for 8 min. After the temperature cycling, the product was treated with *Dpn*I, which digested the parental DNA template and selected for the synthesized DNA containing mutations., The plasmid DNA of each mutant was prepared using the EndFree Plasmid Maxi Kit (Qiagen). The reporter assay using the mutants was performed by the same method as mentioned above. The assay was performed three times for each construct, and results of the assays were indicated after the normalization of SEAP activity to β-gal activity.

2.5. EMSA

Double-stranded oligonucleotides were designed to cover the region of the hBD-2 promoter exhibiting the strongest SEAP activity. They were synthesized (Bex, Tokyo, Japan), and end-labeled with [γ-^{32}P]dATP (Amersham Bioscience, Tokyo, Japan) using MEGA-LABEL Kit (Takara, Otsu, Japan). The labeled DNA was separated from unincorporated [γ-^{32}P]dATP using QIAquick Nucleotide Removal Kit (Qiagen), eluted in DNase-free water, and kept at 4 °C until used for EMSA.

Nuclear extracts were prepared from HeLa cells as described previously [16] with minor modifications. Briefly, 6×10^6 cells were stimulated with 100 ng ml^{-1} *E. coli* LPS for different periods (1, 2, 4, and 6 h). The cells were washed twice with PBS at 4 °C, and recovered using a cell scraper (Becton Dickinson, San Jose, CA, USA). Nuclear protein was extracted from the cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (PIERCE, Rockford, IL, USA) according to the manufacturer's instructions. The amount of protein was measured using Protein Assay Kits (Bio-Rad Laboratories, Hercules, CA, USA) according to the method described previously [17]. Four micrograms of the extract was incubated at room temperature for 30 min in a reaction buffer containing 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM $MgCl_2$, 4% glycerol, 0.05 mg ml^{-1} poly(dI–dC), and 10 fmol labeled probe in a total volume of 11 μl. The DNA–protein complexes were mixed with 1 μl of 10 × loading buffer (Gel Shift Assay Systems, Promega), and then they were analyzed by electrophoresis on a 5% polyacrylamide gel using 0.5× Tris–borate–EDTA running buffer (45 mM Tris–HCl, 45 mM sodium borate, and 1 mM EDTA). The gel was dried under vacuum and visualized by autoradiography. For competition experiments, the nuclear extracts were preincubated on ice for 30 min with a 500-fold molar excess of unlabeled consensus oligonucleotides for NF-κB, NF-IL6, and AP-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

3. Results

3.1. Putative transcription factor binding sites in hBD-2 promoter

The 1.4-kbp DNA fragment isolated contained the 5′-upstream sequence of the hBD-2 cDNA. The nucleotide sequence matched completely with that of the hBD-2 genome (GenBank Accession No. AF040153). Computational analysis of the hBD-2 promoter sequence revealed the typical TATA-like box and potential DNA-binding sites specific to mammalian gene regulatory proteins (Fig. 1).

3.2. Deletion mutagenesis of hBD-2 promoter and SEAP assays

To determine which element of the hBD-2 promoter is important for LPS-induced transcription, a series of 5′ truncated fragments of the 1.4-kbp hBD-2 promoter linked to pSEAP2-Basic plasmids were used for promoter assay. As shown in Fig. 2, the maximum basal promoter value was detected in the 1.4-kbp promoter (pro 1319), and the deletion between −1371 and −329 (pro 329) decreased the value by 80%. The maximum LPS-induced value was detected in the 1.4-kbp promoter (pro 1319), and deletion from position −1019 to −329 (pro 1019, pro 704, and pro 329) resulted in a moderate decrease in the value; however, further deletion between −1371 and −39 (pro 39) decreased the value by 90%. The construct pro 329, in which similar consensus binding sites for AP-1, NF-IL6, and NF-κB were included (Fig. 1), exhibited the highest ratio of LPS-induced value to basal promoter value. Therefore, we investigated further the role of each binding site in the regulation of hBD-2 transcription by LPS.

The promoter activity of the hBD-2 mutant in the pSEAP2-Basic vector was assayed in the HeLa cells followed by incubation for 24 h with or without LPS. Mutation of the two NF-κB binding sites in tandem markedly reduced the LPS-induced value, whereas mutation at the NF-IL6 site resulted in an elevation of the basal promoter value (Fig. 3).

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                                              a gaaatttcct tttataaaaa tgtcattaag gatcttggct gcacaatatc gttaccagct tcctttaaat -1301
                                                                                                                      STAT
-1300 ccacttctgg cctgccagga atcaggttct tcagaacctg acattttaaa tgaagaggtc aggcagttca tgaggaaagc ctcattgtcc ccatgtctct -1201
          AP-2  STAT                                                          NF-IL6
-1200 gtcactgctg caccccctgag acatcacaga catggacact ggggcctgct tgtttctcaa actgccctta gatcgaaaga gggaggaacc aggatgaatg -1101

-1100 ccactcattt tcccaagaaa ggccctctcc tgagtgcccg ggatggggct ctgtccattg cctggggccg ccaattgcta ctctgggtta cggaggaagg -1001
                                                    AP-2              AP-2
-1000 acagggtcct gagagacacc agagacctca cacagccctg aaaacatggg gctccttcat aagtgtttcc catcaccaac agggagacca cgtggaggcc -901
                                                                            STAT
-900  ttgcagcccc actcggtgct tctccaccaa atcccaaggg cagtgacgct gacgtctgtg gaaagcagag aaagccctgg ctcccaaagc cctgaagtcc -801
                                     AP-2                  NF-IL6
-800  ctgtggagct gacattccct gagtgacggt gtgaatggaa ggaactcaag tgcgggtggt aggccacctc ctggcccagg cctgggtgaa ctctgagggg -701

                                                                                 AP-2   AP-2
-700  acacatgtag tcacaatccc atcctcccat tctccttctc agaggaagga agtgggcatc catctgcctc atctctctcc cgtggggaag atggggagtt -601
                                                                                        AP-2       NF-κB
-600  tcaggggaac tttcacataa atttcaccag ctcagatctc ctgtgaggat ggggcccacc atgctcccgg tgctgccaga ggccctgagc cctcccaggg -501
                 STAT       NF-IL6                                                                   AP-2
-500  gtccctgggt ttgagccagc cctgtatcat ccccaggagc tgaatgtcag agcaatggat agaattagat ggaaagagct ctcaatttga cctgagactg -401
                 AP-1   AP-2
-400  tccccagata ctcaggaaaa acaggacgtc gcacagagtg ggcagcaggt gagtggcagg ttataggtcc tgagtttgag tttgttctca cgtgagacag -301

-300  acccagcccc tcactccatt cacacactgg gttttaaatg gtgcaagata ggagcaattt tctggtccca agagcaggag gaagggattt tctggggttt -201
        AP-2                                 NF-IL6                                                 NF-κB      NF-κB
-200  cctgagtcca gatttgcata agatctcctg agtgtgcatt gttctttgag gaccattctc tgactcacca ggtaagtggc tgaattctaa cctctgtaat -101
                                                                          AP-1
-100  gagcattgca cccaatacca gttctgaact ctacctggtg accagggacc aggacctta taaggtggaa ggcttgatgt cctccccaga ctcagctcct -1
                                                                      TATA like box
+1    ggtgaagctc ccagccatca gccatgaggg tcttgtatct cctcttctcg ttcctct
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Fig. 1. Nucleotide sequence of 5′-flanking region of hBD-2 gene. Putative transcription factor binding sites (threshold score, >0.82) are underlined, and the TATA-like box is indicated.

