Stress-induced phosphorylation of caveolin-1 and p38, and down-regulation of EGFr and ERK by the dietary lectin jacalin in two human carcinoma cell lines

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Abstract We have examined the A431 (human epidermoid carcinoma) and HT29 (human colorectal carcinoma) cellular responses evoked by lectins of dietary origin, Jacalin of *Artocarpus integrifolia* (native jacalin; nJacalin), peanut agglutinin (PNA) of *Arachis hypogaea*, and recombinant single-chain jacalin (rJacalin), which has the same protein backbone but ~100-fold less affinity for carbohydrates than nJacalin. All three lectins (nJacalin, rJacalin, and PNA) are cytotoxic inhibitors of proliferation of A431 cells. However, cells recover once jacalin but not PNA have been removed from the growth medium. Treatment of nJacalin results in morphologically visible cell rounding while retaining the membrane integrity when treated at 40 $\mu\text{g ml}^{-1}$, but treatment with PNA did not induce such changes. The observed cell rounding was found to be due to stress as the phosphorylation of caveolin-1 (at tyr^{14}), p38 but not c-Jun N-terminal kinase were up-regulated, while PNA did not up-regulate the phosphorylation of the same. Jacalin also down-regulated the phosphorylation of the epidermal growth factor receptor and extracellular signal regulated kinase in contrast to PNA, which failed to down-regulate the same. Confocal microscopic studies reveal that jacalin is not internalized, unlike the lectin of *Agaricus bisporus*. Analysis of the proteins that bind to an nJacalin-sepharose column revealed the binding of six to eight proteins, and significant among them is a protein at ~110 kDa, which appears to be oxygen-regulated protein 150 (ORP150) (endoplasmic reticulum chaperone) as identified by its isoelectric point, two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis and mass spectrometric analysis. This 110-kDa band is detectable with anti-Hsp70 antibody because ORP150 has homology with Hsp70. Confocal microscopic studies reveal the presence of Hsp70-like proteins on the surface of A431 cells as revealed by immunostaining with anti-Hsp70 antibody. Moreover, overexpression of ORP150 in A431 cells has resulted in a dramatic protection of A431 cells against jacalin-induced toxicity, confirming that the jacalin-induced cytotoxicity is mediated through ORP150, and impairment of ORP150 functions with the help of jacalin makes the cells more susceptible to death due to stress. Our studies suggest that the cellular responses, as a consequence of lectin binding, may not be exclusively mediated by carbohydrate binding property alone, but other factors such as protein-protein interactions may also contribute to the observed cellular responses.

INTRODUCTION

To date, several plant lectins have been exploited to understand the nature of glycosylation on cell surface molecules due to their distinct carbohydrate binding property, while the consequences of their binding to mam-

malian cell surfaces is beginning to be understood in detail. It has been noted that plant lectins also affect the proliferation of cells, probably through binding to specific carbohydrate moieties, thereby affecting the function of mammalian cells (Sanford and Harris-Hooker, 1990; Yu et al 1993, 2001; Ryder et al 1994a, 1998; Jordonson et al 1999; Kelsall et al 2002; Batterbury et al 2002). Although several lectins have been shown to be mitogenic to T cells, or B cells, or both, the effect of the lectins on cells of other

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lineage vary. For example, peanut agglutinin (PNA) stimulates proliferation of human intestinal epithelial cells both in vitro and in vivo (Ryder et al 1992, 1994b, 1998), whereas native jacalin (nJacalin) from *Artocarpus integrifolia* seeds and the lectin of *Agaricus bisporus* (ABL) (edible mushroom) are noncytotoxic inhibitors of proliferation of human colon cancer cells (Yu et al 1993, 2001). *Vicia faba* agglutinin stimulates differentiation of undifferentiated LS174T human colon cancer cells. Native jacalin is mitogenic to T cells and has been shown to up-regulate extracellular signal regulated kinase (ERK) phosphorylation in CD4⁺ T cells (Pineau et al 1990; Blasco et al 1995; Tamma et al 2003), although it is a noncytotoxic inhibitor of proliferation of HT29 cells and it also down-regulates ERK phosphorylation in the same cells (Yu et al 2001, 2004). If one compares the different cellular responses of PNA, ABL, and nJacalin on HT29 cells, even though these lectins have nearly the same sugar specificity (ie, toward the human malignancy-associated Thomsen-Friedenreich disaccharide [TF disaccharide: Gal β 1-3GalNAc α]), their biological effect on the same cell differs. This suggests that the effect of plant lectins on the cellular response may be governed by other factors in addition to their sugar-binding property.

In this regard, the study of plant lectins with respect to understanding mammalian cell signaling pathways appears to be necessary, because plant lectins are an abundant part of the human diet. It has been observed that some lectins resist digestion and may also remain active in the colon, whereas some lectins affect the function of gastrointestinal cells (Brady et al 1978; Jordinson et al 1999). These observations raise several important questions: Why do the same cells respond differently to different lectins despite similar carbohydrate specificity? Is there any role for a protein backbone in the elicited cellular response? Is the cellular response merely due to stress, and if it is, what are the pathways and molecules involved? A detailed understanding of cellular stress orchestrated by plant lectins on mammalian cells is required to delineate the pathways that will shed light on cellular stress and its eventual consequences (ie, to survive, proliferate, or die).

