Enhancement of Dectin-2 Gene Expression by Lignin–Carbohydrate Complex from Lentinus edodes Mycelia Extract (LEM) in a Mouse Macrophage-like Cell Line

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Abstract. Background: Lignin fractions of Lentinus edodes mycelia extract (LEM) have shown anti-HIV and immunopotentiating activity. However, the action point of lignin–carbohydrate complex has not been elucidated. In order to elucidate their action point, DNA microarray analysis was performed, using mouse macrophage-like J774.1 cells. Materials and Methods: RNA was isolated with Qiagen RNeasy Plus Mini kit, hybridized with GeneChip MouseGene 1.0 ST arrays, and scanned with Affymetrix GeneChip Command Console software. Results: One of the seven lignin–carbohydrate fractions isolated from LEM (Fr4) enhanced the expression of dectin-2 (4.2-fold) and toll-like receptor (TLR)-2 (2.5-fold) prominently, but only slightly modified the expression of dectin-1 (0.8-fold), complement receptor 3 (0.9-fold), TLR1, 3, 4, 9 and 13 (0.8- to 1.7-fold), spleen tyrosine kinase (Syk)b, zeta-chain (TCR) associated 70 kDa protein kinase (Zap70), Janus tyrosine kinase (Jak)2 (1.0- to 1.2-fold), nuclear factor (Nf)b1, Nfkb2, reticuloendotheliosis viral oncogene homolog (Rel)a, Relb (1.0- to 1.6-fold), Nfkbia, Nfkbib, Nfkbje, Nfkbil2 and Nfkbiz (0.8- to 2.3-fold). On the other hand, lipopolysaccharide did not affect the expression of dectin-2 or TLR-2. Conclusion: These data suggest the significant role of the activation of the dectin-2 signaling pathway in the action of lignin–carbohydrate complex on macrophages.

Lentinus edodes mycelia extract (LEM) has shown anti-hepatopathic (1-3), antitumour (4-8), immunopotentiating (9, 10), antivascularisation (11), anti-arteriosclerosis (12), skin-protective (13, 14) and undocumented activities, such as improvement of hepatic function, menorrhagia, melancholia, nausea and vomiting. Lentinan (15) and KS-2 (16), polysaccharides isolated from L. edodes have shown immunopotentiating and antitumour activities. Lignin fractions of LEM have shown anti-HIV (17) and immunopotentiating (18) activity. However, the action point of these lignin fractions has not been elucidated. We recently isolated seven lignin-carbohydrate fractions from LEM, and among them, fraction 4 (Fr4) showed the highest anti-HIV activity and most potently inhibited NO production by lipopolysaccharide (LPS)-stimulated mouse macrophage-like cells (RAW264.7, J774.1) (19). Fr4 was found to be composed of lignin precursors such as vanillic acid, syringic acid, p-coumaric acid and ferulic acid, with trace amounts of flavonoids and tannins, and negligible amount of LPS, confirming the authenticity of Fr4 as lignin (20). Preliminary DNA microarray analysis suggested that Fr4 may affect immune response-related gene expression, but may not affect the expression of as many genes as LPS did (20). Dectin-1 and dectin-2 have been identified as the receptors for β-glucan and α-mannan, respectively (21, 22). We previously reported that the polysaccharide portion of lignin-carbohydrate complex from pine cone extract of Pinus parviflora Sieb et Zucc is comprised of arabinose, mannose, galactose and glucose (23), suggesting that lignin–carbohydrate complex may bind to dectin-1 or dectin-2. In order to elucidate the action point of Fr4, DNA microarray analysis of the effect of Fr4 at different doses and in combination with LPS was performed for J774.1 cells.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco’s modified Eagle’s medium (DMEM), GIBCO BRL, Grand Island, NY, USA; fetal bovine serum (FBS), JRH, Bioscience, Lenexa, KS, USA, LPS from Escherichia coli (serotype 0111:B4); dimethyl sulfoxide (DMSO), Wako Pure Chem. Ind., Osaka, Japan.

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Preparation of Fr4. Fr4 was isolated from LEM by alkaline extraction and acid precipitation, as described previously (20).

