Normal Human Fibroblasts Express Pattern Recognition Receptors for Fungal (1→3)-β-D-Glucans

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Fungal cell wall glucans non-specifically stimulate various aspects of innate immunity. Glucans are thought to mediate their effects via interaction with membrane receptors on macrophages, neutrophils, and NK cells. There have been no reports of glucan receptors on non-immune cells. We investigated the binding of a water-soluble glucan in primary cultures of normal human dermal fibroblasts (NHDF). Membranes from NHDF exhibited saturable binding with an apparent dissociation constant (K_d) of 8.9 ± 1.9 μM of protein per ml and a maximum binding of 100 ± 8 resonance units. Competition studies demonstrated the presence of at least two glucan binding sites on NHDF. Glucan phosphate competed for all binding sites, with a K_d of 5.6 μM (95% confidence interval [CI], 3.0 to 11 μM), while laminarin competed for 69% ± 6% of binding sites, with a K_d of 3.7 μM (95% CI, 1.9 to 7.3 μM). Glucan (1 μg/ml) stimulated fibroblast NF-κB nuclear binding activity and interleukin 6 (IL-6) gene expression in a time-dependent manner. NF-κB was activated at 4, 8, and 12 h, while IL-6 mRNA levels were increased by 48% at 8 h. This is the first report of pattern recognition receptors for glucan on human fibroblasts and the first demonstration of glucan binding sites on cells other than leukocytes. It also provides the first evidence that glucans can directly modulate the functional activity of NHDF. These results provide new insights into the mechanisms by which the host recognizes and responds to fungal (1→3)-β-D-glucans and suggests that the response to glucans may not be confined to cells of the immune system.

Glucans are (1→3)-β-D-linked polymers of glucose that are part of the outer cell wall of saprophytic and pathogenic fungi as well as certain bacteria (37). Glucans are also released from the microbial cell wall as exopolymers (14, 23, 28, 37). Numerous studies have demonstrated that (1→3)-β-D-glucans will activate a wide array of innate host defenses (4, 50). This is due, in part, to the ability of these carbohydrate ligands to activate a wide array of innate host defenses (1–3, 26, 27), neutrophils (34, 43), and NK cells (7, 43, 44). Binding of (1→3)-β-D-glucan in human and murine monocytes and macrophages is specific, saturable, and susceptible to displacement by other (1→3)-β-D-glucans (3, 26, 27). We have reported the binding and uptake of a variety of water-soluble (1→3)-β-D-glucans and control polymers with different physicochemical properties in order to investigate the relationship between complex polymer structure and receptor binding (26). We observed that there are multiple glucan receptors on human monocytes, that these receptors can distinguish between (1→3)-β-D-glucan polymers, and that large affinity differences (24 μM to 11 nM) exist between glucan polymers derived from various sources (26). We also observed that certain glucans appear to interact nonselectively with glucan binding sites, while other glucans preferentially interact with only one site (26). The physical and/or functional basis for this selectivity among glucan receptors is not clear.

To the best of our knowledge, there have been no reports of glucan-specific binding sites on cells other than leukocytes, nor is there evidence that glucans can directly activate cells other than immunocytes. In this study we investigated whether normal human dermal fibroblasts (NHDF) expressed receptors for glucan and, if so, whether interaction of a highly purified, water-soluble glucan with fibroblasts activated immunoregulatory pathways (NF-κB and NF–interleukin 6 [NF–IL-6]) in immune cells.
tory and/or proinflammatory intracellular signaling pathways. We focused on fibroblasts for several reasons. First, glucans have been reported to accelerate early wound repair (5, 6, 33). Of greater significance, glucans have been shown to increase collagen deposition in rodent skin wounds and intestinal anastomoses (6, 33). We speculated that the effect of glucan on collagen biosynthesis was indirect, i.e., that glucan stimulated macrophage release of wound growth factors which modulated fibroblast collagen biosynthesis (5, 6, 33). While this was a reasonable hypothesis the data did not preclude a direct interaction of the ligand with fibroblasts. Second, fibroblasts are known to respond to microbial recognition patterns, such as lipopolysaccharide (LPS) (29, 38, 39, 46) and lipoteichoic acid (38). Recently, Killcullen et al. (16) have reported that local application of Staphylococcus aureus peptidoglycan increases wound collagen accumulation. van Tol et al. have reported that bacterial peptidoglycan will directly stimulate in vitro expression of collagen α1 (I) and cytokine mRNA from rodent intestinal myofibroblasts (41). Finally, fibroblasts have been reported to express Toll-like receptors, which are crucial signal transducing molecules for LPS, lipoteichoic acid, and other microbial recognition patterns (39, 46). Taken together these data indicate that fibroblasts can recognize and, in some cases, directly respond to macromolecular structures from microbes.