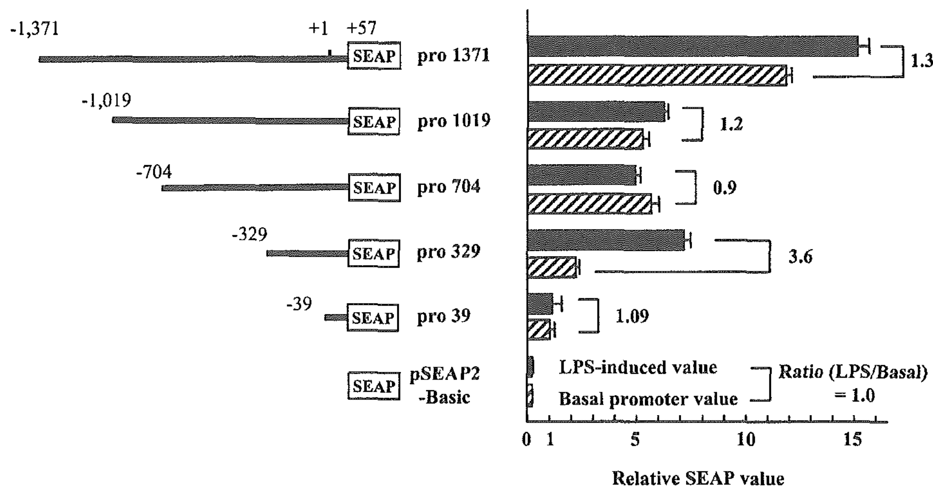

Fig. 2. Promoter activity of hBD-2. (Left) Schematic diagram of the five hBD-2 reporter constructs containing promoter fragments of different lengths cloned into the pSEAP2-Basic vector. The numbers in the names of the constructs indicate their respective lengths in nucleotides. (Right) The relative SEAP value is indicated as a fold increase in the SEAP activity for each construct relative to that without LPS for the pro 39 construct. LPS-induced value, the cells were incubated with LPS; basal promoter value, the cells were incubated without LPS. Error bars indicate SD of three independent assays.

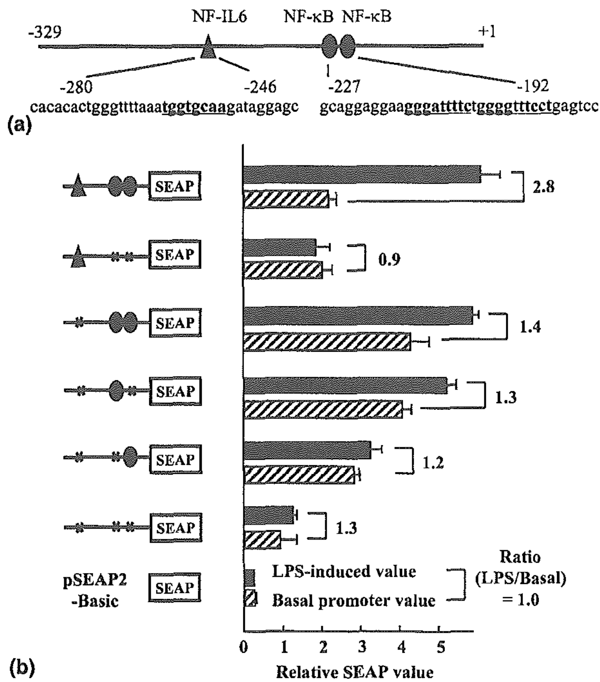

Fig. 3. Effect of mutations in NF-κB and NF-IL6 binding sites on hBD-2 promoter activity. (a) Schematic representation of the hBD-2 promoter including NF-κB and NF-IL6 binding sites. The transcription start site is numbered +1. Consensus sequences for NF-IL6 and NF-κB are indicated in bold and with underlines. (b) Promoter activity of hBD-2 mutants. (Left) Schematic diagram of the mutant. The wild-type or mutant construct was transfected into HeLa cells (5×10^6), and the cells were incubated for 24 h with or without LPS. The point mutations generated are as follows: 5′-gcaggaggaa**ggCattttctggCgtttcct**gagtcc-3′; 5′-cacacactgggttttaaa**GggtTcaG**gataggagc-3′; 5′-cacacactgggttttaaa**GggtTcaG**gataggagc-3′ and 5′-gcaggaggaa**ggCattttctggCgtttcct**gagtcc-3′; 5′-cacacactgggttttaaa**GggtTcaG**gataggagc-3′ and 5′-gcaggaggaa**ggCattttc**tggggtttcctgagtcc-3′; 5′-cacacactgggttttaaa**GggtTcaG**gataggagc-3′ and 5′-gcaggaggaagggattttct**ggCgtttcct**gagtcc-3′. Capital letters show nucleotides replaced for mutation. Right: The relative SEAP value is indicated as a fold increase in the SEAP activity for each construct relative to that without LPS for the pro 329 construct containing point mutations for NF-IL6 and NF-κB (SEAP). The pro 329 construct is shown in Fig. 2. LPS-induced value, the transfected cells were incubated with LPS; basal promoter value, the transfected cells were incubated without LPS. Error bars indicate SD of three independent assays.

3.3. Oligonucleotides and their specific interaction with nuclear extract

Six kinds of 60-bp oligonucleotides covered the similar consensus binding sites for AP-1, NF-IL6, and NF-κB in the hBD-2 promoter region (Fig. 4(a)) which exhibited the highest ratio of LPS-induced value to basal promoter value (Fig. 2). They overlapped each other for 10 base pairs.

