Human β -defensin-2 induction in human foreskin keratinocyte by synergetic stimulation with foods and *Escherichia coli*

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Abstract

We established a real-time quantitative RT-PCR assay that permits rapid and sensitive screening of foods that increase the human β -defensin-2 (hBD-2) mRNA level in human foreskin keratinocyte (HFK) cells. The range of hBD-2 mRNA concentrations suitable for the assay was between 8×10^{-18} M (39-cycle amplification) and 8×10^{-11} M (13-cycle amplification) as calibrated with standard hBD-2 cDNA. With this assay system, it was found that the stimulation of HFK cells by the addition of yeast powder at 5 g l^{-1} to the culture medium resulted in about 40 times increase in hBD-2 mRNA level, though stimulation with *Escherichia coli* attained the same level of induction. The active component of yeast was insoluble in water. Simultaneous co-stimulation of HFK cells with *E. coli* and grains, such as amaranth, millet, soybean and sesame, boosted hBD-2 mRNA induction significantly (6.1, 2.5, 3.3, and 3.3 times, respectively) above the level attained with *E. coli* alone. The results of successive fractionations of amaranth grain powder by ether extraction and amylase digestion showed that the boosting activity of amaranth grain resided in its insoluble fraction. Significant boosting of hBD-2 mRNA induction in epithelial cells with foods opens a new possibility of developing functional foods that can protect the human body against microbial infection at the oral cavity, skin, and respiratory tract among others.

Abbreviations: hBD-2 – human β -defensin-2; HFK – human foreskin keratinocyte; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; FAM – 6-carboxy-fluorescein; TAMRA – 6-carboxy-tetramethylrhodamine; TLRs – Toll-like receptors; PAMPs – pathogen-associated molecular patterns; ΔR_n – fluorescence generation; Ct – threshold amplification cycle

Introduction

Human defensins, the peptides responsible for innate immunity, are known to play a pivotal role in front defense against bacterial infections. The family of defensins is characterized by the cationic nature of the peptides with six cysteine residues that form three intramolecular disulfide crosslinks,

and is classified into α and β subfamilies. α -Defensins, first found in 1985 (Ganz et al. 1985), now have six members (Lehrer et al. 1993). Four of them, termed human neutrophil peptide-1 to -4 (HNP-1 to -4), are localized in the azurophilic granules of neutrophils. The other two α -defensins, termed human defensin-5 and -6 (HD-5 and -6), are distributed in the secretory granules of intes-

tinal Paneth's cells as well as in epithelial cells of the female genital tract (Quayle et al. 1998). All α -defensins are expressed constitutively.

In 1995, a β -defensin, the tertiary structure of which is characteristically different from that of α -defensins, was found in human hemofiltrate (Bensch et al. 1995). The structural difference of β -defensin was ascribed mainly to the different combinations of cysteine residues for intramolecular disulfide crosslinks. Human β -defensin-1 (hBD-1) is constitutively expressed in the kidney, pancreas, urinary and respiratory tracts, and several other epithelial tissues (Bensch et al. 1995; Zhao et al. 1996; Valore et al. 1998), whereas hBD-2, a peptide consisting of 41 amino acid residues, was found in human skin (Harder et al. 1997) and was later shown to occur also in the lung, oral mucosa, and saliva among others (Bonass et al. 1999; Dunsche et al. 2001; Krisanaprakornkit et al. 2002). In recent years, two additional human β -defensins, hBD-3 and hBD-4, have been identified, and their tissue specific expressions in the skin and nasal mucosa (hBD-3) and the testis and stomach pyloric antrum (hBD-4) have been reported (Garcia et al. 2001; Harder et al. 2001).

