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Inhibitory Effect of Fucoidan on the Adhesion of Adenocarcinoma Cells to Fibronectin

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Abstract. *Fucoidans inhibit tumour cell adhesion to various substrata, but their mechanisms of action are not fully understood. Using ^{3}H-fucoidan, we observed that fucoidan binds to fibronectin, this binding being saturable and sensitive to ionic strength and pH. The interaction occurred on at least four different sites along the polypeptide chain, two of them being the heparin-binding sequences. Moreover, when MDA-MB-231 tumour cells were exposed to DTAF–fucoidan, internalization occurred and punctuated vesicles were observed in the perinuclear region. The treated cells also showed a different morphology with a cytoskeleton devoid of vinculin and a reorganiztion of the repartition of the integrin-α5 subunit on the cell surface. Based on these data, we hypothesize that fucoidan inhibits the adhesion of MDA-MB-231 cells to fibronectin i) by blocking the protein's heparin- and cell-binding domains, ii) by modulating the reorganization of the integrin α5 subunit and iii) by down-regulating the expression of vinculin.*

Several polysaccharides have been shown to be potent inhibitors of experimental metastasis (1-4). Among them, fucoidans are able to inhibit metastasis of rat mammary adenocarcinoma 13762MAT by interfering with the passage of tumour cells across the vascular endothelium (1-3). They also disrupt the adhesion of cancer cell lines to different substrata in a dose-dependent and non-toxic fashion (1, 5-7). Using standard adhesion and chemo-invasion assays, we previously demonstrated that fucoidans are able to inhibit MDA-MB-231 cell invasion through matrigel (7), their inhibitory activities being correlated to their charge density, their molecular weight and their carbohydrate structure (5, 7).

Cell surface receptors belonging to the integrin family have been demonstrated to be involved in the invasion and the metastasis of tumours. Usually, cell adhesion to fibronectin involves the most common integrin $\alpha_5\beta_1$ (8) and cell surface proteoglycans. Several types of polysaccharides including heparin have already been demonstrated to inhibit the attachment of cells to a fibronectin-coated surface, mostly through a direct interaction with the adhesive protein (9).

In this study, we attempted to determine the precise cellular mechanism involved in the inhibitory activity of fucoidan, how this polysaccharide acts on the interaction between cells and the extracellular matrix (ECM) and, more precisely, fibronectin. We proposed that fucoidan inhibits cell adhesion to the ECM by blocking the fibronectin cell-binding domain. Its direct interaction with the cell membrane also modulates cell-matrix adhesion through the reorganization of the integrin α_5 subunit on the surface rather than by down-regulation of the expression of this protein.

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Materials and Methods

Materials. Crude heparin (batch H108, 173 IU/mg) was provided by Choay-Sanofi Laboratories, France. High molecular weight (HMW) fucoidan, extracted from the brown seaweed *Ascophyllum nodosum*, was prepared according to the method of Colliec *et al.* (10). Tritiated polysaccharides were prepared as previously described with [^{3}H] sodium borohydride (11). Biotin-labelled and 5-([4,6-dichlorotriazin-2-yl]-amino) fluorescein (DTAF)-labelled polysaccharides were prepared using the procedure developed by Soeda *et al.* (2) and Prigent-Richard *et al*. (12), respectively. Fibronectin was purchased

from Becton-Dickinson (Le Pont de Claix, France). The R-phycoerythrin (R-PE) -conjugated mouse anti-human monoclonal antibody against the α_5 and the R-PE -conjugated mouse anti-human monoclonal antibody against the β_1 integrin subunits, the R-PE-conjugated mouse anti-human IgG and the 7-amino-actinomycin D (7-AAD - VIA-probe™) were purchased from Pharmingen International (Le Pont de Claix, France). The mouse anti-human monoclonal antibodies against vinculin were from Novo Castra Laboratories (Paris, France). The Alexa Fluor® 488 goat anti-mouse IgG conjugate and the FITC conjugated-phalloidin were purchased from Molecular Probes (Montluçon, France). All other chemicals, unless otherwise indicated, were purchased from Sigma Chemical Co. (St Quentin Fallavier, France). The human breast adenocarcinoma cell line MDA-MB231 was obtained from Pr. F. Calvo (Hôpital Saint Louis, Paris, France). All culture plastics were from Corning Costar (Rixheim, France).

