Peanut agglutinin appearance in the blood circulation after peanut ingestion mimics the action of endogenous galectin-3 to promote metastasis by interaction with cancer-associated MUC1

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Peanut agglutinin (PNA), which accounts for ~0.15% of the weight of the common peanut, is a carbohydrate-binding protein that binds the oncofoetal Thomsen–Friedenreich (TF) disaccharide (galactoseβ1,3N-acetylgalactosamine-) that is overexpressed by ~90% of human cancers. Previous studies have shown that PNA is highly resistant to cooking and digestion and rapidly enters the human blood circulation after peanut ingestion. This study investigates the hypothesis that PNA appearance in the circulation after peanut ingestion may mimic the actions of endogenous TF-binding human galectin-3 in metastasis promotion. It shows that PNA at concentrations similar to those found in blood circulation after peanut ingestion increases cancer cell heterotypic adhesion to the blood vascular endothelium and enhances the formation of tumour cell homotypic aggregates, two important steps in the metastasis cascade, and enhances metastasis in a mouse metastasis model. These effects of PNA are shown to result from its interaction with the cancer-associated TF disaccharide on the transmembrane mucin protein MUC1, causing MUC1 cell surface polarization that reveals underlying cell surface adhesion molecules. Thus, PNA appearance in the blood circulation after peanut ingestion mimics the actions of endogenous galectin-3 and promotes cancer cell metastatic spread by interaction with cancer-associated TF/MUC1. As metastasis accounts for the majority of cancer-associated fatality, regular consumption of peanuts by cancer patients would therefore be expected to have an adverse effect on cancer survival.

Introduction

There is a large body of epidemiological evidence linking diet and colorectal cancer causation (1) but less is known of the influence of diet on the progression of this cancer once established. Recent studies have however shown that a higher intake of a Western dietary pattern (high intakes of meat, fat, refined grains and sugar desserts) (2) and a higher dietary glycemic load after cancer diagnosis (3) were associated with increased risk of recurrence and mortality among patients with stage III colon cancer although mechanisms are unclear.

Peanut agglutinin (PNA) is a lectin that accounts for ~0.15% of the weight of the common peanut (Arachis hypogaea) (4). It is a tightly globular protein that is highly resistant to cooking and digestion and has been shown in our previous studies to stimulate proliferation of colonic epithelial cells (4–6) and also rapidly appears in the blood circulation, still intact and biologically active, after ingestion of peanuts (7). PNA binds the oncofoetalThomsen–Friedenreich (TF) (Galβ1-3GalNAcβ-) antigen and shows relative specificity for cancerous or precancerous epithelia in tissue sections (8). Over-expression of TF is one of the commonest glycosylation changes in cancerous and precancerous epithelium (8,9), and it is a natural ligand of the human galactoside-binding galectin-3 (10).

Galectin-3 is commonly overexpressed in cancer and facilitates cancer progression and metastasis by interaction with various galactoside-terminated cell surface glycans (11–13). The levels of circulating galectin-3 is increased up to 31-fold in the bloodstream of cancer patients (12,14–18) and patients with metastasis have higher levels of circulating galectin-3 than those with only localized tumours (14,19). Recent studies by us and others have shown that the increased circulation of galectin-3 in cancer is an important promoter of cancer cell metastatic spread. Galectin-3 binds to the TF antigen on the large transmembrane mucin protein MUC1 (10,20), which is itself commonly overexpressed by cancer cells (21). This induces clustering of MUC1 on cell surface, thus revealing underlying adhesion molecules which allows adhesion of the disseminating tumour cells to vascular endothelium (20) and also increases homotypic aggregation of the tumour cells allowing the formation of tumour micro-emboli that prolongs the survival of tumour cells in the circulation (22). Galectin-3 has also shown to be able to interact with the TF antigen expressed by cancer-associated MUC4 and this, like the galectin-3-MUC1 interaction, increases cancer cell adhesion to endothelium (23). Extracellular galectin-3 is seen also to promote endothelial secretion of metastasis-promoting cytokines (24) and enhance cancer cell migration (25), proliferation (26) and angiogenesis (27). Galectin-3 is now being explored by several biotech companies as a promising target for therapeutic strategies to prevent metastasis (28).

As PNA is a TF-binding lectin and appears rapidly in the circulation after ingestion of peanuts (7), we speculated that circulating PNA might mimic the actions of circulating galectin-3, thus promoting cancer metastasis. In this study, we provide in vitro and in vivo evidence that supports this hypothesis.

Materials and methods

Materials

PNA and non-enzymatic cell dissociation solution (NECDS) were from Sigma. O-glycanase was obtained from Prozyme (Oxford, UK). The Vybrant DiO and Dil Cell-labelling Solutions were from Molecular Probes (Cambridge, UK). Calcein (AM) cell labelling solution was obtained from Invitrogen.