To examine the interaction of *cis*-acting elements of the hBD-2 promoter with HeLa nuclear factors, the ^{32}P-labeled oligonucleotides were used as probes for EMSA. The #2 oligonucleotides, of which sequence encompassed the two NF-κB consensus binding sequences, bound proteins in the nuclear extract (Fig. 4(b)). The DNA–protein complex was increased by stimulation with LPS, and was the same size as the complex of the NF-κB probe and nuclear extract (Fig. 5(a)). No apparent complex was detected for other probes (#1, 3, 4, 5, and 6) whereas oligonucleotides for NF-κB or NF-IL6 bound with the nuclear extract (Fig. 4(b)). We further analyzed whether the consensus sequence for NF-κB was critical for formation of the DNA–protein complex. In the competitive EMSA, the complex of radio-labeled #2 probe and extract was competed by a 500-fold molar excess of unlabeled

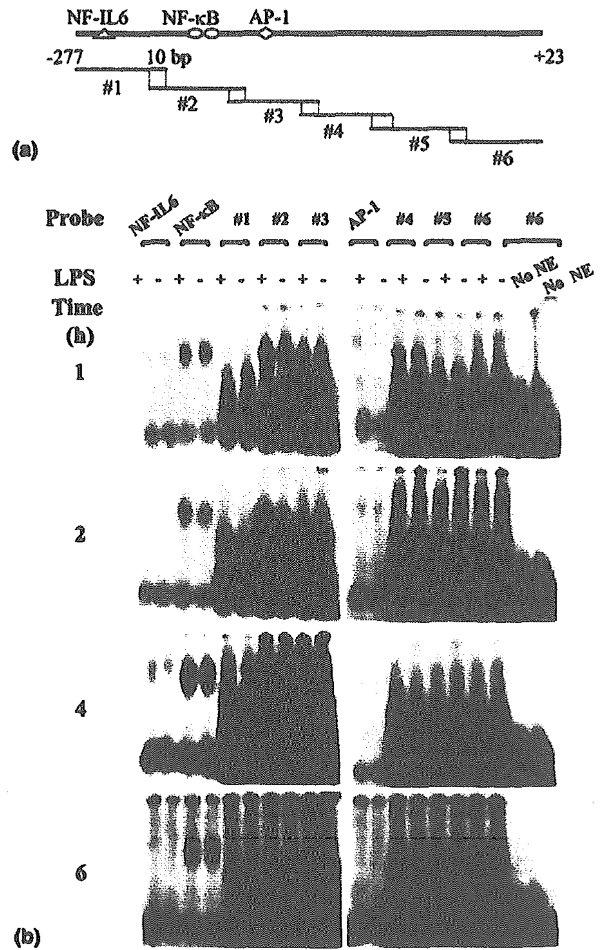

Fig. 4. Synthetic oligonucleotides and their interaction with nuclear factors. (a) Schematic representation of synthetic oligonucleotides used for EMSA. The sequence positions corresponding to the wild-type oligonucleotides span −277 to −218 (#1), −227 to −168 (#2), −177 to −118 (#3), −127 to −68 (#4), −67 to −18 (#5), and −27 to +23 (#6) in the hBD-2 gene. (b) EMSA using a series of oligonucleotides as shown in (a). The ^{32}P-labeled double-stranded oligonucleotides were incubated with nuclear extract prepared from HeLa cells incubated for 1, 2, 4, and 6 h with or without LPS. Three independent assays were performed, and a typical result is shown. No NE, sample without nuclear extract.

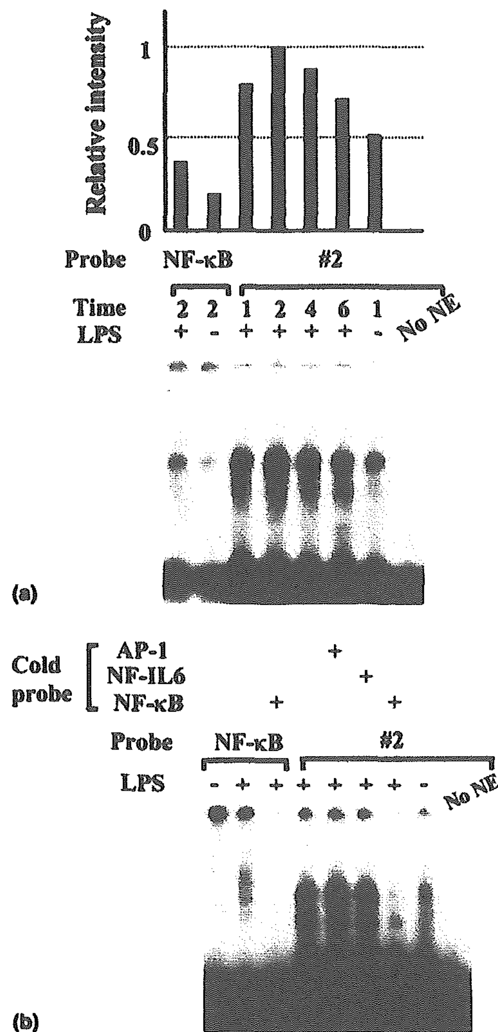

Fig. 5. Interaction between #2 oligonucleotides and nuclear factors. (a) EMSA using #2 and NF-κB oligonucleotides. No NE, sample without nuclear extract. (b) Competitive EMSA. A 500-fold molar excess of cold probe was added to each sample prior to formation of the DNA–protein complex. No NE, sample without nuclear extract.

NF-κB probe whereas the DNA–protein complex was not affected in the case of the other unlabeled probes (Fig. 5(b)).

4. Discussion

In this study, we have performed a functional analysis of the hBD-2 promoter to gain insight into the mechanism for the regulation of hBD-2 transcription in a stratified epithelial cell line, HeLa. Using a series of sequentially deleted hBD-2 promoters (−1371 to +57) ligated into pSEAP2-Basic plasmid for expression in HeLa cells, the minimal promoter region in response to LPS was found to be located in the sequence from position −392 to −39 relative to the transcription start position (Fig. 2). The region contained similar consensus binding sites for transcription factors, one site for NF-IL6 and two sites for NF-κB, and the responsiveness to LPS was markedly reduced by point mutations at the NF-κB binding sites (Fig. 3). These findings suggest that the NF-κB binding sites contribute to LPS-mediated hBD-2 transcription in HeLa cells. Similar regulation by the NF-κB binding sites has been shown in the murine macrophage cell line RAW264.7 [18]. Interestingly, mutation in the NF-IL6 binding site in the region between −392 and −39 resulted in an elevation of the basal promoter activity, and the mutation retained the moderate LPS-induced activity (Fig. 3(b)). The result suggests that the NF-IL6 binding site represses the hBD-2 transcription in the HeLa cells without LPS, and abolishment of the binding site released the repression of the promoter activity.

EMSA indicated that LPS induced binding of the 60-bp oligonucleotides encompassing the two NF-κB consensus binding sequences with nuclear factors prepared from HeLa cells (Figs. 4(b) and 5(a)). Competitive EMSA using unlabeled oligonucleotides showed that the consensus sequence for NF-κB was essential for the binding to the nuclear factors (Fig. 5(b)). Moreover, the promoter region containing the two NF-κB binding sites had the highest responsiveness to LPS in reporter gene activity (Fig. 2). Together, these results suggest that the two NF-κB binding sites contribute to LPS-mediated hBD-2 transcription in HeLa cells. In contrast to NF-κB, no apparent DNA–protein complex was detected for the 60-bp probes encompassing the consensus sequence for NF-IL6 (Fig. 4(b)). This may be due to the different DNA binding activity between the two probes, because the #2 probe contained two sites for NF-κB, whereas the #1 probe did one site for NF-IL6 (Fig. 4(a)).