Western blot analysis. Thermolysin digestion of human fibronectin was performed according to Zardi *et al*. (13). Samples containing 30μg of degraded fibronectin were reduced by 5 mM dithiothreitol (DTT) and separated by SDS-PAGE 7.5% and 12.5%, according to Laemmli (14). The proteins were transferred electrophoretically to nitrocellulose sheets and stained with Ponceau Red to detect the protein fragments. Destained nitrocellulose sheets were blocked with 5% non-fat dry milk for 1 h and then incubated with the biotinylated fucoidan or biotin-heparin for 1 h. Fibronectin fragments bound to biotin-polysaccharides were revealed using an alkaline phosphatase-based assay by chemiluminescence and autoradiographed on Kodak film.

Fucoidan-MDA-MB-231 cell interactions. Two x 10^5 cells were incubated for 1 h at 4˚C in PBS containing different concentrations of DTAF-sulphated polysaccharides. After 2 washing in cold phosphate-buffered saline (PBS), the cells were resuspended in 500 μl of ice-cold PBS and analysed using a flow cytometer (Epics-XL, Beckman Coulter). The fluorescent intensity of 10^5 cells was evaluated and the data were expressed as the specific channel fluorescence intensity. Non-specific binding was determined with 100-fold excess of unlabelled polysaccharides and specific binding was calculated for each point by subtracting non-specific binding from the total binding. The results were analysed by Scatchard plot analysis. The internalization of polysaccharides into cells was studied using DTAF-fucoidan, as previously described (15).

Integrin expression on the cell surface. Cells were incubated at 37˚C with different concentrations of fucoidan for 20 min, 1 h, 2 h or 24 h. After washing with cold PBS, the cells were incubated for 30 min at 4˚C with R-PE anti-human α_5 (0.06 μg), anti-β_1 (2 μg) or PE-IgG2a as an isotype control. The expression of the α_5 or β_1 integrin subunits in fucoidan-treated and untreated MDA-MB-231 cells was analysed by flow cytometry (Epics-XL, Beckman Coulter).

Immunofluorescence and confocal laser imaging. Two x 10^5 cells were allowed to adhere to a fibronectin-coated Lab-tek chamber for 20 min, 2 h, 8 h or 24 h, in the presence or in the absence of fucoidan (500 μg/ml). After washing, they were fixed with 4% paraformaldehyde for 20 min and washed twice with PBS. Then the cells were incubated overnight in the dark at 4˚C with R-PE-conjugated anti-α_5 (0.06 μg) or anti-β_1 (2 μg) antibodies in PBS. Actin fibres were revealed using 5 Units/ml of FITC-conjugated phalloidin.

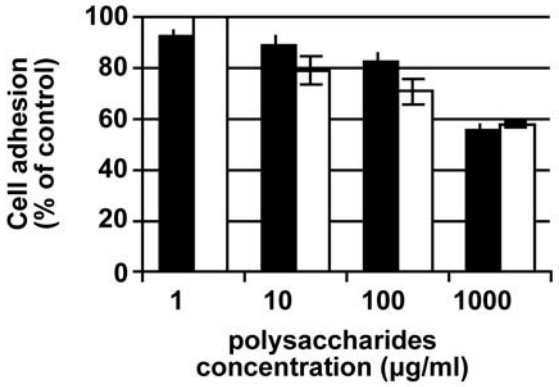

Figure 1. *Effects of polysaccharides on MDA-MB-231 cell adhesion to fibronectin-coated wells. MDA-MB-231 cells (30,000 cells) were added to the substrate with or without polysaccharide: native fucoidan (■) or heparin (□). After 20-min incubation in adhesion buffer, unbound cells were washed away and the attached cells determined using colorimetric assay. Results are presented as percentage of cells attached to fibronectin compared to the control, normalized to 100% in the absence of polysaccharide (mean ± SD, n=3).*

For studying the effect of fucoidan on vinculin, cells were seeded in Lab-tek chambers previously coated with fibronectin for 1 h. Incubation with the anti-vinculin antibody, used at a final dilution 1:20, was performed overnight in the dark at 4˚C. After three washes with PBS, the anti-vinculin antibody was visualized using a Fluor® 488 anti-IgG (1:50 in PBS). Then, all samples were rinsed in PBS and mounted in Mowiol (Calbiochem, Meudon, France). The cells were observed under confocal microscopy (Leitz, Germany).