Cells

HT29-5F7 cells, kindly provided by Dr Thecla Lesuffleur (INSERM U560, France), are a subpopulation of HT29 cells selected following treatment of HT29 cells with 5-fluorouracil (29). HT29-5F7 cells express high levels of MUC1 and MUC5B but extremely low (hardly detectable) levels of MUC4 (29). MUC1-expressing SW620 human colon cancer cells were obtained from the European Cell Culture Collection via the Public Health Laboratory Service (Porton Down, Wiltshire, UK). The cell lines were last authenticated by DNA profiling (DNA Diagnostics Center, London) in May 2014. Human microvascular lung endothelial cells (HMVEC-Ls), EGM-2 endothelial growth media and supplements were from Lonza (Slough, UK). HMVEC-Ls that had been passaged less than five times were used in the experiments.

Assessment of cell aggregation by PNA

Two aliquots of cell suspension were incubated, separately, with DiO and Dil Cell Labelling Solution before washing and incubation with/without PNA (1–8 µg/ml) in a rotating incubator at 37°C for 1.5 h followed by analysis with flow cytometry, as described previously (22).

Assessment of the effect of PNA on cancer cell adhesion to endothelial cells

HT29-5F7 or SW620 cells were released with NECDS and labelled with Calcein AM for 30 min at 37°C (20). The cells were washed and incubated with various concentrations of PNA with/without 100 µg/ml TF-Threonine (Thr) for 30 min
at 37°C before application to HMVEC-L monolayers for 1 h at 37°C. After three washes with phosphate-buffered saline (PBS), cell adherence to HMVEC-L was measured using a fluorescence microplate reader (Infinite 200, TECAN).

MUC1 siRNA suppression and assessment of MUC1 cell surface localization
Sub-confluent HT29-5F7 cells were incubated with 100 nM MUC1 siRNA or non-targeting siRNA (Thermo scientific Dharmacon, Loughborough, UK) for 72 h at 37°C before lysis with sodium dodecyl sulphate-sample buffer and cellular MUC1 expression was quantified by immunoblotting with B27.29 anti-MUC1 antibody. To assess the effect of PNA on cell surface MUC1 distribution, HT29-5F7 cells were released by NECDS and incubated (10⁴ cells/ml) with 4 µg/ml PNA or 4 µg/ml control bovine serum albumin (BSA) in serum-free Dulbecco's modified Eagle's medium for 1 h at 37°C and then applied to poly-lysine coated slides for 30 min. After gentle washing, the cells were fixed with 2% paraformaldehyde, blocked with 5% normal goat serum/PBS, probed

Fig. 1. PNA increases human colon cancer cell homotypic aggregation. The presence of PNA induces dose-dependent increase of human colon cancer HT29-5F7 (A) and SW620 (B) cell aggregation. Representative flow cytometry plots from the cell aggregation assessments of human colon cancer HT29-5F7 cells in the presence or absence of 8 µg/ml PNA are shown in (C). The cell population shown in top right of each bivariate correlation plot is the cell population containing both DiO- and DiI-labelled cells that are defined in this study as cell aggregates. **P < 0.01, ***P < 0.001.

Fig. 2. PNA-mediated increase of cancer cell aggregation is associated with increased cell resistance to anoikis and increased cell survival under anchorage-independent conditions. HT29-5F7 cells were treated with or without 2.5 µg/ml PNA or BSA for 1 h followed by culture of the cells in suspension for 2 days. After passing through the cell strainers, the FITC–Annexin-V cell surface binding (A and B) of the strained (single) cells and cell aggregates and the cell viability (C) were assessed. The data are presented as mean ± SEM of triplicate determinations from two independent experiments. Representative flow cytometry plots from the anoikis assessments of HT29-5F7 cells are shown in (A). Annexin-V-positive and PI (propidium iodide)-negative (early apoptotic, at the bottom right in each bivariate correlation plot) cells are considered as apoptotic cells. *P < 0.05, **P < 0.01.
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with B27.29 anti-MUC1 antibody followed by fluorescent-labelled secondary antibody. The percentages of cells showing a continuous or discontinuous rim of MUC1 in 500 randomly selected cells were counted using an Olympus B51 fluorescent microscope.

Assessment of the effect of PNA on cell viability and anoikis (programmed cell death in response to loss of cell–cell or cell–extracellular matrix contacts)

HT29-5F7 cells were released by NECDS and cultured in suspension in poly-2-hydroxyethyl methacrylate-coated plates in the presence of 2.5 µg/ml PNA or 2.5 µg/ml BSA for 48 h at 37°C (22). The cell suspensions were then passed three times through 40 µm cell strainers. The viability of the cells that did (strained) and did not (aggregates) pass through the strainers was measured using CellTiter-Glo (Promega) and the apoptotic (anoikis) cells were measured by FITC–Annexin-V cell surface binding (Cambridge Biosciences, Cambridge, UK) assessed by flow cytometry.