The present study examines the effect of jacalin on A431 and HT29 cells. To investigate the effects on cellular signaling, we chose nJacalin, recombinant jacalin (rJacalin, which has about 100-fold less affinity for target carbohydrates in comparison to nJacalin), and PNA. We examined the effects of all three lectins on cell proliferation, membrane integrity, and phosphorylation status of stress markers such as caveolin-1 and p38, and c-Jun N-terminal kinase (JNK) along with epidermal growth factor receptor (EGFr) phosphorylation, which is responsible for proliferation. Our studies indicate that the jacalin lectin exerts reversible stress on A431 cells (ie, it induces the

phosphorylation of caveolin-1 and p38 but not JNK, whereas PNA, which has very similar specificity to that of jacalin, did not induce the same). Our results suggest that the jacalin-modulated effects might be due to other factors apart from its sugar-binding property.

MATERIALS AND METHODS

All the reagents used were of analytical grade and all experiments described here were carried out independently at least three times. ORP150 construct in pCINEO vector was a generous gift from Dr Kentaro Ozawa, Department of Neuroanatomy, Kanazawa University Medical School, Ishikawa, Japan. Human recombinant transforming growth factor- α (TGF α), fetal bovine serum (FBS), and methyl- α -galactose were obtained from Sigma (St Louis, MO, USA). Dulbecco modified Eagle medium (DMEM) was from GibcoBRL, Life Technologies (Gaithersburg, MD, USA). Anti-EGFr (sc-120) mouse monoclonal antibody, anti-EGFr (sc-03) rabbit polyclonal antibody, anti-ERK1 (sc-94) rabbit polyclonal antibody, anti-phospho-ERK (sc-7383) mouse monoclonal antibody, anti-phospho-p38 (sc-7973) mouse monoclonal immunoglobulin M (IgM) antibody, anti-p38 (sc-535) rabbit polyclonal antibody, anti-p-JNK (sc-6254) mouse monoclonal antibody, anti-JNK2 (sc-7345) mouse monoclonal antibody, anti-p-Tyr (sc508) rabbit polyclonal antibody, goat anti-mouse IgM horseradish peroxidase (HRP; sc-2064) antibody, mouse anti-rabbit IgG HRP (sc-2357), anti-Hsp70 (K-20) goat polyclonal antibody (sc-1060), anti-goat IgG HRP (sc-2020) antibody, and anticaveolin-1 (sc-894) rabbit polyclonal antibody used for immunodetection were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated anti-mouse antibody and a chemiluminescence detection kit were purchased from New England Bio-Labs. Anticaveolin-1-phospho-tyr¹⁴ mouse monoclonal antibody was from BD Biosciences. Recombinant protein G agarose beads (Invitrogen, Life Technologies). Vector shield antifade mounting medium (Vector Laboratories, Burlingame, CA, USA) protein estimations were carried out with a Bradford protein estimation kit from Bio-Rad (Hercules, CA, USA).

Purification of nJacalin, rJacalin, and PNA

Native jacalin (*Artocarpus integrifolia*) lectins from jackfruit seed as well as PNA from peanut (*Arachis hypogaea*) were purified by affinity chromatography as described earlier (Agrawal and Goldstein, 1967; Sastry et al 1986; Sastry and Suroliya, 1986), and rJacalin was purified as described by Sahasrabuddhe et al (2004).

Cell culture

A431 cells were cultured in DMEM buffered with 2.5 mM *N*-(2-hydroxyethyl) piperazine-*N'*-2-ethanesulfonate (HEPES) supplemented with 5% fetal calf serum and antibiotics (streptomycin sulfate and penicillin G).

Development of A431-ORP150 cell line

The A431-ORP150 cell line was established by selection of stable transfectants (pCI-ORP150 plasmid) and G418 by the lipofectamine method and selected with G418 (1200 $\mu\text{g ml}^{-1}$) for 4 wk.

MTT assay

To assay cell viability after lectin treatment, A431 or HT29 cells were counted and seeded at 5×10^3 cells per well into 96-well plates and allowed to adhere for 24 h at 37°C. Cells were treated with various concentrations (50 to 3.123 $\mu\text{g ml}^{-1}$) of nJalalin, rJalalin, or PNA for 24 h in triplicate. For recovery after lectin treatment, the cells were further incubated for an additional 24 h in the absence of lectin. Thereafter, medium was decanted and 50 μl of MTT (1 mg ml^{-1}) in modified Eagle medium (MEM) (without phenol red) was added to each well and incubated for 4 h at 37°C. Formazan crystals were solubilized in 50 μl of *iso*-propanol by incubating with shaking at room temperature for 10 min. Absorbance was measured at 570 nm using 630 nm as the reference filter. Absorbance given by untreated cells was taken as 100% cell growth.

Trypan blue staining of cells

The A431 or HT29 cells were mildly trypsinized with 10 $\mu\text{g ml}^{-1}$ for a few minutes and the trypsin was inactivated with FBS. The cells were thoroughly washed with plain medium followed by Dulbecco phosphate-buffered saline (DPBS). The cells are counted with a hemocytometer and equal cells were treated with nJalalin, rJalalin, and PNA or, for the control, with DPBS. After 3 h the cells were stained with trypan blue (0.2% in DPBS) for 45 s and the stained cells as well as total cells were counted.

Light microscopic observation

To investigate the morphological changes after lectin treatment, cells were seeded at a density of 1×10^6 cells per plate into 60-mm plates and allowed to adhere for 8 h in a CO_2 incubator to obtain monodispersed, well-adhered cells. The cells for the experiment were washed with DPBS and treated with 50 $\mu\text{g ml}^{-1}$ concentrations of nJalalin, rJalalin, and PNA along with DPBS-treated con-

trol and incubated for 1 h at 37°C before microscopic observation.