Cell culture. Mouse macrophage-like J774.1 cells (RIKEN Bioresource Center, Tsukuba, Japan) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate under a humidified 5% CO2 atmosphere (20). Cells were inoculated at 0.25×10^6/ml (10 ml) in a 8.5 cm-dish and incubated for 36 hours. Near confluent cells were treated for 24 hours without (referred to as ‘Control’) or with 125 or 250 μg/ml Fr4 (referred to as ‘Fr125’ or ‘Fr250’), or 0.1 μg/ml LPS (referred to as ‘LPS’) or combination of 250 μg/ml Fr4 and 0.1 μg/ml LPS (referred to as ‘Fr250+LPS’). In some experiments, the cells were treated with RLT plus buffer (RNeasy Plus Mini Kit; Qiagen Inc., Tokyo, Japan).

Assay for TNF production. The tumor necrosis factor (TNF) released into the culture medium was determined by ELISA (Quantikine ELISA kit, R&D systems, Minneapolis, MN, USA).

RNA isolation and preparation. Total RNA was isolated from cell samples according to the manufacturer’s instructions using a Qiagen RNeasy Plus Mini kit (Qiagen Inc.). The quality of the RNA was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc, Santa Clara, CA, USA). Total RNA was used with Ambion’s ArrayControl RNA (Ambion, Inc., Austin, TX, USA) spikes, and in vitro synthesised polyadenylated Bacillus subtilis T3 RNA spikes, and amplified according to the Eberwine procedure (24) using Ambion’s MessageAmp™ kit.

Array hybridization. The five prepared RNA samples, namely Fr250, Fr125, LPS (as positive control for Fr250 and Fr125), Fr250+LPS, and Control (as negative control for Fr250 and Fr125), were submitted to GLab Pathology Center Co, Ltd (Hokkaido, Japan), and the labeling, hybridization, washing, and scanning were performed there. For hybridization, GeneChip MouseGene 1.0 ST arrays (Affymetrix Inc, Tokyo, Japan) were used, which measure the expression of at least 28,000 genes (using T3 RNA spikes, Bacillus subtilis, and 1,880 pg/ml (LPS+Fr250). The RNA was prepared from these cells for the subsequent DNA microarray analysis.

Extraction of differentially expressed genes. Normalized raw data were analyzed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

Results and Discussion

TNF production. Fr4 dose-dependently stimulated the production of tumour necrosis factor-α (TNF-α) by mouse macrophage-like J774.1 cells. The TNF-α concentration in the culture medium was elevated from 132 pg/ml (Control) to 706 pg/ml (Fr125), 881 pg/ml (Fr250), 1,741 pg/ml (LS) and 1,880 pg/ml (LPS+Fr250). The RNA was prepared from these cells for the subsequent DNA microarray analysis.
Classification of each treatment using PCA. Figure 1 shows a visualization of the five treatments in the space of the first and second principal components of PCA. The horizontal axis indicates the first component and the vertical axis indicates the second component. The cumulative proportion from first to second components was 96.3%. Similarity between the gene expression pattern under treatment with Fr125 and Fr250 was recognized.

Classification of each treatment using HCL method. Figure 2 shows the results of hierarchical clustering for 1,314 genes and 5 treatments. The dendrogram on the right indicates the gene expression patterns with Fr125 and Fr250 were the most similar, and those of LPS and Fr250+LPS were also similar, and then that of Control was more similar to Fr125 and Fr250 than LPS and Fr250+LPS.

Gene expression pattern of Dectin-1 and Dectin-2 family. The expression patterns of Dectin-1 and Dectin-2 family genes (28) which are strongly suggested as receptors of polysaccharides of the cell walls in the fungus (Figures 3 and 4). For example, ‘Fr125/Control’ on the x-axis denotes the expression of each gene under Fr125 treatment in comparison to that with Control. The dotted lines represent FC in gene expression rate of above 2.00 and below 0.50 (Figures 3-7), changes which were considered significant.

For Dectin-1 family genes, it was confirmed that there were no differences between the expression of Clec1a, Clec1b, Clec7a (Dectin-1), Clec9a, Clec12a and Clec12b genes with each treatment and that of Control.