Effect of PNA on metastasis in vivo in a mouse metastasis model

Female Balb/c nude mice (6–8 weeks old), obtained from Charles River Laboratories (Margate, Kent, UK), were housed in specific pathogen-free conditions and were maintained and used in accordance with the animal care protocol approved by the University of Liverpool. Eighteen animals were randomly divided into two equal groups and were subjected to intravenous tail vein injection with 100 µl PBS (control group) or 20 µg PNA in 100 µl PBS (treatment group) in the left lateral vein and immediately followed by a second tail vein injection of 2 × 10⁶ SW620 cells in 100 µl PBS in the right lateral vein. The animals were killed 6 weeks later by cervical dislocation and tumour metastases in the lungs, liver, kidney, brain and spleen were examined. The weight and the numbers of visible metastatic foci on the surface of lungs were recorded and lung tumours were further confirmed by histology of H&E stained lung sections.

Statistical analysis

Statistical analyses were by one-way analysis of variance followed by Bonferroni test for single comparison or Newman and Keuls test for multiple comparisons (PASW17.0) where appropriate.

Results

PNA increases human colon cancer cell homotypic aggregation that prevents cancer cell anoikis and increases cell survival.

PNA at concentrations similar to those found in the sera of people after eating 200 g peanuts (7) caused dose-dependent increase of homotypic aggregation of MUC1-positive human colon cancer HT29-5F7 (Figure 1A and C) and SW620 cells (Figure 1B). At 4 µg/ml, PNA induced 41 ± 5% (mean ± SEM, P < 0.0001) and 11 ± 2% (P = 0.0026) increased aggregation of HT29-5F7 and SW620 cells, respectively, when cultured in suspension. The PNA-induced increase in cell aggregation resulted in 53 ± 12% (P = 0.0046) reduction of anoikis when compared with that obtained from control BSA-treated cells (Figure 2A and B). Furthermore, the viability in cell aggregates from PNA-treated cells was increased by 206 ± 34% (P = 0.03) compared with BSA-treated cells (Figure 2C).

The lesser effect of PNA on aggregation of SW620 cells in comparison to HT29-5F7 cells may be related to the lower binding of PNA to SW620 (39% stained with biotin–PNA) than HT29 cells (48%) although they express similar levels of cell surface MUC1 (Supplementary Figure S1, available at Carcinogenesis Online).

Fig. 3. PNA increases cancer cell heterotypic adhesion to human microvascular lung endothelial cells. PNA induces dose-dependent increase of HT29-5F7 (A) and SW620 (B) cell adhesion to HMVEC-L monolayers. Data are expressed as mean ± SEM of triplicate determinations from three independent experiments. PNA (4 µg/ml)-mediated HT29-5F7 (C) and SW620 (D) cell adhesion to HMVEC-Ls is prevented by the presence of 100 µg/ml TF-Thr. *P < 0.05, **P < 0.01, ***P < 0.001.
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The presence of PNA at similar concentrations also caused dose-dependent increase of HT29-5F7 (Figure 3A) and SW620 (Figure 3B) cell adhesion to HMVEC-Ls, an effect that was prevented by the presence of 100 µg/ml TF-Thr (Figure 3C and D).

**Fig. 4.** The PNA-mediated cell adhesion requires the expression of TF on MUC1. O-Glycanase treatment reduces HT29-5F7 cell surface TF expression (A) and abolished PNA (4 µg/ml)-mediated HT29-5F7 cell adhesion to HMVEC-Ls (B). Suppression of MUC1 expression by MUC1 siRNA in HT29-5F7 cells (C) abolished PNA-mediated HT29-5F7 cell adhesion to HMVEC-Ls (D). Data are expressed as mean ± SEM of triplicate determinations from two independent experiments **P < 0.01, ***P < 0.001. Representative images of MUC1 immunohistochemistry showing MUC1 cell surface localization in response to treatment of the cells with 4 µg/ml PNA are shown in E, scale bar = 5 µm.

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The PNA-mediated cell–cell interactions require the expression of TF disaccharide on MUC1 and results in MUC1 cell surface polarization