Immunoprecipitation

A431 cells were seeded at a density of 1×10^6 cells per plate into 60-mm plates and allowed to adhere for 8 h at 37°C. Cells were treated with 50 $\mu\text{g ml}^{-1}$ concentrations of nJalalin along with an appropriate control and incubated for 10 min and 20 min at 37°C. After treatment, cells were scraped immediately and centrifuged. The cell pellet was lysed in 500 μl of immunoprecipitation assay (IPA) buffer (10 mM Tris pH 8.0, 150 mM NaCl, 1 mM ethylenediamine-tetraacetic acid [EDTA], 1% Triton X-100, 0.5% deoxycholate, 0.05% sodium dodecyl sulfate [SDS], 1 mM Na_3VO_4 , 1 mM phenyl methyl sulphonyl fluoride, 1 $\mu\text{g ml}^{-1}$ leupeptin, and 1 $\mu\text{g ml}^{-1}$ aprotinin). The cell lysate was centrifuged at 4°C for 10 min at 14 000 $\times g$, and the supernatant was precleared by incubating with recombinant protein G-agarose beads at 4°C for 6 h, followed by centrifugation at 4°C for 1 min, at 14 000 $\times g$. The supernatant was subjected to protein estimation and was divided equally into two tubes. One was subjected to immunoprecipitation by the addition of 2 μg of rabbit polyclonal anticaveolin-1 antibody and the second tube was subjected to immunoprecipitation by the addition of 2 μg of rabbit polyclonal anti-p38 mitogen-activated protein (MAP) kinase antibody. Both tubes were then incubated overnight at 4°C followed by incubation with recombinant protein G agarose beads at 4°C for 2 h. After washing the beads three times with ice-cold IPA buffer containing the detergent and three times with IPA buffer without detergent, the phosphorylation status of caveolin-1 and p38 were visualized after SDS-polyacrylamide gel electrophoresis (PAGE), electrophoretic transfer, and immunoblotting.

Homotypic aggregation assay

A431 cells (1×10^4 cells ml^{-1}) were washed with phosphate-buffered saline (PBS), detached with 0.05% trypsin containing 0.02% EDTA, and resuspended in serum-free growth medium. Cells were allowed to aggregate for 60 min in a 50- μl drop on inverted coverslips in the presence as well as the absence of respective lectins in a humidified chamber. For the lectin inhibition assay, 50 μM methyl- α -galactose was added to the respective cell drop in the presence and absence of lectins and allowed to aggregate for 60 min, and visualized by phase-contrast microscope.

Phosphorylation of EGFr, ERK, p38, and caveolin-1

A431 cells (2.0×10^5) were incubated with an indicated amount of the lectin in a total volume of 50 μl at 37°C

for indicated times. The cells were washed once with DPBS to remove the unbound lectin and finally resuspended in 50 μ l of DPBS. The cells were stimulated at 37°C for 10 min with TGF α (20 ng; 66 nM). The cell pellet was recovered by centrifugation, resuspended in Laemmli sample buffer (20 μ l) containing protease inhibitors (10 μ g ml⁻¹ leupeptin, 2 mM phenylmethylsulfonylfluoride [PMSF] and 2 mM Na₃VO₄), boiled for 10 min, and electrophoresed on 7% SDS-PAGE after centrifugation. The proteins were transferred to a nitrocellulose membrane using 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid buffer (pH 10.5) at 150 mA for 2 h. The blot was subjected to immunodetection with antiphosphotyrosine antibody. The cells treated with TGF α exhibited a slightly diffuse EGFr band because of their phosphorylation (Vandana et al 2003).

Quantitation of phosphorylation

The bands obtained after immunodetection with antiphosphotyrosine antibody or with anti-EGFr antibody were quantified by scanning using Bio-Rad phosphor-imager software. The band intensity of TGF α -stimulated cells was taken as 100%, and the intensity of other bands was expressed as a percentage of TGF α -stimulated lanes.

Fluorescein isothiocyanate labeling of nJacalin

The nJacalin in sodium borate buffer (pH 9.0) (1 mg ml⁻¹ concentration) was mixed in a 1:1 ratio with fluorescein isothiocyanate (FITC). The reaction mixture was kept in the dark for 45 min with gentle shaking at room temperature for labeling to take place. To stop the reaction, twice the molar concentration of glycine to FITC was added and kept for 15 min. Excess fluorescent reagent was removed by gel filtration on a Sephadex G-25 column using PBS.

Confocal microscopy

A431 cells were cultured on glass coverslips, rinsed with PBS, and treated with or without nJacalin-FITC (100 nM) for 60 min at room temperature, followed by washing with PBS thrice. Cells were then fixed in 2% paraformaldehyde in PBS on ice for 10 min, followed by permeabilization with 0.1% Triton X-100 containing 0.5 M NH₄Cl in PBS on ice for 10 min. The cells were blocked in 5% fatty acid-free bovine serum albumin in PBS at room temperature for 30 min. The cells were then incubated with anticaveolin-1 antibody (SC-894), anti-Hsp-70 (SC-1060), or anti-EGFr antibody (SC-120) overnight at 4°C followed by washing with PBS and incubation with anti-mouse tetramethyl rhodamine iso-thiocyanate (TRITC) or anti-goat TRITC-conjugated secondary antibody. The

cells were then visualized with a Zeiss LSM 510 microscope under confocal settings.