Figure 4 shows the expression of Dectin-2 family genes under Fr125, Fr250, LPS, and Fr250+LPS treatment in comparison to Control. The expressions of Clec4d, Clec4e, and Clec4n (Dectin-
genes with both Fr125 and Fr250 were more than twice as high as those of Control. Furthermore, the expression of Clec4n (Dectin-2) in both of those treatments increased by more than threefold as compared with Control. On the other hand, LPS treatment did not change the expression of Clec4n (Dectin-2). This indicates that Clec4n (Dectin-2) was specifically overexpressed under treatment with Fr125 and Fr250.

There were no significant differences between the expression of Clec4n (Dectin-2) with Fr125 and Fr250. In terms of other Dectin-1 and Dectin-2 family genes, and thus the tendency of similarity between Fr125 and Fr250 was recognized (Figures 3, 4). Furthermore, there were no significant differences between the expressions of these genes among Fr250, LPS, Fr250+LPS treatment, and thus
the synergy effect of the combination of Fr250 and LPS on the enhancement of gene expression were not observed (Figures 3, 4).

Expression pattern regarding the Fc receptor family genes. Activated Clec4n by its ligands would control the transcription of cytokines through Fcer1g which is a member of the Fc receptor family, with immune receptor tyrosine-based activation motif (ITAM) domain. Following the phosphorylation of the ITAM of Fc receptor after lectin receptor (Dectin-2) activation, spleen tyrosine kinase (Syk)b (which is a member of non-receptor tyrosine kinase family that has two SH2 domains) is recruited to activate caspase recruitment domain family, member 9 (Card9)-dependent
signaling pathway leading to the activation of NFKb (29). It is also known that Fcer1g co-expresses and co-localizes with Clec4n. Figure 5 shows the expression of Fc receptor family genes, including Fcer1g. There was no difference in the expression of Fcer1g between Fr125, Fr250, and LPS treatments. On the other hand, we observed a tendency for an increase of Fcgr4 expression with LPS treatment.

Expression pattern of integrin family genes. Itgam (CR3), which is a member of the integrin family and expressed in macrophages, is considered as a receptor for β-glucan. Figure 6 shows the expression pattern of integrin family genes Itga5, Itga6, Itgal, Itgam, Itgav, and Itgb5. There was no difference in the expression of Itgam among treatments. It was confirmed that the expression of Itga5 significantly increased only with LPS treatment. On the other hand, the expression of Itga6, Itgav, and Itgb5 decreased with LPS, Fr125, and Fr250 treatments.

Expression pattern of Tlr family genes. Figure 7 shows the expression pattern of toll-like receptor (Tlr) family genes Tlr1, Tlr2, Tlr3, Tlr4, Tlr9, and Tlr13. The expression of Tlr1, Tlr2, and Tlr3 was increased by LPS treatment, but, that of Tlr4 (a known receptor of LPS), Tlr9 and Tlr13 were reduced by LPS treatment. On the other hand, the expression of Tlr2 was increased more by Fr125, and Fr250 treatments, as well as by LPS treatment, than by Control. In the case of the expression of Tlr1, Tlr3, Tlr4, Tlr9 and Tlr13, there was no difference between Control and Fr125, or Fr250 treatments.

Expression pattern of genes for inflammatory cytokines. The expression patterns of genes that are involved in the mammalian immune system, such as those in toll-like receptor signaling pathway, are listed in Table I. The expression of Il1a, Il1b, Il6, Osm, Cs12, and Il12b in LPS treatment was increased more than threefold that in Control. In particular, Il6 gene expression was increased by more than two hundredfold that in Control. The expressions of Il1a, Il1b, Il6, Osm, and Cs12 with Fr250 treatment were increased by more than twofold that of Control, as were the expressions of Il1a, Il1b, and Il6 with Fr125.