Here, we report the existence of specific glucan binding sites on NHDF. Interaction of fungal glucan with fibroblast membrane receptors increases NF-κB activity and proinflammatory cytokine gene expression. This is the first report of pattern recognition receptors for glucans on cells other than leukocytes.

**MATERIALS AND METHODS**

**Carbohydrate polymers.** Water-insoluble (1→3)-β-D-glucan was extracted from Saccharomyces cerevisiae (49). Insoluble glucan was converted to a water-soluble form as described by Williams et al. (49) and chemically characterized as previously described (11, 19, 24, 25, 49). The final product was stored (0.45-mg/ml) in water-insoluble (1→3)-β-D-glucan phosphate was dissolved, 400 ml (355 mg, 4.8 mmol) of freshly prepared solution of 100 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide and 25 mM N-hydroxysuccinimide. The surface was then exposed to DAP-glucan (3 mg/ml) for 7 min. This cycle of activation with 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide–25 mM N-hydroxysuccinimide and exposure to DAP-glucan phosphate was repeated five times. Immobilization of DAP-glucan phosphate was typically about 2,000 resonance units (RU), or 2 ng/mm². After immobilization, the biosensor surface was exposed to 1 M ethanolamine (pH 8.5) for 6 min to inactivate any remaining succinylated carboxyl groups. The BIAcore biosensor contains four flow cells; DAP-glucan was immobilized on three flow cells, while the fourth flow cell served as a control for nonspecific binding to the dextran surface.

**Saturation experiment using the CM-5 sensor chip.** Membranes prepared from NHDF were suspended at concentrations of 0.012 to 12 µg/ml in HEPES buffer in siliconized vials. A continuous buffer flow of 25 µl/min was used to establish a baseline measurement of RU. Assays were performed at 37°C to approximate physiologic conditions. NHDF membranes were injected for 3 min, followed by continuous flow of HEPES buffer for 3 min to allow dissociation of membranes from the carbohydrate surface. At the end of each cycle, the surface was regenerated by consecutive 1-min exposures to Triton X-100 (0.5%) and guanidine hydrochloride (3 M) at 100 µl/min. Regeneration resulted in displacement of ≥90% of the added RU from the surface.

The BIAcore sensorgram displays the interactions between immobilized DAP-glucan and membrane receptors in real time. During the initial flow of buffer, a baseline is established. When the surface is exposed to NHDF membranes, there is an initial rapid change in the sensorgram due to the change in refractive index of the solution. This occurs in each flow cell, including the control which lacks DAP-glucan. This bulk shift is followed by a concentration-dependent interaction between DAP-glucan and membrane proteins. When the membrane preparation is replaced with buffer flowing over the DAP-glucan surface, there is an equal but opposite bulk shift, followed by dissociation of membrane from the DAP-glucan surface.

**Electrophoretic mobility shift assays.** We employed the gel shift and supershift assays to assess the activation of NF-κB as well as the specificity of binding and the contribution of NF-κB components (p50 and p65) to the activity (2, 3, 45, 47).