Results

Fucoidan inhibits cell attachment on fibronectin. Fucoidan was able to inhibit MDA-MB-231 cells adhesion to fibronectin in a dose-dependent manner (Figure 1). This effect was rapid and appeared within the first 10 min of contact, as previously described (5, 7), and it was related to the sulphate content and the molecular weight, since the desulphated fucoidan and a low molecular weight fraction exhibited a weaker effect than the native molecule (data not shown).

Fucoidan binds to fibronectin with high affinity. We first confirmed, by solid-phase binding assays, that fucoidan binds to fibronectin in a dose-dependent and saturable manner and with a higher affinity than heparin (Kd = 1.3 nM *versus* 340 nM, respectively). This interaction was sensitive to ionic strength, pH and sulphate content, but relatively insensitive to temperature. We next analysed the polypeptide pattern of fibronectin degradation products (FDP) bound to biotinylated fucoidan compared to that of heparin. As shown in Figure 2, biotinylated fucoidan reacted

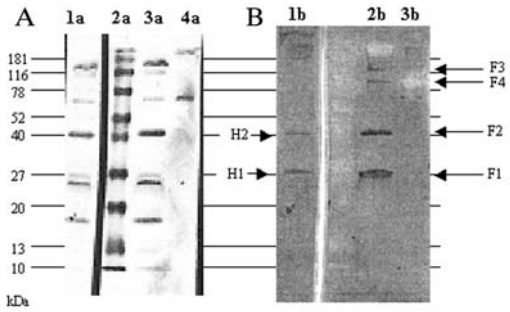

Figure 2. *Comparison of fucoidan-binding with heparin-binding to proteolytic fragments of fibronectin. Panel A: 12.5% SDS-PAGE (reducing conditions) analysis of the thermolysin-digested fibronectin fragments. Lanes 1a and 3a: Fibronectin degradation products produced by incubation of native fibronectin with thermolysin for 4 h; lane 2a: Molecular weight standards; lane 4a: Native fibronectin (control). Panel B: Lane 1b: Fibronectin degradation products incubated with biotinylated heparin; lane 2b: Fibronectin degradation products incubated with biotinyled HMW fucoidan; lane 3b: Native fibronectin incubated with biotinylated fucoidan.*

with two fragments of 29kDa (F1) and 38kDa (F2), which are heparin-binding domains common to HepI (H1) and HepII (H2). Fucoidan also interacted with others fragments, F3 and F4, of 70 to 116kDa respectively, which very probably represent the cell-binding domains.

Fucoidan blocks cell spreading on fibronectin. To determine whether fucoidan interferes with stress fibre formation, we compared the actin organization in MDA-MB-231 cells on fibronectin after various incubation times. Extensive control cell spreading occurred on fibronectin and actin filaments stretched across the entire cell area (Figure 3A). When cells were plated on fibronectin for 20 min to 2 h, in the presence of 500 µg/ml fucoidan, minimal cell spreading occurred and the extensive array seen with control cells was not present (Figure 3B). This effect was still observed after 24 h (data not shown).

Labelling with the anti-vinculin antibody showed that vinculin was present in the cell with typical spots along the membrane. After 2 h of adhesion, a typical intense labelling of vinculin was observed at the periphery of both non-treated (Figure 4A) and, to a lesser extent, of treated cells (Figure 4B). However, when cells were pre-incubated with fucoidan (500 mg/ml) 1 h prior to the adhesion assay and further seeded with fucoidan at the same concentration for 2 h, a lack of vinculin in the lamellae was observed (Figure 4C).

Fucoidan binds to MDA-MB 231 cell membrane and is internalized. Using fluorescent DTAF-fucoidan that retains its full inhibition activity on MDA-MB-231 cells adhesion, analysis of the binding data by the Scatchard method indicated