The expressions of Il1a, Il1b, Il6, Cs12, and Il12b in Fr125 and Fr250 were reduced to less than one seventh level of that with LPS treatment. The findings indicate that Fr125 and Fr250 enhanced the expressions of genes for inflammatory cytokines, such as Il1a, Il1b, Il6, Osm, Cs12, and Il12b, but the effects of Fr125 and Fr250 were lower than those of LPS. The expression of Osm with Fr125, Fr250, and LPS increased by more than twofold compared to Control, but the ratio expression of Osm with Fr250, compared to LPS was 0.8, and no difference between gene expression induced by Fr250 and LPS was confirmed. The expressions of genes for other inflammatory cytokines, such as Cs11, Cs13, Il18 and Il1f6, which are not listed in Table I, with LPS, Fr125, and Fr250 treatments were increased much more than that of Control. In the case of Ifng, Il12, Il17a, Il17b, Il17c, Lif and Tnf, there was no difference among their gene expressions induced with LPS, Fr125 and Fr250.
Expression pattern of genes for anti-inflammatory cytokines. The expression of *Il1rn*, an anti-inflammatory cytokine, with LPS, Fr125 and Fr250 treatments was increased by more than fivefold that in Control, and that of *Il1rn* with Fr250 was reduced to 0.39-fold that with LPS treatment. Meanwhile, the expression of *Tgfb3* in Fr125, Fr250, and LPS treatment was reduced to less than 0.35 times that of Control, and there were no differences in *Tgfb3* gene expression among Fr125, Fr250 and LPS treatments.

In the case of the gene expressions of other anti-inflammatory cytokines, such as *Il4*, *Il10*, *Tgfb1*, *Tgfb2*, and *Il11* and *Il13*, which are not listed in Table I, there were no differences among Fr125, Fr250, LPS, LPS+Fr250, and Control.

Expression pattern of non-receptor tyrosine kinases. We compared the expression pattern of genes for several non-receptor tyrosine kinases, such as *Sykb*, *Zap70*, *Jak2*, *Fes* and *Hck*. *Sykb* contains two SH2 domains and binds to the ITAM domain of FcRgamma, such as Fce1g (28, 30), and *Zap70* contains two SH2 domains and binds to the ITAM domain of TCR (CD3) (31). There was no difference in the expression of *Sykb* among Fr125, Fr250, LPS, LPS+Fr250, and Control. The expression of *Fes* with LPS treatment was reduced to less than 50% that of Control. The gene expression of *Hck*, which contains an SH2 domain, an SH3 domain, and a membrane-associated domain, with Fr125, Fr 250, LPS, Fr250+LPS treatments were more than doubled as compared with Control. The gene expression for *Jak2*, which does not contain SH2 domains but binds to Il6 receptor and activates a transcription factor Stat, in LPS treatment was increased by more than fourfold that of Control.

Expression pattern of *Ikb* family genes. The expression of *Ikb* family genes, which are known as negative regulators for transcription factor Nfkβ, were investigated. LPS stimulated the expression of *Ikbβ2* family genes, except for *Nfκbβ2*. Fr125 and Fr250 showed similar effects, but to a much lesser extent (Table I).

Expression pattern of Rel/Nfκb family genes. It is known that the Rel/Nfκb family, such as *Nfκb1*, *Nfκb2*, Rela, Relb and Rel, regulates the expression of cytokines, and chemokines. LPS stimulated the expression of *Nfκb1*, *Nfκb2* and Rel, whereas Fr125 and Fr250 were much less active (Table I).

Conclusion

The present DNA microarray study demonstrated for the first time that lignin–carbohydrate complex from LEM preferentially induced the expression of *Dectin-2* mRNA. This data suggests the involvement of Dectin-2 signaling pathway in the defense mechanism of lignin–carbohydrate complex. Dectin-2 is a specific receptor for α-mannan, and plays significant role in the regulation of protection of the body from *Candida* infection via induction of differentiation of IL-17-producing T-cells (29). It is expected that the activation of Dectin-2 may be significantly affected by the compositional changes in the lignin–carbohydrate complex. Further structure–activity study is underway in our laboratory.

References

3. Terada H, Ohara T, Yamaguchi Y, Ueda T and Asano K: Protective effects of the *Lentinus edodes* mycelia on carbon

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