Interestingly, the NF-IL-6 binding site is likely to repress the hBD-2 gene activation, in the absence of LPS stimulation, in the HeLa cells, because mutation at the site resulted in an elevation of the basal promoter value (Fig. 3(b)). Diamond et al. [12] has reported that the NF-IL-6 binding site positively regulates both basal and LPS-induced hBD-2 gene activation in the TECs. The mechanisms responsible for the different promoter activity of the NF-IL6 binding site between the HeLa cells and TECs are currently unknown.

After our submission, Vora et al. [19] reported the regulation of hBD-2 expression by Toll-like receptor (TLR) signaling in intestinal epithelial cells (Caco-2, T84, and SW480). Activation of the hBD-2 promoter by LPS stimulation differs in these cell types, and its level depended on expression of both TLR4 and its accessory molecule MD-2. The hBD-2 promoter region located between −938 and −229, containing the consensus sequence for NF-κB, has moderate LPS responsiveness in Caco-2 and T84 followed by cotransfection with

both TLR4 and MD-2. Furthermore, the promoter with a mutation in the NF-κB site exhibits significantly reduced LPS-dependent hBD-2 expression in these transfected cells. In this study, the similar promoter region had the highest level of responsiveness to LPS in HeLa cells (Fig. 2), and the responsiveness to LPS was markedly reduced by point mutations at the two NF-κB binding sites (Fig. 3). Our results are consistent with the findings in the intestinal epithelial cells cotransfected with TLR4 and MD-2. Moreover, TLR4/MD-2 complex has shown to be required for higher responsiveness to LPS in the HeLa cells [20,21]. Taken together, TLR4/MD-2-mediated activation of signaling cascade is likely to be required for expression of hBD-2 in not only these intestinal epithelial cells but also the HeLa cells. In addition, the hBD2 promoter activity, except for the construct pro 329, were poorly upregulated by LPS (Fig. 2). The poor upregulation may be due to the low expression level of TLR4–MD-2 complex in the HeLa cells.

Deletion of the hBD-2 promoter from position −1019 to −329 demonstrated a moderate LPS inducibility but less than full-length promoter (Fig. 2). The similar reduction in LPS inducibility of the hBD-2 promoter has been reported in both Caco-2 and T84 cells [19]. Tandem STAT-like sequences are located in hBD-2 promoter from −1312 to −1281 (Fig. 1), and they may play a role in the regulation of the promoter activity. Because, it has been reported that STAT proteins are key regulatory proteins that bind to two tandem γ-interferon-activated site motifs within an IL-2 response element (positive regulatory region III [PRRIII]) in the human IL-2Rα promoter [22]. Moreover, the formation of a tetrameric Stat5 complex is essential for the IL-2-inducible activation of PRRIII [23]. The tandem STAT-like sequences would act as enhancers of the hBD-2 promoter; therefore, the missing of the sequences may cause the moderate LPS inducibility in the hBD-2 promoter.

In conclusion, the hBD-2 promoter region, containing the consensus binding sites for NF-IL6 and two NF-κBs, exhibited the highest level of responsiveness to LPS in terms of reporter gene activity. The two NF-κB binding sites contributed to the responsiveness to LPS, and both sites were important for formation of the DNA-nuclear factor complex. The mutation in the NF-IL6 binding site resulted in an elevation of the basal promoter activity of the region. These results suggest that the two NF-κB binding sites contribute to LPS-mediated hBD-2 transcription and the NF-IL6 binding site represses LPS-independent hBD-2 transcription in the HeLa cells.

Acknowledgements

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Case Report

Periodontal Treatment in Severe Aplastic Anemia

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Background: *Aplastic anemia (AA) is a rare hematologic disease characterized by hypo-cellular bone marrow. The clinical features include fatigue, increased bruising, and gingival bleeding caused by anemia, leukopenia, and thrombocytopenia. A patient with AA is at high risk for infection because of leukopenia. The risk of systemic infection is especially high in AA patients with severe local infections, including periodontitis. Accordingly, periodontal treatment should include antibiotic prophylaxis to reduce the risk of systemic infection. However, treatment of periodontitis in the AA patient is significantly complicated by the bleeding disorder. We present a case report of the successful periodontal treatment of an AA patient with spontaneous gingival bleeding.*

Methods: *The patient was closely monitored for platelet and neutrophil counts before every treatment. The patient's platelet count was always under 10,000/μl. Therefore, it was necessary to increase platelet counts to over 25,000/μl by transfusion, after which subgingival scaling with anesthesia was performed. When the neutrophil count was less than 2,000/μl, local minocycline chemotherapy was applied to the pockets. Periodontal infection was monitored by detection of bacterial DNA and measurement of serum immunoglobulin (Ig) G titer against periodontal bacteria.*

Results: *Following the physical and chemical treatment, the gingival appearance improved dramatically and the spontaneous gingival bleeding disappeared. Moreover, the IgG titer against periodontal bacteria decreased to normal range and specific periodontal pathogens were no longer detectable in the tested pockets.*

Conclusion: *We believe that the treatment strategy in the present report provides new sight into treatment planning for severely medically compromised patients.* J Periodontol 2005;76:1211-1216.

KEY WORDS

Anemia, aplastic/therapy; neutrophils; patient care planning; platelet count; platelet transfusion.

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Aplastic anemia (AA) is a serious hematologic disease characterized by hypocellular bone marrow that produces an insufficient number of hematopoietic stem cells, leading to deficient production of erythrocytes, granulocytes, and platelets.[1] Although the pathogenesis of bone marrow failure in AA is still unknown, the dominant hypotheses today are that AA results from an immune-mediated mechanism which leads to T-cell activation and release of inhibitory cytokines with subsequent destruction of hematopoietic progenitor cells.[2] The worldwide incidence of AA is estimated to be 2.2 cases per million annually with no gender predilection.[3] The age distribution reflects the two classes of AA, congenital and acquired.[4] The congenital cases are the minority and occur in childhood associated with Fanconi's anemia, *dyskeratosis congenita*, and Diamond-Blackfan syndrome. Acquired AA is usually found in adults, with the incidence dramatically increased after 65 years, and is considered idiopathic.[5] However, multiple etiologic agents have also been implicated, including numerous drugs and chemicals, cancer therapies, and viral infections.[6]

The clinical features of AA include fatigue, increased bruising, epistaxis, and gingival bleeding. Accordingly, gingival bleeding is often the chief complaint when these patients present to the dentist and most of the patients hesitate to brush their teeth because of gingival bleeding. For these reasons, oral hygiene is generally poor, with bacterial overgrowth and generalized inflammation.