Isolation of jacalin-bound proteins of A431 cells

The nJacalin (1 mg) was coupled with 500 mg of CNBr-activated sepharose 4B according to the suggested protocol of the manufacturer. The column was thoroughly washed with 10-bed volumes of PBS (pH 7.4) before use. The matrix and A431 whole cell lysate (7 mg protein) was mixed by rotation for 2 h at 4°C. The beads were then packed into a column (1 $\times$ 15 cm) and washed thoroughly with PBS until no protein was detected in the wash. Native jacalin-sepharose-bound proteins were eluted with 50 mM methyl- α -galactose. The eluate was precipitated with acetone and analyzed by one-dimensional (1-D) or two-dimensional (2-D) electrophoresis.

Mass spectrometric analysis

The jacalin-sepharose eluate was concentrated by freeze-drying and processed by the W.M. Keck Foundation Center for Biomedical Mass Spectrometry at the University of Virginia (Charlottesville, VA, USA). Peptide sequences obtained were searched by conducting a search using the basic local alignment search tool.

RESULTS AND DISCUSSION

The aim of the present study was to understand the nature of cellular responses evoked by plant lectins. In this regard, we investigated the effect of jacalin isolated from tropical jackfruit seed and PNA from peanut on A431 and HT29 cells because the carbohydrate specificity of both lectins is well defined and they both bind to TF disaccharide. We specifically chose TF disaccharide-binding lectins for the present study because TF disaccharide is an oncofetal carbohydrate antigen known to result in an increased expression in malignancy and hyperplasia (Campbell et al 1995; Wolf et al 1988).

We first investigated the effect of jacalin and PNA on cell proliferation/cytotoxicity because they have nearly the same sugar specificity but different peptide backbones. In the present study, jacalin was found to be a noncytotoxic inhibitor of proliferation of HT29 colon cancer cells (tested at 1–50 μ g ml⁻¹) (Fig 1A). The same lectin is, interestingly, cytotoxic to A431 epidermoid carcinoma cells as evidenced in Figure 1B, which amounts to about 70% at 50 μ g ml⁻¹, while it was inhibited at about 50% at 25 μ g ml⁻¹. However, both HT29 and A431 cells have the same magnitude of proliferation after removal of the lectin, as seen in Figure 1C and D, respectively. These data are consistent with an earlier observation by Rhodes and coworkers (Yu et al 2001), who also showed that the

Fig. 1. Treatment of A431 and HT29 with indicated lectins. The MTT assay was performed as described in Materials and Methods. HT29 cells (A and C) or A431 cells (B and D) were incubated in the presence of various concentrations of nJacalin, rJacalin, or PNA for 24 h, and viable cells were estimated by MTT. In all panels, cells without any treatment were considered as 100% viable and viable cells (mean $\pm$ SD, $n = 5$) were expressed as a fraction of untreated cells. Data represent the average of three parallel wells and one of the five independent experiments. Recovery of HT29 (C) and A431

binding of PNA resulted in proliferation of HT29 cells (by about 42%), whereas ABL lectin was found to be a non-cytotoxic inhibitor (by about 37%) of proliferation of the same cells. However, both lectins in the presence of TF disaccharide have no effect on proliferation.

An interesting observation that emerged from this experiment is the cellular responses evoked by recombinant single-chain jacalin (rJacalin). We recently reported that this single-chain rJacalin has ~ 1000 -fold less affinity for TF disaccharide (nJacalin $K_a = 4.0 \times 10^5 \text{ M}^{-1}$ vs rJacalin $K_a = 5.37 \times 10^2 \text{ M}^{-1}$) in comparison to the two-chain nJacalin (Sahasrabudde et al 2004). However, both native as well as recombinant jacalin evoked a nearly identical magnitude of cytotoxic responses as far as their effects on A431 and HT29 cell proliferation are concerned (Fig 1). Hence, it is possible that factors apart from the carbohydrate binding property of jacalin also contribute to the observed cellular responses, because both nJacalin and rJacalin with 100-fold differences in affinity (overall difference for carbohydrates in general) evoked the same magnitude of cytotoxic response. With PNA it was observed that the recovery was insignificant (meaning cytotoxic) for A431 cells and the result for HT29 was the same (proliferation) as that observed by Rhodes and co-workers (Jordinson et al 1999; Yu et al 2001). Thus, the data suggest that jacalin treatment results in reversible cytotoxicity of A431 cells and the cells recover well after the removal of the lectin.

Because it was observed that both jacalin and PNA are toxic to A431 cells, the membrane integrity of A431 cells after lectin treatment were examined by trypan blue dye exclusion. It is clear from Figure 2A,B that post-treatment of jacalin or PNA for 3 h, $>90\%$ of cells are viable at $10 \mu\text{g ml}^{-1}$ concentration. This observation indicates that jacalin treatment does not damage the cell membrane of A431 cells, which is in agreement with a previous report on HT29 cells (Yu et al 2001). A slight damage to the A431 cell surface ($\sim 25\%$) was observed at concentrations of jacalin greater than $100 \mu\text{g ml}^{-1}$ (data not shown). However, jacalin treatment results in morphological changes (ie, cell rounding much akin to changes observed with EGF/TGF α binding to its receptor on A431 cells [Fig 2C]). Within minutes of jacalin treatment, A431 cells begin to round and tend to appear as if they are off the substratum. It is interesting that treatment with PNA did

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(D) cells after lectin treatment: HT29 or A431 cells were treated with $25 \mu\text{g ml}^{-1}$ for 24 h and the lectin was removed from the medium, fresh medium containing 10% FBS was added to wells for 24 h, and the MTT assay was carried out as described in the text. Groups labeled "Lectin treatment" and "Recovery" represent lectin treatment and recovery after lectin treatment, respectively. The results shown are an average of three independent measurements and the error bar represent standard deviations ($n = 5$).