It was shown that hBD-2 effectively kills Gram-negative bacteria and pathogenic yeast (Harder et al. 1997; Singh et al. 1998). The bactericidal activity of hBD-2 is at least 10 times more potent than that of all other α - and β -defensins (Singh et al. 1998).

hBD-2 is inducible when the human body is infected by pathogenic bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*, or produces proinflammatory cytokines (Harder et al. 1997; Singh et al. 1998). However, no report is available as yet concerning the induction of hBD-2 by foods or food-related ingredients, though some amino acids and their analogs are known to be effective in inducing β -defensin in bovine epithelial cells (Fehlbaum et al. 2000).

The inducible nature of hBD-2 prompted us to examine the possibilities of enhancing hBD-2 levels by the intake of particular foods, which may lead to the development of functional foods that can facilitate the prevention of infectious diseases such as sepsis and opportunistic infection. In this paper, the increase in the hBD-2 mRNA level in HFK cells by some foods, particularly by the synergetic stimulation with foods and *E. coli*, is described.

Materials and methods

RNA extraction from stimulated and unstimulated HFK cells

HFK cells were cultured in EpiLife medium supplemented with Human Corneal Growth Supplement (Cascade Biologics, Inc., Portland, OR, USA), which contained bovine pituitary extract, murine epidermal growth factor, hydrocortisone, bovine insulin and bovine transferrin, in humidified atmosphere containing 5% CO₂ at 37 °C.

HFK cells plated onto six-well dishes at a density of 2.5×10^5 cells ml⁻¹ were precultured for 24 h, and incubation was further continued for 16 h in the presence and absence of test materials. Total RNA was extracted from the harvested cells with an RNeasy Mini Kit (QIAGEN K.K., Japan) according to the manufacturer's instruction.

Quantitative PCR

cDNA was synthesized by RT-PCR using the total RNA extracted from HFK cells after cultivations in the presence and absence of test materials. The amounts of hBD-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs were assayed by real-time quantitative PCR using the TaqMan methodology (Holland et al. 1991) with an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Japan). The primers (forward: 5'-ctt cca ggt gtt ttt ggt ggt a-3'; reverse: 5'-gac tgg atg aca tat ggc tcc-3') and the probe (5'-agg cga tcc tgt tac ctg cct taa gag-3') for assaying hBD-2 mRNA by quantitative PCR were designed in accordance with the published hBD-2 sequence (Accession No. Z71389) using Primer Express 1.5 Software (Perkin-Elmer Applied Biosystems, Japan). The probe was dual-fluorescence-labeled, i.e., the 5'-end of the probe was labeled with 6-carboxy-fluorescein (FAM) and the 3'-end with 6-carboxy-tetramethylrhodamine (TAMRA). GAPDH was assayed by quantitative PCR employing TaqMan GAPDH Control Reagents (Perkin-Elmer Applied Biosystems, Japan).

The hBD-2 mRNA levels in HFK cells after cultivation in the presence and absence of stimulants were assessed in terms of the copy number ratio of hBD-2/GAPDH, in order to standardize

the fluctuation of absolute concentrations of cDNAs among the sample preparations to be examined. In addition, the possibilities of hBD-2 cDNA amplification of the genomic DNA origin were excluded by introducing the exon-intron junction sequence into the reverse primer, as was the case for commercial GAPDH primers.

The cDNA amplification by PCR was performed in a reaction mixture containing 5 μ l of cDNA solution, 12.5 μ l of TaqMan Universal PCR Master Mix (Perkin-Elmer Applied Biosystems, Japan), 5 pmol each of the forward and reverse primers, and 3 pmol of the dual-fluorescence-labeled probe, in a total volume of 25 μ l. The thermal cycling of the reaction mixture was carried out under the conditions recommended by the manufacturer, i.e., initial heating at 50 °C for 2 min and at 94 °C for 10 min, followed by 45 cycles of successive denaturation and annealing extension at 94 °C for 15 s and at 60 °C for 60 s, in combination.