Laboratory analysis of peripheral blood reveals anemia, leukopenia, and thrombocytopenia. Above all, the AA patient with leukopenia is in a higher risk group for systemic infection because of neutropenia. It has been reported that bacterial septicemias and fungal infections represent the most frequent cause of death in patients with AA.[1] Moreover, in the patient with thrombocytopenia, there is the possibility that dental treatments, such as subgingival scaling, may cause systemic infection subsequent to the inevitable bacteremia. There have been several epidemiologic reports suggesting an association between periodontitis and other systemic diseases, such as diabetes, cardiovascular, and other diseases.[7-9] Considering the bacterial load in periodontitis, this oral condition might

be a significant source of infection in the AA patient. Clearly, reduction of the bacterial load through the treatment of periodontitis would be a goal in the AA patient. However, uncontrolled bleeding is a significant treatment complication. In this paper, we report a case of AA with periodontitis accompanied by severe spontaneous bleeding from the gingiva. Successful periodontal treatment was accomplished using a combination of chemotherapy and subgingival scaling after platelet transfusion to prevent the bleeding complications.

CASE REPORT

The patient was a 43-year-old Japanese male. At the age of 11 years, he presented to the hospital for hemorrhage on epistaxis and was diagnosed with aplastic anemia. At about 20 years of age, he became aware of gingival swelling and bleeding after brushing, which prompted him to visit his family dentist. He was diagnosed with moderate periodontitis. However, no treatment was performed because of hemostasis difficulty and his problem was not resolved. At 43 years of age, he was hospitalized several times for fatigue and he was treated with whole blood transfusion and steroid therapy. His condition temporarily improved after treatment. A month before his referral to the university, he was hospitalized again and spontaneous gingival bleeding and severe gingival swelling were noted by the physician. He was then referred by his physician to Okayama University Dental Hospital for oral examination and treatment. At the time, he was receiving a platelet transfusion once a week and a clinical trial medicine for AA; immunosuppressive agents, including steroids, had been discontinued.

His medical history was not remarkable except hepatitis C infection acquired at 20 years of age. His serum glutamic-oxaloacetictransaminase (SGOT; normal range: 5 to 40 IU/l) and serum glutamic-pyruvic transaminase (SGPT; normal range: 3 to 35 IU/l) were 30 and 34 IU/l, respectively, at the first visit.

Clinical Examination

A clinical examination was performed to evaluate the following: 1) number of remaining teeth; 2) probing depth at six points around each tooth (mesial-buccal, buccal, distal-buccal, mesial-lingual, lingual, distal-lingual); and 3) alveolar bone loss as shown by radiography. The diagnosis of moderate chronic periodontitis was made based on the history, clinical findings, and radiographs.

Hematology

Blood samples were sent to the Central Clinical Laboratory of Okayama University Medical School Hospital for analysis.

Humoral Immune Responses to Periodontal Bacteria

Humoral immune responses to periodontal bacteria were assayed using an enzyme-linked immunosorbent assay (ELISA) as described previously.[10] The antigens used were sonicates of *Actinobacillus actinomycetemcomitans* ATCC29523, *A. actinomycetemcomitans* SUNY67, *A. actinomycetemcomitans* Y4, *Tannerella forsythensis* ATCC43037, *Capnocytophaga ochracea* S3, *Campylobacter rectus* ATCC33238, *Eikenella corrodens* FDC1073, *Fusobacterium nucleatum* ATCC25586, *Prevotella intermedia* ATCC25611, *P. intermedia* ATCC 33563, *Porphyromonas gingivalis* FDC381, *P. gingivalis* SU63, and *Treponema denticola* ATCC35405.

Microbiological Examination

Bacterial samples were collected from five periodontal pockets. The methods for the isolation and detection of three major periodontal bacteria, *A. actinomycetemcomitans, P. gingivalis*, and *P. intermedia*, have been described previously.[11]

RESULTS

Clinical Findings

Physical examination showed mild facial pallor caused by anemia and subcutaneous bleeding on the right antebrachium. Oral examination revealed severe redness, swelling of the gingiva with spontaneous bleeding (Fig. 1), and petechiae on the upper lip and the tongue (not shown). Periapical radiographic examination[12]

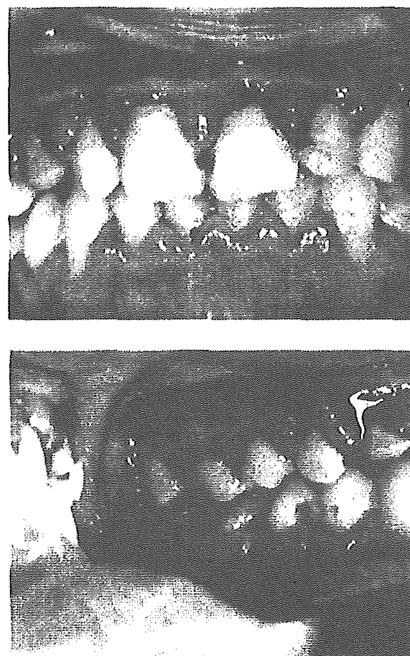


Figure 1.
Gingival appearance at the first visit. Note presence of gingival swelling and spontaneous bleeding.

revealed moderate alveolar bone loss and calculus (Fig. 2). Deep pockets with probing depths ranging from 4 to 9 mm were noted (Table 1).

Hematology

The results of the complete blood counts are shown in Table 2. Leukocytes (including neutrophils), erythrocytes, and platelet counts were decreased remarkably.

Figure 2.
Periapical radiographs at the first visit. Note moderate alveolar bone loss.

Table 1.

Clinical Findings

	First Visit	After Initial Preparation
N teeth	31	31
Mean probing depth		
<3 mm (%)*	0	93.6
4~6 mm (%)	54.8	3.2
>7 mm (%)	45.2	3.2
Bleeding on probing (%)	100.0	12.4
Mean bone score (%)	19.4	19.4

* Percent of sites with periodontal pocket.

Table 2.

Hematology at the First Visit

	Patient	Normal Range
Peripheral blood		
White blood cell ($\times 10^3/\mu l$)	2.9	3.0-9.4
Red blood cell ($\times 10^6/\mu l$)	2.18	3.70-4.90
Hemoglobin (g/dl)	7.2	11.5-14.5
Platelet ($\times 10^3/\mu l$)	8	150-400
Differential leukocyte count (%)		
Segmented cell	17.0	29-70
Stab cell	2.0	0-13
Lymphocyte	68.0	20-52
Monocyte	12.0	0-13
Eosinophilic leukocyte	0	0-11
Basophilic leukocyte	0	0-2

Microbiological Findings and Humoral Immune Responses to Bacteria

P. intermedia was detected in the periodontal pockets examined. Serum IgG titer against nine bacteria out of 13 was elevated greater than two standard deviations above the mean of healthy subjects. The antibody was detected to *A. actinomycetemcomitans* ATCC29523, SUNY67, and Y4; *T. forsythensis* ATCC43037; *C. ochracea* S3; *C. rectus* ATCC33238; *E. corrodens* FDC1073; *P. intermedia* ATCC25611; and *T. denticola* ATCC35405. Serum IgG titers to other periodontal bacteria were within normal ranges.