Briefly, double-stranded consensus binding site oligonucleotides for NF-κB were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). The oligonucleotides were end labeled with [α-32P]ATP (Amersham, Arlington Heights, Ill.) using T4 polynucleotide kinase (Promega, Madison, Wis.). Binding assays were performed in 10 µl of binding reaction mixture containing 10 µg of nuclear proteins and [32P]-labeled NF-κB oligonucleotides. The binding reaction mixture was incubated at room temperature for 20 min and then electrophoresed...
on 4% nondenaturing polyacrylamide gels. The specificity of binding was confirmed by supershift and competition assays, which establish the specificity of the binding reaction as well as the relative contribution of p50-p65 heterodimer. To assess specificity of binding, a 10-fold excess of cold oligonucleotide was added. As an additional control, a 10-fold excess of cold oligonucleotide bearing the AP-1 binding site was added to separate reaction mixtures. To assess the contribution of the NF-κB components (p50 and p65) to the activity observed, we performed a supershift assay in which antibody to p50, antibody to p65, or antibodies to both p50 and p65 were added to separate reaction mixtures. After polyacrylamide gel electrophoresis, the gels were analyzed by phosphorimaging (Bio-Rad Laboratories, Hercules, Calif.) followed by drying and exposure to Kodak X-Ormat film at −70°C.

**RNA isolation and reverse transcriptase PCR.** Total cellular RNA was isolated from control and glucan-treated human dermal fibroblasts using the Ultraspect-II RNA isolation kit (Biotechn, Houston, Tex.). One microgram of total RNA was used for cDNA synthesis with murine leukemia virus reverse transcriptase (Perkin-Elmer Inc., Braunschweig, N.J.) in a 20-μl final volume. The cDNA synthesis reaction was for 15 min at 42°C and 5 min at 99°C. The reaction mixture (2 μl) was subjected to PCR amplification in a mixture (25 μl) that contained a 1 μM concentration of each of two primers, 1.5 mM MgCl₂, a 0.2 mM concentration of each of four deoxynucleotides, and 1.25 U of Taq polymerase (Perkin-Elmer Inc.). The upstream primer for human IL-6 was GAGAATTTGCTCTCTCTCAC AAAGGG. The downstream primer for human IL-6 was TGGACTGCAGGAA CTCCTT. PCR amplification of IL-6-cDNA was performed under the following conditions: 35 cycles of 45 s at 94°C, 35 s at 54°C, and 45 s at 72°C. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was employed as the gene transcript control. The PCR data were imaged and quantified by computer-assisted densitometry and referenced to the gene transcript control.

**NF-κB and IL-6 protocol.** NHDF were incubated with glucan phosphate (1 μg/ml) for various periods of time. NHDF incubated in medium alone served as the control. At each time point, nuclear protein was harvested from control or glucan-treated NHDF. Parallel cultures of NHDF were harvested for total RNA.

**Data analysis.** In saturation (see Fig. 1) and competition (see Fig. 2 and 3) studies, data were normalized to the baseline established at the start of the experiment and analyzed by unweighted nonlinear regression using Prism 3.0 (GraphPad Software, Inc., San Diego, Calif.). Since the bulk shift occurs over approximately 15 s, we estimated the amount of membrane protein bound to the surface by measuring the increase in RU at 30 s after changing from membrane to buffer exposure. For saturation experiments in which the DAP-glucan phosphate surface was exposed to various concentrations of membrane protein, RU values for each concentration of analyte were analyzed using the model RU = (RUₘₐₓ × [analyte])/(Kᵥ + [analyte]) + Kᵥₙₐₛₙₛ × [analyte], where RUₘₐₓ is the maximum binding, Kᵥ is the apparent dissociation constant, Kᵥₙₐₛₙₛ is the constant of linear nonspecific binding, and [analyte] is the protein concentration. Kᵥ values are also accompanied by 95% confidence intervals (CI) for the apparent Kᵥ.

For competition experiments in which the DAP-glucan phosphate surface was exposed to a fixed concentration of membrane protein in the absence or presence of competitor, RU values for each competitor concentration were further normalized to binding in the absence of competitor (100%) and analyzed using models for competitive displacement at a single binding site, a single binding site plus nonspecific binding, and/or two binding sites. The best model was chosen statistically using the sequential F test.

Mean values for NF-κB binding activity at time points up to 48 h (see Fig. 4) were compared by one-way analysis of variance and the least-significant-difference procedure. Probability levels of 0.05 or smaller were considered statistically significant.