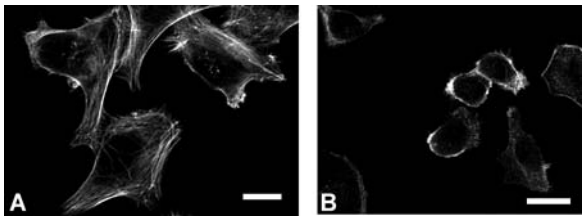

Figure 3. *Actin organization in MDA-MB-231 cells on fibronectin, in the presence or the absence of native fucoidan. Cells were plated for 8 h in the absence (A) or presence (B) of 500 µg/ml fucoidan! Actin fibres were stained by FITC-phalloidin conjugation and observed by confocal microscopy. Scale bar, 30µm. Cells in A are representative of cells with well-organized stress fibres in quantitative stress fibre assays.*

the presence of two specific binding sites, one being highly specific for fucoidan. The major site analysis indicated a Kd of 4 ± 0.5 µM, with 4.8 x 10^{10} sites/cell (n=3) for fucoidan, which is lower than for heparin (Kd 51 µM and 10^8 sites/cell).

We also observed, by confocal microscopy, that fucoidan bound rapidly to the cell membrane and was internalized within the first hour of contact in a time-dependent manner (data not shown). Increasing incubation times resulted in an increased fucoidan internalization. After 24 h of incubation, DTAF-fucoidan was found in endocytotic vesicles that became localized in the perinuclear region (data not shown).

Fucoidan has no effect on α_5 and β_1 integrin subunits expression. By flow cytometry, we measured the expression of the α_5 and β_1 integrin subunits on cells grown in the presence of fucoidan, from 20 min to 24 h. No additional shift was observed when cells were treated with fucoidan for various incubation times and for both integrin subunits studied (data not shown).

Fucoidan compromises α_5 subunit distribution on MDA-MB-231 cell membrane. The distribution of the α_5 subunit appeared to be modified by the fucoidan treatment where they form clusters at the periphery of the cells. In control cells, labelled α_5 formed vesicles that appeared near the nucleus after 20 min (Figure 5A) and then migrated to the periphery of the cell following 2 h of adhesion (Figure 5C). The distribution of the α_5 integrin subunit appeared modified by the fucoidan treatment (Figures 5B and 5D). During the first 20 min of adhesion, fucoidan-treated cells remained round and did not spread (Figure 5B). After 2h, labelled α_5 integrin was present only near the nucleus (Figure 5D). After 8h to 24h, treated-cells did not differ from the control (data not shown). With regard to the β_1 integrin subunit, we observed that fucoidan-treated cells exhibited the same distribution of β_1 at the extremity of the cells as non-treated cells, at all time-points of adhesion examined (data not shown).

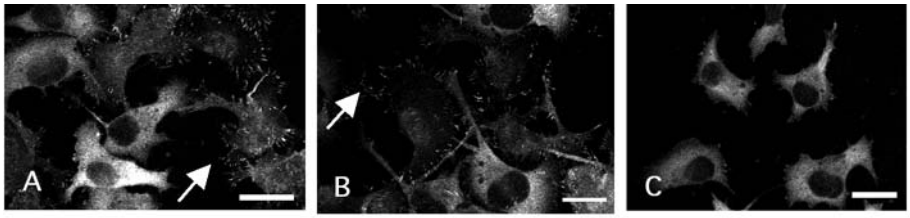

Figure 4. *Effects of fucoidan on the vinculin localization in MDA-MB-231 cells adhering to the fibronectin. Untreated (panel A) and pre-treated for 1 h with 500 μg/ml of fucoidan (panel B), cells were allowed to adhere to the fibronectin for 2 h in the DMEM. MDA-MB-231 cells were also previously incubated with labelled fucoidan for 1 h before adhesion assays (panel C). Scale bar = 30 μm.*

Discussion

Fucoidans are high molecular weight fucose-based polysaccharides extracted from brown algae (4). They interact with many proteins, including growth factors, chemokines and structural proteins of the ECM to influence cell growth, differentiation and the cellular responses to the environment (4, 16). We reported previously that a fucoidan fraction extracted from *Ascophyllum nodosum* was able to inhibit MDA-MB-231 cell invasion through matrigel (7). This inhibitory effect on cell invasion was caused, at least in part, by the blockage of tumour cell adhesion to the ECM and by the increase of the proteolysis of the extracellular membrane (5, 7). Moreover, this fucoidan did not have any effect on cell proliferation.