Fig. 2. Cell trypan blue staining and morphological changes. (A) Trypan blue staining was carried out on lectin-treated A431 or HT29 cells as described in Materials and Methods. A431 cells (A) and HT29 cells (B) were incubated in the presence or absence of n-Jacalin, rJacalin, or PNA for 2 h and 4 h, and viable cells were counted after staining with trypan blue dye. In all panels, the percentage of viable cells was estimated from the cells without any treatment and viable cells after lectin treatment (mean $\pm$ SD, $n = 5$) is expressed as a percentage of untreated cells. The results shown are an average of three independent measurements and the error bar represent standard deviations ($n = 5$). (C) Morphology of A431 cells after lectin treatment ($50 \mu\text{g ml}^{-1}$) was observed as described in the Materials and Methods section. The morphological changes shown are one representative set of observations of six independent measurements.

Fig. 3. Phosphorylation of caveolin-1, p38, and JNK. Kinetics of phosphorylation of caveolin-1, p38, and JNK by nJacalin treatment was carried out as described in Materials and Methods, and probed with indicated antibodies. (A) Time-dependent phosphorylation of caveolin-1 after treatment of nJacalin ($50 \mu\text{g ml}^{-1}$). The top and bottom panels were obtained by probing with anti-phospho-tyr¹⁴-specific and anticaveolin-1 antibodies, respectively. (B) Time-dependent phosphorylation of p38 after treatment of nJacalin ($50 \mu\text{g ml}^{-1}$). The top and bottom panels were obtained by probing with antiphospho-specific p38 and anti-p38-specific antibodies, respectively. (C) Stress-induced phosphorylation of JNK after treatment of nJacalin ($50 \mu\text{g ml}^{-1}$). All panels are one of the representative set out of three independent experiments. (D) Immunofluorescence studies of localization of phosphocaveolin-1, caveolin-1, and jacalin. (A) Nuclear staining with DAPI, (B) surface staining of Tyr¹⁴ phospho Caveolin-1, (C) phase-contrast image, (D) surface staining of jacalin-FITC, (E) merged image of FITC-nJacalin and phosphocaveolin-1 along with nuclear staining, (F) surface staining of caveolin-1.

Fig. 4. Immunofluorescence studies of jacalin on A431 cells. (A) A-431 cells were grown on coverslips and treated with FITC-labeled nJacalin (100 nM) and DAPI ($1 \mu\text{g ml}^{-1}$) for nuclear staining. (A) Nuclear staining with DAPI. (B) Cell surface localization of FITC-labeled nJacalin. (C) Dual staining of nJacalin and DAPI. (D) Phase-contrast image. (B) Dual staining of nJacalin and EGFr. A-431 cells are treated with FITC-nJacalin (100 nM) followed by staining for EGFr-specific antibody and DAPI ($1 \mu\text{g ml}^{-1}$). (A) Nuclear staining with DAPI, (B) cell surface staining of FITC-nJacalin, (C) cell surface staining of EGFr, (D) merged image of FITC-nJacalin and EGFr along with nuclear staining.

not result in the same changes to the extent of those observed for nJacalin, although both lectins have similar carbohydrate specificity (Fig 2C). With rJacalin, the observed changes are somewhat intermediate between nJacalin and

PNA. At a gross level this observation suggests that these two lectins (nJacalin and PNA) might have different modes of binding to target cells and that factors apart from sugar specificity might be responsible for the observed morphological changes. These changes may also reflect cellular stress.

It is well known that stress induces morphological changes, and in particular, changes in the cytoskeleton induce phosphorylation of caveolin-1 and p38. For example, NIH3T3 cells after treatment with 600 mM sucrose show visible changes in morphology (ie, cell rounding and phosphorylation at tyr^{14} of caveolin-1, an important lipid raft constituent implicated in cellular signaling [Volonte et al 2001]). For this phosphorylation, changes in cytoskeleton are necessary but a change in the volume of cells is not. We therefore examined the phosphorylation status of caveolin-1, p38, and JNK (another stress marker) after jacalin treatment to assess the stress response. It is clear from Figure 3 that jacalin treatment of A431 cells resulted in sustained phosphorylation of caveolin-1 and p38 while there was no effect on JNK. This observation is in contrast to the sucrose treatment of NIH3T3 cells in which the phosphorylation of caveolin-1 was rather transient (ie, the phosphorylation has reached a maximum at around 10 min and it was completely diminished by 20 min [Volonte et al 2001]). While p38 phosphorylation was sustained until 30 min after jacalin treatment, similar to sucrose treatment (Fig 3B). However, we could not observe any detectable level of caveolin-1 phosphorylation upon PNA treatment of A431 cells (data not shown). These observations once again suggest a differential mode of interaction between jacalin and PNA to target cells despite similar carbohydrate specificity. Immunostaining for Tyr^{14} phosphorylated caveolin-1 after jacalin treatment did not indicate any dramatic change in the distribution of phosphorylated caveolin-1 (Fig 3D).