**RESULTS**

NHDF membranes bind to a glucan-coated biosensor surface. The NHDF membranes were injected over a concentration range of 0.012 to 12 μg of protein per ml. This was followed by exposure of the surface to a continuous flow of buffer (3 min) to allow dissociation of the analyte from the ligand. The bulk shift occurs over 15 s in the control flow cell, so we chose to measure responses 30 s after the end of the analyte injection (Fig. 1). The binding response as a function of the NHDF membrane protein concentration was proportional to the immobilized DAP-glucan attached to the flow channels. The apparent Kᵥ was 8.9 ± 1.9 μg of protein/ml, and the maximum binding was 100 ± 8 RU. Nonspecific binding was not significant at the protein concentrations which were used.

**Glucan phosphate completely inhibits binding of NF-κB to a glucan-coated sensor surface.** Glucan phosphate completely inhibited the binding of NHDF membranes to a glucan-phosphate biosensor surface with characteristics of a single binding site (Fig. 2). The Kᵥ for the inhibition was 5.6 μM (95% CI, 3.0 to 11.0 μM), corresponding to a concentration of 0.88 μg of glucan phosphate per ml.

Laminarin partially inhibits binding of NHDF membranes to a glucan-coated sensor surface. Using a cell-based receptor ligand assay, we have observed that laminarin will bind to a subset of (1→3)-β-D-glucan receptors on the human U937 monocyte cell line (26). We have confirmed and extended this observation using surface plasmon resonance and U937 membranes (E. Lowe et al., submitted for publication). In the present study, laminarin partially inhibited the binding of NHDF membranes to the glucan phosphate biosensor surface with characteristics of a single binding site (Fig. 3). Laminarin could inhibit only 69% ± 6% of the binding of NHDF membranes to glucan phosphate. The Kᵥ for the inhibition by lamia-
narin was 3.7 μM (95% CI, 1.9 to 7.3 μM), corresponding to a
concentration of 0.028 μg/ml. The failure of laminarin to completely inhibit the interaction of NHDF and immo-
ibilized DAP-glucan phosphate suggests the presence of two types of
binding interactions, one of which is not inhibitable by laminarin.

Coincubation with glucan phosphate increases NHDF NF-κB activity and IL-6 mRNA expression. Glucan stimulated NF-κB nuclear binding activity and IL-6 mRNA expression in a time-
dependent manner. NF-κB was activated at 4 (102%), 8 (131%), and 12 (85%) h (Fig. 4). NDHF were treated for 8 h
with glucan phosphate (1 μg/ml). Reverse transcriptase PCR
data indicate that IL-6 mRNA levels were increased by 48% at
8 h (Fig. 5).

DISCUSSION

A number of significant observations have emerged from
this study. First and foremost, we found that NDHF express
membrane receptors for (1→3)-β-D-glucans. To the best of our
knowledge, this is the first report of glucan-specific receptors
on cells other than leukocytes. Second, interaction of glucan
with membrane receptors on NDHF activates NF-κB, an im-
portant intracellular signaling pathway which is associated with
regulation of cytokine and chemokine gene expression. This is
consistent with previous studies which have shown that glucans
stimulate transcription factor activation in macrophages and
neutrophils (1–3). Third, glucan receptor interactions in
NHDF resulted in increased IL-6 mRNA expression. Previous
studies have reported increased cytokine gene upregulation in
macrophages treated with glucans (13, 18, 31). However, other
studies have reported activation of immunocyte NF-κB by glu-
cans without cytokine gene upregulation (45), while still other
investigators have reported cytokine downregulation in re-
response to glucan and proinflammatory stimuli (36, 47, 48). It is
not clear whether this relates to the glucan used, the cytokines
examined, the cells or tissues studied, or other factors.