hBD-2 cDNA standard

A standard hBD-2 cDNA for calibrating the quantitative PCR was prepared as follows: First, cDNA was synthesized by RT-PCR using the total RNA obtained from unstimulated HFK cells. The cDNA was amplified by PCR using a pair of hBD-2 specific primers with multicloning sites for *Hind* III, *Nde*I and *Xba*I included at the 5'-termini (indicated by capital letters): 5'-GCA AGC TTC Ata tga ggg tct tgt atc tcc tct tct cgt-3' and 5'-GCT CTA Gat cat ggc ttt ttg cag cat ttt gtt cca-3'. The PCR product was digested with restriction enzymes and cloned into the pRc/CMV vector (Invitrogen), and the sequence was confirmed. A similar cDNA construct cloned into the vector was prepared for GAPDH in the same manner (Accession No. M33197). These cDNA constructs were used as standards for quantitative PCR.

Preparation of test samples

Saccharomyces cerevisiae (KANEKA Yeast Red), donated by KANEKA Corp., Japan, was suspended in water to a final concentration of 100 g l⁻¹. The yeast suspension, as well as its

soluble and insoluble fractions obtained by centrifugation at 15,000 $\times$ g for 20 min, was autoclaved to examine the effects of inducing hBD-2 mRNA level on HFK cells. Lyophilized *E. coli* (SIGMA) was suspended in water at a concentration of 10 g l⁻¹ and sterilized by autoclaving before its addition to HEK cell culture.

Pulverized grains, of amaranth, millet, soybean and sesame, were similarly suspended in water at a concentration of 100 g l⁻¹ and autoclaved. An aqueous suspension of steamed amaranth grain powder (100 g l⁻¹) was extracted with an equal volume of ether, and the aqueous and ether layers were separated. For the ether layer, ether was evaporated and the residual material was suspended in water. Both the water- and ether-soluble materials in water were autoclaved and assayed for the activity of boosting hBD-2 mRNA induction. The aqueous layer was incubated at 50 °C for 3 h with 0.05 g l⁻¹ liquefying amylase (EC 3.2.1.1) from *Bacillus subtilis* with gentle stirring to reduce its viscosity. After the amylase treatment, the incubated mixture was centrifuged at 15,000 $\times$ g for 20 min and both the supernatant and precipitate were examined for the boosting activity.

Results

Establishment of hBD-2 mRNA detection system

We reconfirmed, in terms of mRNA, that sterilized *E. coli* cell powder induces significant levels of hBD-2 in cultured HFK cells (Harder et al. 1997), and that the induction effect of *E. coli* dose-dependent (data not shown). By considering the hBD-2-inducing effect of *E. coli* as positive control, we established a real-time quantitative RT-PCR assay that permits rapid and sensitive screening of foods that increase the hBD-2 mRNA level in HFK cells. The range of hBD-2 mRNA concentrations suitable for the assay was evaluated with a series of sequentially diluted solutions containing known concentrations of standard hBD-2 cDNA, which had been cloned and inserted into a plasmid vector. Representative results of the real-time quantitative PCR assay with different concentrations of standard hBD-2 cDNA are shown in Figure 1. Since the fluorescence is generated by the 5'-3' exonuclease activity of Taq polymerase, which splits FAM and TAMRA

Figure 1. Typical results of real-time quantitative PCR assay with standard hBD-2 cDNA. The assay was performed with standard hBD-2 cDNA of different concentrations (8×10^{-18} – 8×10^{-11} M), which were prepared by serial 10-fold dilutions. The relationship between fluorescence generation (ΔRn) and amplification cycle is shown (a). The logarithm of applied cDNA concentration was plotted against the threshold amplification cycle (C_t) required to reach an arbitrary ΔRn level ($\Delta Rn = 2 \times 10^{-1}$, i.e., about 20% of the maximal velocity of the 5′–3′ exonuclease reaction; indicated by a horizontal solid line in (a)) (b). Results of triplicate measurements were superimposed.