The data presented so far clearly indicate that jacalin is reversibly toxic to A431 cells, although it does not damage the cell membrane; rather, it induces cellular stress by merely binding to the cell surface. In a recent report, it was documented that jacalin down-regulated the ERK signaling in HT29 cells (Yu et al 2004). We therefore investigated the pathway upstream of ERK, because it is important to know how jacalin can influence the signal from the cell surface. First, it is necessary to ascertain by confocal microscopy whether jacalin is present on the cell surface or internalized inside the cell because the ABL lectin, which also binds to the TF disaccharide, has been shown to be internalized (Yu et al 1999). As seen in Figure 4A, it is clear that jacalin is present on the cell surface, but we could not detect any internalized jacalin. It is also clear that jacalin binding did not result in large disturbances on the cell surface, because the distribution of EGFr on A431 cell surface remained same (Fig 4B).

Fig. 5. Down-regulation of EGFr and ERK signaling by nJacalin. Down-regulation of phosphorylation on EGFr and ERK was monitored after treatment of jacalin and peanut lectins as described in Materials and Methods. (A) Concentration Kinetics of phosphorylation on EGFr after the indicated concentration of jacalin treatment followed by stimulation with TGF α (20 ng/reaction). In all panels lanes designated with a U and an S indicate unstimulated cells and cells stimulated with TGF α (20 ng/reaction), respectively. The top and bottom panels were obtained by probing with antiphosphotyrosine and anti-EGFr, respectively. (B) Time kinetics of phosphorylation of EGFr was carried out as described above: cells stimulated with a 40 $\mu\text{g ml}^{-1}$ concentration of nJacalin for 5, 10, 15, 20, 25, and 30 min followed by stimulation with TGF α (20 ng). The top and bottom panels were obtained by probing with antiphosphotyrosine and anti-EGFr, respectively. (C) Concentration kinetics of phosphorylation of EGFr after treatment with rJacalin at various concentrations as mentioned. Cells were treated with Jacalin at 5, 10, 20, 40, and 80 $\mu\text{g ml}^{-1}$ followed by stimulation with TGF α (20 ng). (D) Time kinetics of phosphorylation on EGFr after treatment of r-Jacalin as described above. Cells stimulated with 40 $\mu\text{g ml}^{-1}$

The observations described above raise the question of how jacalin can down-regulate ERK phosphorylation. We attempted to examine the phosphorylation status of the upstream molecule of ERK (ie, EGFr). The data shown in Figure 5A,B clearly show that jacalin causes dose- and time-dependent down-regulation of the EGFr phosphorylation signal. The level of phosphorylation in untreated cells after starvation was taken as the basal level of phosphorylation. Treatment of cells with TGF α (20 nM) resulting in stimulation of EGFr phosphorylation was taken as the positive control. Cells treated with various concentrations of jacalin followed by stimulation with TGF α (20 ng) showed a decrease in EGFr phosphorylation level with an increase in the concentration of jacalin. It was observed that at 1.25 to 5 $\mu\text{g ml}^{-1}$ of jacalin there is no marked decrease in phosphorylation, but at the 10 to 20 $\mu\text{g ml}^{-1}$ concentration there was slight loss of phosphorylation signal, and at 40 $\mu\text{g ml}^{-1}$ concentration of jacalin, EGFr phosphorylation signal was undetectable. It is also clear from Figure 5A–E that the amount of EGFr is same as in control or mock-treated lanes, but it did not carry the Tyr phosphorylation signal.

The time course of EGFr phosphorylation in A431 cells was monitored over 30 min after treatment of nJacalin using 40 $\mu\text{g ml}^{-1}$ for various time periods. From Figure 5B it is very clear that from 5 min to 20 min of jacalin treatment the signal is comparable to that of the positive controls; the decrease is observed by 25 min and is completely undetectable by 30 min. It is also clear from Figure 5C,D that rJacalin parallels nJacalin. These data suggest that jacalin sugar-binding property alone might not influence the jacalin-mediated signaling. To further confirm whether or not the absence of EGFr phosphorylation is related to sugar specificity, we repeated the experiment with PNA. It is clear from Figure 5E that PNA does not down-regulate EGFr phosphorylation. Both nJacalin and rJacalin have down-regulated the phosphorylation of ERK as seen Figure 5F,G, however, the phosphorylation status of ERK is the same as that of EGFr for PNA treatment, as seen in Figure 5E. This observation is consistent with the observation by Rhodes and coworkers who showed that jacalin has a negative modulatory effect on ERK

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rJacalin for 15 and 30 min followed by TGF α stimulation. (E) Phosphorylation status of EGFr of A431 cells after jacalin or peanut lectin treatment. Cells were treated with 40 $\mu\text{g ml}^{-1}$ nJacalin or peanut agglutinin for 30 min followed by stimulation with TGF α (20 ng) as described in Materials and Methods. (F) Phosphorylation status of ERK of A431 cells after lectin treatment was carried out as described above for EGFr. Cells stimulated with nJacalin or PNA for 30 min followed by stimulation with TGF α . (G) Phosphorylation status of ERK of A431 cells after rJacalin treatment by following the same procedure as that in (F). In all panels, top panels were obtained by probing with antiphosphotyrosine and bottom panels with the indicated antibodies.