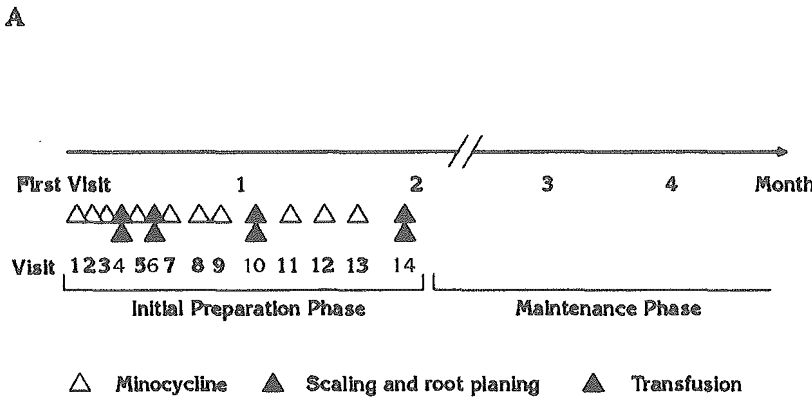
Diagnosis and Therapy

Based on the clinical and microbiological findings and humoral immune response to periodontal bacteria, we diagnosed this patient with moderate chronic periodontitis and severe AA. Since at the first visit the neutrophil count was low at 500/μl, a 2% minocycline slow release ointment,† that has been used for periodontal treatment for more than a decade in Japan and suggested to be effective for periodontal disease,[13] was applied to the pockets after 0.2% iodine irrigation for a few weeks (Fig. 3). The neutrophil count was highly variable over time (from 500 to 3,000/μl) and when the count was over 2,000/μl, subgingival scaling was performed after platelet transfusion. The platelet count was under 10,000/μl before every treatment and rose to 50,000/μl after transfusion, but never higher. The platelet count decreased again to less than 10,000/μl in a few days. Therefore, the neutrophil and platelet counts were examined before each subgingival scaling appointment. When the platelet count was under 25,000/μl or the neutrophil count was under 2,000/μl, minocycline was applied to the periodontal pockets. We also advised the patient to continue brushing carefully with a soft dental brush and an interdental brush and to use chlorhexidine mouthrinse every day, which helped reduce plaque and gingival inflammation. Following therapy, the gingival appearance improved dramatically (Fig. 4). The spontaneous bleeding from the gingiva disappeared even after brushing. Moreover, the IgG titer against all bacteria except *A. actinomycetemcomitans* SUNY67 decreased to normal range and *P. intermedia* could no longer be detected in the sulcus.

At the time of this manuscript preparation, 3 years had passed since our initial evaluation. Even though the platelet count is still very low (around 5,000/μl) and

† Periocline, Sunstar Corp, Osaka, Japan.

A



B

Period	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Platelet ($x10^3/\mu l$)	8.0	12.0	0.7	26.0	0.8	27.0	2.0	1.1	1.3	27.0	5.0	1.8	11.0	30.0
Neutrophil ($x10^3/\mu l$)	0.5	0.4	0.3	2.3	4.0	2.4	1.3	1.0	1.0	2.6	2.6	4.1	1.9	7.1

Figure 3.
*Schematic presentation of the outline of periodontal treatment **(A)** and platelet and neutrophil counts at each time point **(B)**. Each platelet and neutrophil count in panel B corresponds to the time period in panel A.*

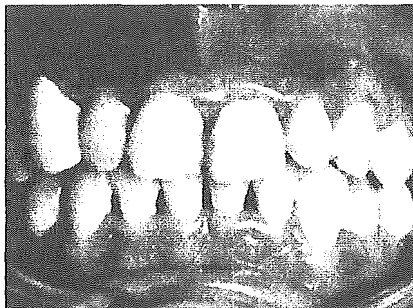


Figure 4.
Gingival appearance after the treatment; note disappearance of gingival swelling and spontaneous bleeding.

his AA has not improved, his gingiva is in good condition without any bleeding or acute swelling.

DISCUSSION

In this case report, we present periodontal treatment that was provided to a patient with severe AA. At the initial visit, the neutrophil and platelet counts were 500/μl and 8,000/μl, respectively. The gingiva showed severe swelling with spontaneous bleeding caused by infection (Fig. 1).

The objective of periodontal treatment for an AA patient is to prevent or manage the most common complications: infection and hemorrhage. Hemorrhage may be induced by infection locally or systemically due to bone marrow depression.[14] Accordingly, oral bacteria must be removed to reduce both infection and hemorrhage. However, depending on the severity of the AA, periodontal treatment can be life threatening. Therefore, before instituting any therapy, the dentist should perform a complete hematological assessment in conjunction with the physician to determine leukocyte and platelet counts. In this case, the platelet count was always less than 10,000/μl and, even after platelet transfusion, the count did not increase more than 50,000/μl. Platelet count decreased rapidly to less than 10,000/μl in a few days. Since the patient had been receiving transfusion for an extended period, the rapid reduction in platelet count was due to the so-called transfusion resistance. Accordingly, subgingival scaling had to be performed within 2 days of the transfusion. Moreover, the neutrophil count of this patient was highly variable (500 to 3,000/μl) between appointments. The low neutrophil counts raised concerns of the inability to eliminate the organisms of the bacteremia caused by dental manipulation.

The normal range of platelet counts is 150,000 to 450,000/μl. It is recommended that a platelet count of >50,000/μl is required for invasive procedures such as subgingival scaling or oral surgery.[15] In the case, however, we pursued subgingival scaling with anesthesia when the platelet count was more than 25,000/μl after transfusion, on the advice of his physician. Even after transfusion, a count of >50,000/μl was never obtained. If the platelet count was less than 25,000/μl at any appointment, no scaling was performed and minocycline ointment was administered subgingivally.

The leukopenic host has a weak immune response to bacteria. The leukopenia is often subsequent to steroid or other immunosuppressive agent administration, which might cause the subjects to be more susceptible to infection.[16] There is a real threat of opportunistic infection from the normal oral flora, periodontal pathogens, or mixed flora from odontogenic infection. It is, therefore, imperative that the practitioner who encounters an AA patient be cognizant not only of platelet counts, but also other leukocyte counts as

well, particularly neutrophils. The risk of death subsequent to infection is as great as the risk of hemorrhage. In this case, the neutrophil count was 500/μl at the initial visit, indicating a weak innate immune response. Topical application of minocycline was used to reduce the bacterial load as an initial step. Subsequently, subgingival scaling was pursued when both the neutrophil and platelet counts were more than 2,000/μl and 25,000/μl, respectively. It has been reported that if the neutrophil count is less than 2,000/μl, prophylactic antibiotics should be given before invasive dental procedures.[17] Dental professionals should recognize that patients receiving steroids or other immunosuppressive agents are more susceptible to infection.[16] Therefore, dental treatment for AA patients should be undertaken in a hospital setting, using universal precautions for infection control.