moieties from the probe bound to hBD-2 cDNA, the fluorescence intensity reflects amplified hBD-2 cDNA concentrations. As the amplification proceeds and the substrate concentration increases, the velocity of the 5′–3′ exonuclease reaction, i.e., the fluorescence generation speed, becomes maximal and reaches a plateau (Figure 1a). The results indicate that the higher the concentration of cDNA applied in the assay, the fewer the amplification cycles required before fluorescence becomes detectable. A linear relationship ($r^2 = 0.995$) was observed between amplification cycle and applied cDNA, when the amplification cycle required to reach an arbitrary ΔRn level ($\Delta Rn = 2 \times 10^{-1}$, i.e., about 20% of the maximal reaction velocity of 5′–3′ exonuclease) was plotted

against the logarithm of cDNA concentration (Figure 1b). These results indicate that the assay can determine hBD-2 mRNA level accurately at concentrations between 8×10^{-18} M (39-cycle amplification) and 8×10^{-11} M (13-cycle amplification). Thus, the hBD-2 mRNA levels in HFK cells with and without stimulation were determined and calibrated with the standard hBD-2 cDNA.

Effects of food-related microorganisms on hBD-2 induction

In search of food-related microorganisms that can increase the hBD-2 mRNA level, we screened various microorganisms and found that among the food-related microorganisms examined, yeast can most significantly induce hBD-2 mRNA level in HFK cells. The addition of yeast powder to a final concentration of 5 g l^{-1} in the culture medium resulted in about 40 times higher level of hBD-2 mRNA induction in HFK cells, though *E. coli* attained the same level of induction (data not shown). In order to identify the component(s) in yeast that is responsible for hBD-2 mRNA induction, yeast powder was extracted with water and separated into soluble and insoluble fractions by centrifugation, and the fractions were examined for induction activity. The results of the assay showed that the activity of hBD-2 mRNA induction resides essentially in the insoluble fraction (Figure 2), indicating that the insoluble component of yeast, such as the cell wall, is responsible for the stimulation of HFK cells. Since it is known that hBD-2 induction in human cells occurs in response to infections by living microorganisms (Wada et al. 1999) as well as contact with sterilized microbial cells and their insoluble fraction as shown above (Figure 2), it is most feasible that the material(s) responsible for hBD-2 induction locate at the surface of microorganisms, rather than in the cytoplasm or secreted from it.

*Synergetic co-stimulation of HFK cells with grains and *E. coli**

We also screened various foods of non-microbial origin that can induce hBD-2 mRNA in HFK cells. It was found that the simultaneous co-stimulation of HFK cells with *E. coli* and powders of

Figure 2. The effects of yeast powder on hBD-2 mRNA induction in HFK cells. Heat-inactivated yeast powder, as well as its water-soluble and -insoluble fractions, was examined for activity of hBD-2 mRNA induction. The total RNA extracted from HFK cells after 16-h stimulation with each test material was subjected to reverse transcription. The resulting cDNA was quantified for hBD-2 and GAPDH by the Taq-Man PCR method. The amount of hBD-2 cDNA determined was standardized with that of GAPDH cDNA in each test, and the induction effect was expressed in terms of increment fold as compared with the non-stimulated level. The stimulation with *E. coli* was shown for comparison. The stimulation effects with (a) no additive, (b) 0.75 g l^{-1} *E. coli*, (c) 5 g l^{-1} whole yeast, (d) 5 g l^{-1} water-soluble fraction of yeast, and (e) 5 g l^{-1} water-insoluble fraction of yeast, are shown. The error bar indicates the standard deviation for triplicate measurements.