Fig. 6. Inhibition of homotypic aggregation of A431 cells by jacalin. A homotypic aggregation inhibition assay was performed as described in Materials and Methods. A431 cells were incubated in the presence or absence of nJacalin, rJacalin, and PNA, and in the presence or absence of their selective sugar methyl- α -galactose at a 50 mM concentration for 1 h. Cell aggregation inhibition as well as aggregation after sugar addition to the reaction along with lectin were observed and captured directly from phase-contrast microscopy. (A, C, E, and G) represent photos of control cells without any treatment of lectin, nJacalin, rJacalin, and PNA, respectively. (B, D, F, and H) represent photos of cells in the presence of a 50 mM concentration of methyl- α -galactose (ie, control cells without treatment of lectin, nJacalin, rJacalin, and PNA, respectively). The results shown are an average of three independent measurements.

phosphorylation at 30 $\mu\text{g ml}^{-1}$ (Yu et al 2004). We also rule out the possibility of direct interaction between jacalin and the carbohydrate moieties of EGFr because the EGFr has been shown to contain carbohydrate chains that are representative of a high mannose type, which jacalin does not bind with high affinity (Stoscheck et al 1985). In addition, the interaction between any mannose/fucose binding receptors present on the A431 cells and the carbohydrate chains of jacalin (jacalin is mildly glycosylated at Asn74; Capon, 1990) is also ruled out as the recombinant jacalin, which parallels native jacalin, is expressed in *Escherichia coli* and devoid of any glycosylation.

These data together raise an interesting aspect of lectin-cell interaction, as the observed effects of cytotoxicity, stress responses, and modulation of EGFr and ERK signals described here could be due to involvement of factors other than the carbohydrate-binding property probably through a jacalin peptide backbone with some cell surface molecules. This argument is supported by the fact that native and recombinant jacalin, which have identical peptide backbone, evoked similar cellular responses in contrast to that of PNA.

During cancer metastasis, multicellular homotypic and heterotypic aggregates are formed to aid colonization. This process helps them to survive and proliferate at the site of attachment. This attachment is mediated by the β -gal binding of the Galectin family of lectins, probably through binding to the terminal β -gal residues of the TF disaccharide. In contrast to normal cells, this core TF disaccharide is well exposed in transformed cells. In the present study we provided several forms of evidence regarding the ability of jacalin and peanut lectin, which have been shown to modulate signals differently with respect to target cells. Because both the lectins are specific to TF disaccharide, we investigated whether or not the binding of jacalin and PNA to A431 cells evinces any significant differences in homotypic aggregation of A431 cells. The data shown in Figure 6 indicate that both jacalin and PNA parallel each other (ie, they both inhibit the homotypic aggregation) and preincubation with Me- α -Gal, (which prevents the lectin from binding to β -gal residues) restores the homotypic aggregation of A431 cells. These data grossly indicate that there is no measurable difference as far as their recognition toward the β -gal portion of TF disaccharide moiety is concerned. It is relevant to mention that both jacalin and PNA have similar affinities toward galactose by in vitro affinity measurements (Sastri and Surolia 1986; Sastri et al 1986; Kabir 1998).

To investigate the putative proteins that jacalin or PNA can bind on the A431 cell surface, we passed the A431 cell lysate through jacalin- and PNA-immobilized sepharose columns. After extensive washing with PBS, bound proteins were eluted with 50 mM Me- α -Gal and the pattern of the proteins is shown in Figure 7A, lane 1. The

Fig. 7. 1-D and 2-D gel analysis of jacalin-bound proteins of A431 cells. A-431 cell lysate were run on an NHS sepharose coupled with native jacalin matrix and purified with 50 mM Me α Gal. The fractions thus purified were run on 1-D and 2-D SDS-PAGE. (A) Lane 1: Intracellular jacalin binding profile of A431 cell lysate after jacalin-NHS sepharose affinity purification. Lane 2: Intracellular PNA binding profile of A431 cell lysate after PNA-NHS sepharose affinity purification. Lane 3: Ligand blotting of elute from A431 cell lysate after jacalin-NHS sepharose affinity purification. Lane 4: Probing of elute from A431 cell lysate after jacalin-NHS affinity purification with anti-HSP70 antibody. (B) 2-D electrophoresis profile of A431 cell lysate after jacalin-NHS sepharose affinity purification. Black arrows indicate protein spots obtained. Horizontal axis shows isoelectric point and vertical axis shows molecular weight in kilodaltons.

jacalin-sepharose column yielded a very clear protein profile in comparison to PNA-sepharose despite the passage of an identical amount of whole A431 cell lysate. Although bands in the PNA-agarose are observed, they are faint (Fig 7A, lane 2). In jacalin-sepharose, around eight prominent bands in the molecular weight range of 40–200 kDa were observed, whereas in the PNA column, we could observe faint bands at ~60 kDa.

A direct jacalin lectin blot of the jacalin-sepharose column eluate was performed to further authenticate the bands, and the most prominent among the detected bands is a protein at 110 kDa and at 68–70 kDa (Fig 7A, lane 3). In a recent report, it was observed that TF disaccharide binding lectin ABL (which is homologous to n-Jacalin in sugar specificity) binds to oxygen-regulated protein-150 (ORP150) of HT29 cells. It is possible that the

Table 1 Peptide sequences obtained by mass spectrometry of jacalin-sepharose eluate

Sequence	Mass	Residues in ORP150
TVLSANADHMAQIEGLMDDVDFK	2520.2	318–340
AEAGPEGVAPAPEGEK	1508.7	670–685
EKAANSLEAFIFETQDK	1941	737–753
LPATEKPVLLSK	1295.8	878–889
EVQYLLNK	1006.6	901–908