We observed that binding of glucan by NHDF membranes
was saturable, dose dependent, and specific. Competition for
this interaction by glucan phosphate was complete and had a
$K_D$ of 5.2 μM (95% CI, 0.8 to 7.1 μM) for the human U937
promonocytic cell line (unpublished observation). Interest-
ingly, laminarin competition for this interaction was able to
displace a maximum of 69% \pm 6% of the interaction, with a $K_D$ of 3.7 \mu M (95% CI, 1.9 to 7.3 \mu M). Laminarin is equipotent with glucan phosphate based on molar concentration, but its lower molecular weight makes it more potent (28 versus 880 ng/ml). The fact that laminarin cannot completely inhibit the interaction of NHDF membranes with immobilized glucan phosphate suggests the presence of at least two different binding sites for glucan phosphate on NHDF membranes. Laminarin selectively interacts with one site, while glucan phosphate interacts with both sites. We have reported similar results using the human U937 promonocytic cell line, where laminarin displaced 61% \pm 4% of binding, with a $K_D$ of 2.6 \mu M (95% CI, 1.7 to 4.2 \mu M), and glucan phosphate completely inhibited binding in a dose-dependent manner (Lowe et al., submitted). The similarity in the affinities of these interactions in human promonocytes and fibroblasts suggests that similar receptors are present in both cell types. The data also strengthen the contention that there are multiple binding sites for glucans.

While there are numerous reports that monocytes, macrophages, neutrophils, and NK cells express membrane pattern recognition receptors for ($1\rightarrow3$)-\beta-D-glucan, the precise nature of the glucan receptor(s) is the subject of controversy. Di Renzo et al. (7), Thornton et al. (40), and Vetvicka et al. (43) have reported that the type 3 complement receptor (CR3 [also known as CD11b or CD18]) is a glucan binding site on macrophages, neutrophils, and NK cells. The glucan binding is reported to be through one or more lectin sites located outside the CD11b I domain (40, 43, 44). Duan et al. (8), Di Renzo et al. (7), and Vetvicka et al. (43, 44) have reported a \beta-glucan binding lectin on NK cells which contributes to NK cell-mediated cytotoxicity. Zimmerman et al. reported that lactosylceramide binds PGG-glucan (a proprietary glucan) and that this glycosphingolipid may be a leukocyte glucan binding moiety (52). Dushkin et al. (9) and Vereschagin et al. (42) have reported that a carboxymethylated glucan binds to the macrophage scavenger receptor. We have reported the presence of two glucan binding sites on U937 cells which stimulate intracellular signaling pathways culminating in the activation, translocation, and nuclear binding of immunoregulatory and proinflammatory transcriptional activator proteins (3, 26). Our data suggest that neither of these sites is CR3 (26). Michalek et al. have extended this observation by reporting that PGG-glucan also binds to a site distinct from CR3 (22). Whether PGG-glucan and the glucans described in this study bind to the same site is not known. CR3 is a \beta integrin which is leukocyte restricted (35), and it is involved in the recognition of microbial molecular patterns, such as LPS (32). However, fibroblasts have not been reported to express CR3, and thus the binding and functional activation of fibroblasts by glucan cannot be attributed to a CR3-dependent mechanism. This does not diminish the potential importance of CR3 as a leukocyte binding moiety for glucans; rather, it reinforces the notion that there are multiple glucan binding sites and it indicates that glucan receptors are not sequestered solely in leukocytes, suggesting that these receptors may be more widespread than previously thought. Whether there are glucan receptors on cells other than fibroblasts, macrophages, neutrophils, and NK cells remains to be established. In addition, it is not clear whether the two binding sites which we have identified on human monocytes and fibroblasts activate the same or different signaling pathways within the cell. We are currently investigating both of these questions.

In conclusion, we have identified at least two specific glucan binding sites on NHDF. The interaction of the glucan ligand with NHDF results in the activation of proinflammatory intracellular signaling pathways and upregulation of cytokine gene expression. This is the first report of a glucan binding site on cells other than leukocytes. The potential ramifications of these data are significant because they force us to reexamine the current hypotheses regarding the mechanisms by which both recognize and respond to these fungal cell wall carbohydrates. By way of example, glucans have been reported to exert a plethora of nonspecific effects on immune function (4, 50). The presumed mechanism was that glucans interact with leukocytes and other elements of innate immunity, resulting in either a primed or activated state (1, 12, 36). The systemic effects were attributed to release of proinflammatory and/or immunoregulatory mediators which serve as second messengers, i.e., the systemic effects were indirect. While this is a reasonable explanation for the observed effects, the present data suggest that glucans may also directly interact with and modify the functional state of cells such as fibroblasts. Since fibroblasts are present in many organ systems, it is reasonable to speculate that some of the nonspecific effects which have been ascribed to glucans may be more direct than previously thought.

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