certain grains, such as amaranth, millet, soybean and sesame, could increase hBD-2 mRNA level much more than that attained with *E. coli* powder alone. The increase in hBD-2 mRNA levels by the co-stimulation with $0.2\text{--}0.5 \text{ g l}^{-1}$ amaranth, millet, soybean and sesame powders were 6.1, 2.5, 3.3, and 3.3 times, respectively, which are higher than that achieved by *E. coli* powder alone (Figure 3, solid bars). The synergetic stimulation of HFK cells with *E. coli* and grain powders resulted in an hBD-2 mRNA level 25–61 times higher than that without stimulation, whereas the stimulation with *E. coli* powder alone enhanced the mRNA level by only about 10 times. However, the above grain powders had no effect of hBD-2 mRNA induction in HFK cells when examined in the absence of *E. coli* powder (Figure 3, open bars). These results indicate that the grain powders have an activity of boosting the microorganism-dependent hBD-2 mRNA induction.

Fractionation of active material(s) in amaranth

An attempt was made to characterize the active material(s) contained in amaranth powder that can boost hBD-2 induction, since amaranth was the most effective among the grains examined (Figure 3). Since it was reported that the major components of amaranth grain are starch (56–78%) and lipid (6.5–12.5%) (Guzman-Maldonado and Paredes-Lopez 1998), amaranth powder was successively subjected to ether extraction, followed by amylase digestion after autoclaving, to fractionate the active material(s). The ether extraction of amaranth powder yielded oil components, which accounted for approximately 10% of the powder's weight. It was shown, however, that the ether-extractable materials had no activity of boosting hBD-2 mRNA induction in HFK cells, when examined by the co-stimulation with *E. coli* (Figure 4a). When examined by the co-stimulation with *E. coli* (Figure 4a). When the ether-unextractable residue, which accounted for 90% of the weight as measured after lyophilization, was suspended in water and autoclaved, it became highly viscous due to solubilized starch, and the high viscosity interfered with the separation of insoluble materials by centrifugation. The amylase treatment effectively reduced the viscosity of the autoclaved ether-unextractable materials, and precipitates were successfully separated by centrifugation. The ether-unextractable materials before and after amylase treatment, were assayed for the activity of boosting hBD-2 mRNA induction in HFK cells in the presence of *E. coli* powder. The assay showed that the ether-insoluble fraction before and after amylase treatment had the co-stimulation activity of enhancing hBD-2 mRNA induction by 2.3 and 3.5 times, respectively, as compared to that stimulated with *E. coli* alone. Similarly, the soluble and insoluble fractions of the amylase-treated ether-insoluble materials showed 3.7 and 1.0 times the hBD-2 mRNA induction activity by co-stimulation, respectively, as compared to that stimulated with *E. coli* alone. These results indicate that the boosting activity of the grain powder resides essentially in the insoluble residue (Figure 4b). The amaranth powder had a tendency of increasing the activity of boosting hBD-2 mRNA induction after ether extraction and amylase treatment (Figure 4).

Figure 3. Effects of stimulation with grain powders on the level of hBD-2 mRNA in HFK cells. HFK cells were cultured with the autoclaved suspension of pulverized amaranth (a), millet (b), soybean (c), and sesame (d) grains, in the presence (solid bars) and absence (open bars) of 0.5 g l⁻¹ sterilized *E. coli* powder. The amount of hBD-2 cDNA determined was standardized with that of GAPDH cDNA in each test as described in Figure 2. The error bar indicates the standard deviation for triplicate measurements.

Discussion

Assay performance

We established a real-time quantitative RT-PCR assay that permits rapid and sensitive screening of foods that have the activity to enhance the hBD-2 mRNA level. Although a method of quantifying hBD-2 mRNA by real-time PCR has already been reported (Lehmann et al. 2000), the method presented here provides improved sensitivity and accuracy with excellent reproducibility. It can assay a wide range of mRNA concentrations between 8×10^{-18} and 8×10^{-11} M (Figure 1), which is 500 times more sensitive than the method of Lehmann. The introduction of an exon-intron junction sequence into the reverse primer elimi-

nated the possibilities of amplifying the genomic hBD-2 DNA and permitted simple assay procedures without DNase treatment. Calibration with standard hBD-2 cDNA warranted the reproducibility and accuracy of the assay. The only drawback of the assay using primary HFK cells, which can lead to possible fluctuations of the absolute hBD-2 mRNA amount in the cell from one experiment to another, was ruled out by standardization, i.e., by taking the ratio of hBD-2 mRNA/GAPDH mRNA in each experiment. Although attempts were made to select a cell line that expresses a constant hBD-2 mRNA level, the hBD-2 mRNA levels in all cell lines examined were much lower than that in HFK cells. We are now trying to immortalize HFK by keeping the hBD-2 expression level high.