In the current study, we attempted to elucidate how fucoidan can reduce the interaction between the tumour cell and the ECM. Because its pre-incubation with ECM proteins prior to addition into the adhesion assay system alters the amplitude of the adhesive response, we considered the possibility that fucoidan blocks cell attachment on fibronectin by masking one of its cell-binding sites previously located within the third heparin-binding domain (17). We first confirmed, in a solid-phase assay, that fucoidan binds to fibronectin, in a saturable manner, as revealed by Scatchard analyses (Kd of 1.3nM), this affinity being 250 times higher than that of heparin. These results are in good agreement with previous data showing that neither heparin, nor chondroitin sulphate or crude dextrans, were able to displace fucoidan bound to fibronectin (7). This interaction is specific and arises from a direct binding to the heparin-binding sequences HepI and HepII and probably the cell-binding domain in fibronectin, although it has not been demonstrated. Further investigation would be useful to confirm this hypothesis, using fibronectin recombinant fragments. Considering the possibility that the anti-adhesive activity of fucoidan stems from its ability to form ionic complexes with fibronectin and alteration of the secondary structure of the protein, we determined the CD spectra of fibronectin incubated with fucoidan using circular dichroism analyses. We observed that fucoidan did not modify the conformation of fibronectin (α-helix domain) in our experimental conditions like heparin (18) (data not shown).

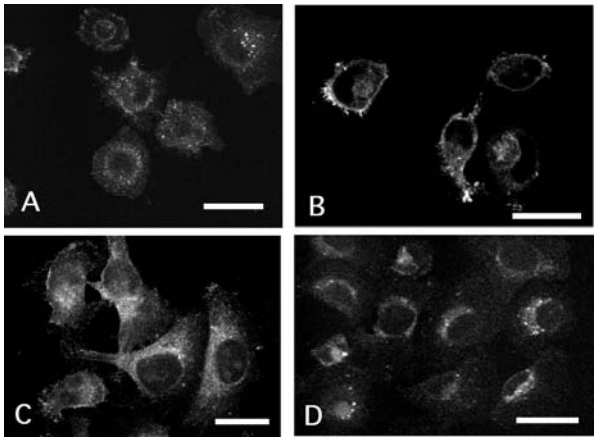

Figure 5. *Effects of native fucoidan on integrin α_5 distribution on MDA-MB-231 cell surface. Either untreated (left lane A and C) or pre-treated (right lane B and D) for 1 h with 500 μg/ml of native fucoidan, the cells, were allowed to adhere to the fibronectin for 20 min in the adhesion buffer (A and B) or 2 h in DMEM (C and D). The distribution of the α_5 integrin subunit was revealed by direct immunofluorescence and was observed by confocal microscopy. Scale bar = 30 μm.*

Fluorescence microscopy of MDA-MB-231 cells cultured on fibronectin in the presence of fucoidan showed a lesser spread morphology as compared to the morphology of non-pretreated cells. Our data suggest involvement of fucoidan in the observed reorganization of the integrin α_5 subunit on the surface of the cells, which constitutes, with the β_1 subunit, the major fibronectin receptor that recognizes the RGD located within the cell-binding domain of fibronectin. As a matter of fact, untreated cells displayed vesicles containing the α_5 subunit located near the nucleus after 20 min of adhesion, which migrated to the periphery of the cell as shown after 2 h of adhesion. In contrast, the distribution of the α_5 subunit appeared modified by the fucoidan treatment, where clusters were formed at the periphery of

the cells even after 2-h incubation. In addition, no changes in the quantitative expression of both the α_5 and β_1 subunits were detected. Further studies are underway to clarify the molecular mechanisms underlying this activity, studying the interaction of fucoidan with the proteins involved in the integrin-mediated cell adhesion like filamin and talin, since these proteins interact with the β_1 integrin cytoplasmic domain and are instrumental in the formation of focal adhesion complexes and cell spreading.

The fact that fucoidan interferes with MDA-MB-231 cell adhesion and spreading on fibronectin suggests that the integrin signalling pathway of the cell is abrogated. This is supported by our observation that fucoidan compromises focal contact and stress fibre formation. We found that DTAF-fucoidan interacts with membrane sites in a saturable manner and is internalized within the first hour of contact. By fluorescent microscopic studies, we also observed that fluoro-labelled fucoidan was incorporated into endocytotic vesicles, which migrated to the perinuclear region, suggesting an important role of this compound at this site. Further investigation will be useful to determine whether fucoidan is able to compete with one or several of the cell-associated sulphate proteoglycans, like the transmembrane syndecans, for binding to fibronectin and to displace them from cells.

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