prominent band at ~110 kDa could be ORP150 itself. We also carried out 2-D electrophoresis of the jacalin eluate to identify the isoelectric point (pI) value of the 110 kDa band. The observed pI value (~4.9–5.15) and molecular weight (~110 kDa) (as seen in 2-D electrophoresis; Fig 7B) are in agreement with the pI and molecular weight values of ORP150 (from 2-D gel) published by Calvert et al (2003). Because ORP150 has sequence homology with Hsp70, the jacalin-sepharose column eluate was also probed with anti-Hsp70 antibody. The ~110 kDa band was clearly recognized by the anti-Hsp70 antibody (Fig 7A, lane 4). Mass spectrometric analysis of the jacalin eluate confirmed the presence of ORP150, because we could observe five peptides, as shown in Table 1. In view of the homology between Hsp70 and ORP150, we examined whether or not any proteins that have homology to Hsp70 are present on the A431 cell surface. We immunostained the A431 cell surface with anti-Hsp70 antibody without permeabilization and could clearly see the presence of Hsp70-like proteins on the A431 cell surface (Fig 8A). It is important to mention that the negative control coverslips were prepared exactly as those of the experimental slides in the absence of anti-Hsp70 antibody, and no detectable staining was observed despite higher gain settings in confocal microscopy. This observation is consistent with several reports that positively identified the presence of Hsp70 on various cancer cell surfaces (Shin et al 2003; Gastpar et al 2004). In order to further strengthen our conclusion regarding the role of ORP150 in jacalin-mediated cytotoxicity, we developed a stable cell line of A431 cells that overexpress ORP150 and examined them for jacalin-induced cytotoxicity. It is clear from Figure 8B that the A431-ORP150 cell line is highly resistant to jacalin-induced cytotoxicity in comparison to normal A431 cells.

These data suggest that jacalin-induced cytotoxicity is mediated through ORP150, and that impairment of ORP150 functions with the help of jacalin, makes the cells more susceptible to death due to stress. Moreover, it has been reported that ORP150 has sialyl-(2→3)-Gal β (1→3)GalNAc α (sialyl-TF disaccharide chain), which might also be responsible for binding to jacalin (Yu et al 2002). In view of the same magnitude of cytotoxicity evoked by rJalalin, it is possible that the initial recogni-

Fig. 8. (A) Immunofluorescence studies. A-431 cells were grown on coverslips and probed with anti-HSP70 antibody without permeabilization. (A) Phase-contrast image of negative control without anti-HSP70 antibody. (B) Immunostaining with only secondary anti-goat-TRITC labeled antibody. (C) Phase-contrast image of cell probed with anti-HSP70 antibody and respective anti-goat-TRITC labeled antibody. (D) Cell surface staining of anti-HSP70 and respective secondary TRITC-labeled antibody. (B) MTT assay with A431-ORP150 cells. MTT assay was performed as described in Materials and Methods. A431 and A431-ORP150 cells were incubated in the presence of various concentrations of nJacalin for 24 h, and viable cells were estimated by MTT. In all panels, cells without any treatment were considered as 100% viable and viable cells (mean $\pm$ SD, $n = 5$) are expressed as a fraction of untreated cells. Data represent the average of three parallel wells and one of the five independent experiments.

tion between jacalin and ORP150 could be due to carbohydrate binding, which might be further strengthened by protein-protein interactions. These data also suggest that jacalin might interact with ORP150 and this could be one of the proteins that might have played a role in the jacalin-mediated cytotoxicity of A431 cells.

Four important points have emerged from this study. First, lectins with similar carbohydrate specificity evoke different cellular responses, especially stress, depending upon the lineage of the cell line. For example, HT29 colon cancer cells are less susceptible to jacalin than A431 cells. In sharp contrast, PNA is irreversibly toxic to A431 cells, although it stimulates HT29 cells. Second, this cellular response could not be entirely due to high affinity interaction between lectin and carbohydrate, but other factors such as lectin-protein interactions may also contribute to the observed cellular responses as nJacalin, and PNA have elicited different stress responses. Hence, the involvement of the lectin backbone in the elicited responses cannot be completely ruled out. This possibility derives sufficient strength from our observation that PNA, which has a similar sugar specificity to that of jacalin, neither induced the caveolin-1 phosphorylation nor its associated, morphologically visible, cell rounding or down-regulation of EGFr/ERK phosphorylation. In contrast, rJacalin, which has the same protein backbone as that of nJacalin but less affinity for carbohydrates, is as efficient as nJacalin in its ability to inhibit EGFr-mediated signaling and cytotoxicity. If the observed cellular responses orchestrated by nJacalin are solely due to sugar-binding affinity alone, higher concentrations of rJacalin would have been required to achieve the same magnitude of responses to that of nJacalin. The interaction of jacalin with the target cell surface molecules through protein-protein or another mode of interaction, in addition to carbohydrate binding, cannot be completely ruled out because it was shown earlier that jacalin can interact with deglycosylated CD4 of T-lymphocytes through protein-protein interactions (Lafont et al 1996). Third, it appears that jacalin induces the novel cellular stress pathway on A431 cells, probably through the caveolin-1 $\rightarrow$ c-src $\rightarrow$ p38 pathway, which is akin to the effect of high concentrations of sucrose on NIH3T3 cells as reported by Lisanti and coworkers (Volonte et al 2001). Fourth, our data suggest that jacalin-induced cytotoxicity is probably mediated through the endoplasmic reticulum chaperone ORP150, because overexpression of ORP150 in A431 cells abolished jacalin-induced cytotoxicity. Hence, lectins or other proteins that bind/interact with cell surface chaperones such as ORP150 and their role in inducing cytotoxicity or proliferation will be of considerable importance to understanding the nature of interactions and their functional ramification inside the cell. A similar opinion was also expressed by Yu et al (2001), who suggested that it is not always possible to predict the biolog-

ical consequences of lectin-cell interactions based on carbohydrate specificity criteria alone. Also, it is necessary to identify the role and function of TF disaccharide-containing proteins expressed in cells that mediate the eventual biological effects.

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