Figure 4. Effects of stimulation with amaranth fractions on hBD-2 mRNA enhancement in HFK cells. The ether-extractable and -unextractable fractions of amaranth powder were suspended in water and examined for their activity to enhance hBD-2 mRNA level in HFK cells, where the stimulation of the cells was performed in the presence (solid bars) and absence (open bars) of 0.5 g l⁻¹ sterilized *E. coli* powder. The effects of (a) no addition, (b) 0.2 g l⁻¹ amaranth powder, (c) ether-extractable fraction, corresponding to (b), and (d) ether unextractable fraction, corresponding to (b), are shown (a). The ether-unextractable fraction of amaranth powder was subjected to amylase digestion, and the soluble and insoluble fractions were examined for their activity to enhance hBD-2 mRNA level in HFK cells in the presence (solid bars) and absence (open bars) of 0.5 g l⁻¹ sterilized *E. coli* powder. The effects of (e) no addition, (f) ether-unextractable fraction before amylase treatment, (g) ether-unextractable fraction after amylase treatment, (h) insoluble fraction after amylase treatment and (i) soluble fraction after amylase treatment, are shown (b). The error bar indicates the standard deviation of triplicate measurements.

hBD-2 induction with food-related materials

The use of microorganisms in food fermentation, such as yeast (*S. cerevisiae*) in baking, lactic acid bacteria (*Lactobacillus acidophilus*) in yogurt production, and natto bacteria (*B. subtilis* var. *natto*) in soybean fermentation, has a long history in various food cultures of the world. It is feasible that such food-related microorganisms may play an important role in host defense by stimulating hBD-2 expression, particularly in the oral cavity and gastrointestinal tracts where hBD-2 producing cells contact directly with such microorganisms in foods. In this context, we screened various food-related microorganisms that may have the activity of inducing hBD-2 mRNA, and it was found that the stimulation of HFK cells with yeast powder at a concentration of 5 g l⁻¹ could induce 40 times as much hBD-2 mRNA as that without stimulation. This induction activity of yeast was significant, though *E. coli* attained the same level of induction. Studies of hBD-2 induction conducted thus far have been focused on the effect of pathogenic microorganisms, and it is known that Gram-negative bacteria are particularly effective for this induction, as well as pathogenic yeast (*C. albicans*) to some extent (Harder et al. 1997). It is emphasized in this study that edible yeast, and not only pathogenic microorganisms, can induce hBD-2 mRNA, which suggests that yeast is a promising

candidate component for the development of functional foods that can facilitate human body protection against microbial infections. Essentially the activity in yeast that induces hBD-2 was found in the insoluble fraction, suggesting that the component responsible for this activity locates in the cell wall (Figure 2). Since it is known that hBD-2 is induced in human cells in response to infections by living microorganisms (Wada et al. 1999), the results of the above-described fractionation experiments consistently indicate that the component responsible for hBD-2 induction locates at the surface of microorganisms, rather than in cytoplasm or secreted from it.

Another very important finding in the present work is that certain grains, such as amaranth, millet, soybean and sesame, significantly boost hBD-2 mRNA induction in human cells with the co-stimulation with *E. coli*. The boosting effect of grain powders accounted for 2.5- to 6.1-fold hBD-2 mRNA induction as compared to that attained with *E. coli* alone (Figure 3). On the other hand, those grain powders had no activity of inducing hBD-2 mRNA in the absence of *E. coli*. These results indicate that these grains can participate in the protection of the human body against pathogenic bacteria by boosting hBD-2 expression.