P. intermedia was detected from this patient's periodontal pockets by microbiological examination and the patient showed an elevated immune response against the bacterium. After treatment, *P. intermedia* was not detected and the IgG titer against the bacterium reduced remarkably. We speculate that periodontal disease in this patient was caused in part by *P. intermedia*. Interestingly, despite the chronic low neutrophil counts, bone loss was moderate and the major clinical manifestations were edema, erythema, and spontaneous gingival bleeding. There have been many reports of a relationship between leukopenic disease and periodontal disease.[18-20] The condition of the soft tissue coupled with relatively little bone loss suggests invasion of bacteria and direct destruction of soft tissues rather than the robust innate and acquired immune response usually seen in chronic periodontitis.

Because the neutrophil and platelet number was very low, irrigation with iodine and minocycline chemotherapy were used to control the flora in the periodontal pockets. We believe that iodine irrigation is quite effective because it penetrates into the pocket without any bleeding. We used local minocycline delivery to avoid the possible side effects of systemic antibiotics and an appearance of bacteria resistant to such antibiotics. It is also recommended that AA patients continue careful brushing and flossing, unless there is spontaneous oral bleeding.[21] We recommended the use of chlorhexidine mouthrinse every day at home, as it has been shown to be safe and effective in reducing plaque and gingival inflammation in patients with other blood dyscrasias.[22]

All dentists, regardless of specialty, play a role in the diagnosis of AA because oral manifestations are common and often the first noted. When gingival or mucosal bleeding without a local cause is detected, a blood dyscrasia should be part of the differential diagnosis. Previous case reports have described submucosal hemorrhage, gingival swelling, herpetic lesions, spontaneous gingival bleeding, pallor, and periodontal disease in patients with AA.[23-25]

In conclusion, AA patients often visit periodontists or other dentists with a complaint of gingival or mucosal bleeding. A careful workup is necessary in conjunction with a physician to diagnose and treat such patients. In this paper, we have described what we believe to be a rational and systematic approach to the management of the periodontal patient with AA.

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Isolation and Expression of *FIP-2* in Wounded Pulp of the Rat

ABSTRACT

Pulpal wound healing followed by cavity preparation may involve reactionary or reparative dentinogenesis in relation to the cavity position; however, little is known about the molecular responses. We aimed to isolate and analyze genes induced or suppressed in the wounded pulp to identify molecular processes involved in the pulp responses to injury. Twenty-three cDNAs were isolated by cDNA subtraction between healthy and wounded pulp of rats. By library screening, we identified rat 14.7K-interacting protein *(rFIP)-2A* and *B* genes homologous to human *FIP-2*, being involved in regulating membrane trafficking and cellular morphogenesis. RT-PCR analysis showed induction for only *rFIP-2B* in the wounded pulp. *In situ* hybridization analysis revealed that both *rFIP-2s* were expressed strongly in condensing mesenchymal cells of the palatal process and surrounding Meckel's cartilage, but not in intramembranous chondrogenic cells. Thus, up-regulated *rFIP-2B* expression may play a role in regulating membrane trafficking or cellular morphogenesis of these embryonic and wounded pulpal cells.

KEY WORDS: *FIP-2*, wounded dental pulp tissue, subtractive hybridization, *in situ* hybridization.

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A supplemental appendix to this article is published electronically only at http://www.dentalresearch.org.

INTRODUCTION

Close regulation of odontoblast differentiation and subsequent secretory activity is critical for dentinogenesis during both embryogenesis and tissue repair (Smith and Lesot, 2001). After injury to the mature tooth, the fate of the odontoblast can vary according to the intensity of the injury. Milder injury can result in up-regulation of functional activity, leading to focal secretion of a reactionary dentin matrix, while greater injury can lead to odontoblast cell death. The regulation of odontoblast death after cavity preparation may be important for reparative dentinogenesis, because dentinogenesis in the damaged pulp may start after the elimination of apoptotic cells (Kitamura *et al.*, 2001). In general, reparative dentinogenesis is observed 1 wk after a moderate wound, such as the formation of a cavity whose depth is half the thickness of the dentin, whereas the induction of apoptosis and the elimination of apoptotic cells are observed within 3 or 4 days after wounding occurred (Sveen and Hawes, 1968; Taylor and Byers, 1990; Kitamura *et al.*, 2001). Many genes may be expressed differentially in the pulpal healing process; however, little is known about these genes.

In this study, we assumed that specific genes would be up-regulated or down-regulated in the pulpal healing following an experimental wound. We aimed to isolate wound-inductive (WIN) and wound-suppressive (WSP) cDNA and to analyze their mRNA expression to identify molecular processes involved in the rat pulp responses to injury.

MATERIALS & METHODS

Mechanical Wound and Tissue Preparation

The experimental protocol was approved by the Okayama University Dental School Review Board for animal care (no. 1-005-117). Twenty Wistar rats (male, 10-12 wks old), each weighing from 300 to 350 g, were used. The rats were deeply anesthetized with an intraperitoneal injection of 5% sodium pentobarbital (Nembutal, Dianippon Pharmaceutical Co., Suita, Japan) at a dose of 30 mg/kg, and a cavity depth of half the thickness of the dentin was prepared in the maxillary first molar (Fig. 1Aa). The cavities were left exposed to the oral environment for 1 wk, and then the teeth were extracted with the animals under the anesthesia mentioned above. For a control, a maxillary first molar without a cavity was extracted from the opposite side of the mouth in the same rat.

To avoid contamination of the gingival tissue and periodontal ligaments, we broke coronal dentin using dental forceps, and, using a dental curette, recovered pulp tissue from the chamber for further analysis.

Reciprocal Subtractive Hybridization, Cloning, and Homology Search

Subtractive hybridization and cloning were performed as described by Myokai *et al.* (2003), and as described in detail in the Appendix. Total RNA was isolated from the pulp tissue by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The target and driver single-stranded (ss) cDNAs bound to the beads were synthesized

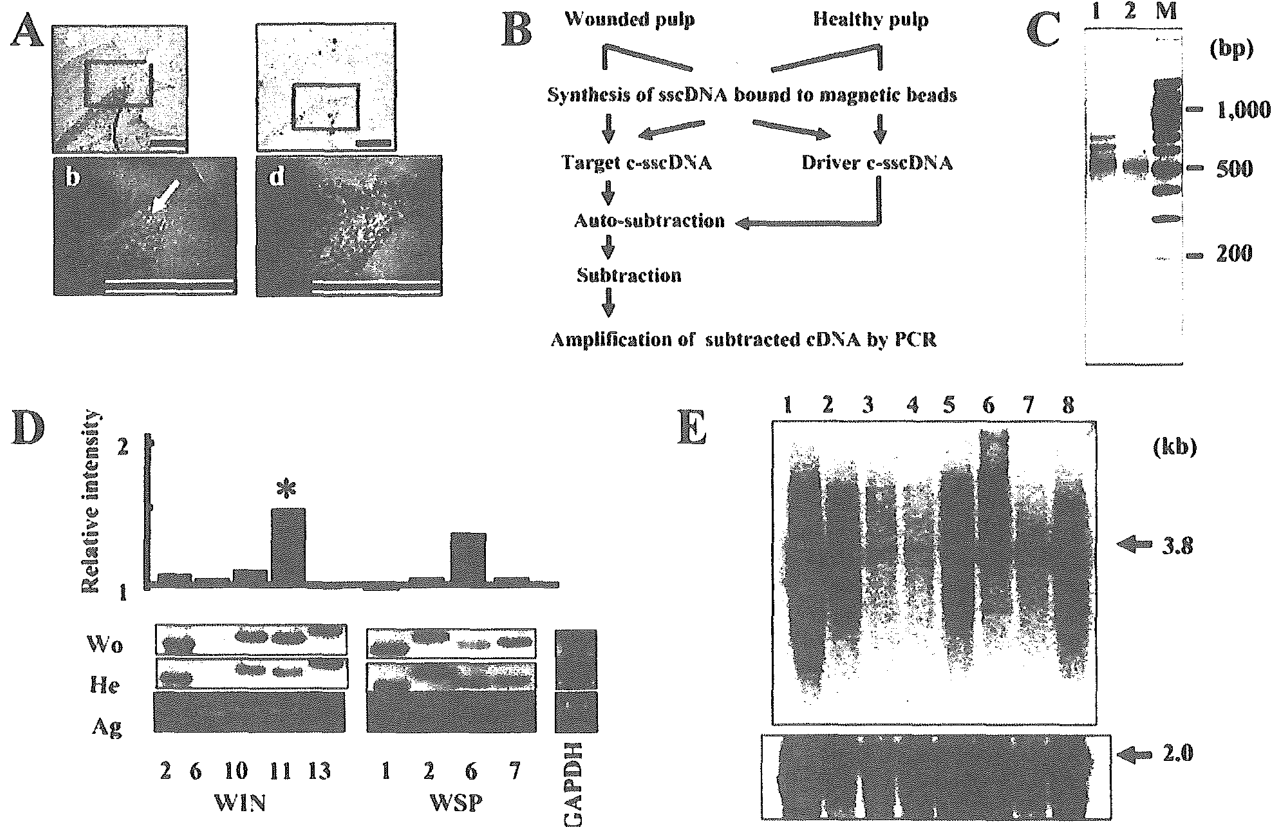

Figure 1. Histology of pulp, detection of cDNAs, and reverse Northern analysis. **(A)** Histology of pulp tissues. The wounded and healthy teeth were fixed with PBS containing 4% paraformaldehyde, demineralized with 10% EDTA for 4 wks, and embedded in paraffin. Hematoxylin and eosin staining was performed on serial sections 7 μm thick. The pulps 1 wk after cavity preparation (a,b) and healthy pulp (c,d) are shown. Pulpal cells underneath the cavity are indicated by the arrow, and neither reparative dentin formation nor apparent disruption of the odontoblast layer is observed (a,b). Bar equals 300 μm.

(B) Procedure of subtractive hybridization. The target and driver single-stranded (ss) cDNAs bound to the beads were synthesized from the total RNA (100 ng) isolated from the wounded and healthy pulp tissues. The target complementary sscDNA (c-sscDNA) was synthesized from the target sscDNA-beads with an *Eco*RI-dT primer (5'-GGCGAATTCTGCAGTTTTTTTTTTTTTT-3'). After auto-subtraction, the target c-sscDNA was subtracted twice from the driver sscDNA beads. The subtracted c-sscDNA was amplified by polymerase chain-reaction (PCR) with use of the *Eco*RI-dT primer, and was displayed on a 3% agarose gel. The procedure is described in detail in the Appendix.

(C) Display of amplified cDNAs. The wounded pulp cDNA subtracted from the healthy pulp cDNA was used as WIN cDNA, while the healthy pulp cDNA subtracted from the wounded pulp cDNA was used as WSP cDNA. The subtracted cDNA was amplified, and the product underwent gel electrophoresis. Lanes: 1, wound-inductive (WIN) cDNA; 2, wound-suppressive (WSP) cDNA; M, 100-bp DNA ladder.

(D) Messenger RNA expression of genes in pulp tissues. The cDNAs (WIN-2, 6, 10, 11, 13, WSP-1, 2, 6, 7, GAPDH) underwent electrophoresis on an agarose gel (Ag). They were then transferred to a membrane, and hybridized with the probe from wounded pulp tissue (Wo) and healthy pulp tissue (He). Relative signal intensity (each cDNA/GAPDH) is shown in the upper panel. *WIN-11 was used as a probe for screening of the cDNA library. Two independent hybridizations were performed, and a typical result is shown.

(E) *WIN-11* mRNA in adult rat tissues. *WIN-11* mRNA was detected in the tissues shown by an arrow in the upper panel, while *β-actin* mRNA was detected in the tissues shown by an arrow in the lower panel. Lanes: 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis. Three independent hybridizations were performed, and a typical result is shown.

from total RNAs of both wounded and healthy pulp (Fig. 1B). The complementary (c)-sscDNA was synthesized from the sscDNA beads. The target cDNA was subtracted and amplified by polymerase chain-reaction (PCR), and cloned into the *Eco*RI site of a pUC118 plasmid vector (Takara, Otsu, Japan). The plasmid containing a cDNA insert longer than 250 bp was prepared with the use of Qiagen Plasmid Miniprep Kits (Qiagen, Hilden, Germany), and sequenced by the dideoxy sequencing procedure (Sanger *et al.*, 1977) in an Automatic 377 sequencer (Perkin-Elmer, Foster City, CA, USA). A nucleotide homology search was performed in the Rat Genome Database (http://www.rgd.mcw.edu/), the Rat Genome Assembly (http://www.hgsc.bcm.tmc.edu/), and both BLASTN and BLAST EST homology programs in GenBank DNA databases (final searches in October 30, 2003).

Reverse Northern and Northern Hybridization

Reverse Northern hybridization was performed as described previously (Ohira *et al.*, 2004) and in the Appendix. Briefly, the cDNAs isolated by subtraction were subjected to gel electrophoresis in duplicate, then transferred to nylon membranes. Total RNAs from the wounded and healthy pulps were reverse-transcribed with the use of oligo $(dT)_{12\text{-}18}$ primer, and labeled with $[\alpha\text{-}^{32}P]$ dCTP. The membranes were hybridized with the probe, and the signals were visualized in a BioImaging Analyzer (BAS 2000; FUJI,