The results of successive fractionations of amaranth grain powder by ether extraction and amy-

lase digestion showed that essentially the boosting activity in amaranth grain resides in the insoluble fraction (Figure 4a, b). All attempts to solubilize the active component in amaranth grain with various solvents such as dimethylsulfoxide were unsuccessful, and further studies should be carried out to characterize the active component.

hBD-2 induction through activation of the NF- κ B pathway

Since human Toll-like receptors (TLRs) were found as homologues of the *Drosophila* Toll receptor (Medzhitov et al. 1997), which are indispensable for immune responses, TLRs have been considered to be key molecules for innate immunity rather than part of the acquired immune system. It was also reported that TLRs recognize pathogen-associated molecular patterns (PAMPs), which alert the potential presence of pathogenic microorganisms, such as LPS (Ingalls et al. 1999), β -glucans (Czop and Kay 1991), certain mannose-containing carbohydrates (Fraser et al. 1998), peptidoglycan (Jin et al. 1998), and bacterial DNA (Heeg et al. 1998).

Although the expression and functions of TLRs has been demonstrated in various kinds of myeloid cells, little is known about the expression and function of TLRs on non-leukocytes. However, Song et al. (2002) recently reported that human keratinocytes are capable of expressing functional TLRs. It is known that TLR signal transduction is mediated by way of an intranuclear transcription factor NF- κ B (Belvin and Anderson 1996) and the hBD-2 gene has a NF- κ B response element within the upstream promoter region (Liu et al. 1998). Thus, the induction of hBD-2 by stimulation with microorganism coincides with the activation of the NF- κ B pathway (Becker et al. 2000). The successful hBD-2 mRNA induction with edible yeast suggests that it is most plausible that some PAMP-like components in non-pathogenic microorganisms are recognized by TLRs in a similar manner as pathogenic microorganism.

In contrast, since grain powders had no activity of inducing hBD-2 mRNA in the absence of *E. coli* (Figure 3), these grains would not be recognized by TLRs. A possible explanation for the grain powders activity of boosting, hBD-2 mRNA enhancement is that the grains activate signal

Figure 5. Putative cellular pathways of food-mediated defensin expression. The diagram shows the putative pathways involved in food-mediated hBD-2 expression. The schematic was assembled using gene expression data from food and bacterial treatment and previous literature.

transduction molecules, such as MyD88, IRAK, and TRAF6, which transduce the signal of *E. coli* recognition from TLRs to the NF- κ B downstream in cascade (Figure 5). Another possibility is the activation of the MAP-kinase pathway via the activation of transcription factor AP-1 through molecules such as p38, JNK and ERK, rather than through NF- κ B. A recent study of Krisanaprakornkit et al. (2002) showed that hBD-2 is induced through the MAP-kinase pathway under NF- κ B blockade.

Possible applications

The results of the present study indicate that the amaranth, millet, soybean and sesame grains have no activity of inducing hBD-2 mRNA in the absence of microorganisms, but can enhance the expression of hBD-2 mRNA during microbial infection. The above-described findings concerning the induction and boosting of hBD-2 mRNA with foods in HFK cells opens a new possibility of developing functional foods that can protect the human body against microbial infection at the oral cavity, skin, and respiratory tracts among others, though it is necessary to confirm the effects *in vivo*. The daily intake of such grains would be good for

human health by keeping the body ready for prompt induction of high levels of hBD-2 mRNA in response to bacterial infections. In addition, it is plausible that the daily intake of foods containing such grains and edible microorganisms such as yeast, which in combination have a synergetic induction effect, would facilitate the effective induction of hBD-2 mRNA in intestinal epithelial cells. The development of functional foods along this line is under way in our laboratory.

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