To test whether the PNA-mediated increase of cancer–endothelial cell adhesion and cancer cell aggregation was through a similar mechanism as shown previously for galectin-3, we analysed the effects of cell surface TF and MUC1 expression in PNA-associated cell–cell interactions. It was found that removal of the cell surface TF by treatment of HT29-5F7 cells with O-glycanase specific for unsubstituted TF, which caused 74% reduction of cell surface TF expression (Figure 4A), abolished the effect of PNA on cell adhesion to HMVEC-Ls (Figure 4B). Suppression of cellular MUC1 expression by siRNA, which caused 53% reduction of MUC1 expression in HT29-5F7 cells (Figure 4C), was seen also to cause 76% reduction of PNA-mediated cell adhesion (Figure 4D). Moreover, loss of confluent circumferential MUC1 cell surface staining indicating clustering was significantly commoner (53%, 265 of 500 cells) in the PNA (4 µg/ml)-treated than the control BSA-treated HT29-5F7 cells (39%, 196 of 500 cells) (P < 0.001, Chi-square test, Figure 4E). Together, these results indicate that PNA-mediated increase of cancer cell–endothelial adhesion and cancer cell–cell aggregation acts through a similar mechanism as shown for circulating galectin-3 (10,20,22) that is, by interaction with the TF-MUC1 thus inducing MUC1 cell surface polarization with consequent exposure of the smaller cell surface adhesion molecules.
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Having found that PNA, like galectin-3, promotes two important steps in metastasis—cancer cell–endothelial adhesion and cancer cell–cell aggregation, we then tested the influence of the presence of PNA in the circulation on metastasis in a mouse model. The animals were injected intravenously with 20 µg PNA immediately followed by injection of human colon cancer SW620 cells into a different tail vein. Injection of 20 µg PNA should yield a concentration of around 10 µg/ml in the mice. Humans have up to PNA 5 µg/ml in venous blood 1 h after ingestion of 200 g peanuts (7). The animals were killed after 6 weeks and examined for metastasis in various organs. Metastases were found only in the lungs and 134% (134 ± 38%, mean ± SD, P < 0.01) more metastatic nodules were observed in the PNA-treated animals than in the control animals (Figure 5A–C). We found that pretreatment of HMVEC-L cells with PNA (up to 10 µg/ml) for 1 h before washing and addition of cancer cells has no significant effect on subsequent adhesion of either HT29-5F7 or SW620 cells to HMVEC-Ls in comparison with BSA-treated control (Supplementary Figure S1, available at Carcinogenesis Online), indicating that short-term interaction of the injected PNA with vascular endothelium in the in vivo model used here likely makes minimal contribution to the observed effect of PNA on metastasis.

Discussion

This study shows that PNA at concentrations similar to those found in the human blood circulation after peanut ingestion increases human colon cancer cell heterotypic adhesion to human lung vascular endothelium and cancer cell homotypic aggregation in vitro and enhances metastasis in vivo in mice. Like the metastasis-promoting human galectin-3, PNA binds to the TF disaccharide on cancer-associated MUC1, causing MUC1 cell surface polarization leading to exposure of underlying cell surface adhesion molecules.

The lungs are the second commonest site for metastases from colon cancer, affecting 10–15% of patients with advanced colorectal cancer (30). Lung metastasis after tail vein injection of cancer cells, as employed in this study, represents a much simpler model for testing of our hypothesis than animal models of liver metastasis which usually require intra-splenic injection.

PNA binds highly specifically to the TF disaccharide which is expressed in up to 90% of human cancers but is rarely expressed on healthy adult tissues due either to its concealment by other sugar residues or differential expression of alternative core O-glycan structures (31). The increased expression of TF disaccharide is one of the commonest glycosylation changes in cancer (31) and probably results most commonly from Golgi disarray (32,33). Two cell membrane proteins have been shown to carry the unsubstituted TF structure, the high-molecular-weight splicing variant of the cell adhesion molecule MUC1.

Fig. 5. Circulating PNA enhances metastasis in a mouse metastasis model. Representative images show gross appearance of metastases on the surface and on H&E-stained microscopic sections of the lungs from control and PNA treatment animals (A). Mean tumour number on the lung surface in control (n = 9) and PNA-treated (n = 9) animals are shown in (B). (C) Correlation between lung surface tumour nodules and lung weights. **P < 0.001 by two-tailed t-test (normally distributed data with equal variance), scale bar = 500 µm.
CD44(v6) (34) and the transmembrane mucin protein MUC1 (35), both of which are associated with cancer metastasis (36–38). In gastric and colorectal adenocarcinomas, the unsubstituted TF antigen is predominately expressed by cancer-associated MUC1 (35,39,40). As a large and heavily glycosylated transmembrane mucin protein, MUC1 is expressed on the apical surface of most normal epithelia and is overexpressed (up to 10-fold) and aberrantly glycosylated in cancer cells (21). Compared with the expression of MUC1 by normal epithelium, cancer-associated MUC1 loses its apical polarization and becomes expressed over the entire cell surface (40) and also loses its complex O-glycosylation and becomes modified with short oligosaccharides including the galectin-3-PNA-binding ligand TF disaccharide. Thus, PNA appearance in the blood circulation interacts with the MUC1-associated TF on disseminating tumour cells and increases their adhesion to the vascular endothelium and also increases the formation of tumour micro-emboli, two very important steps in the cancer metastasis cascade.

It is noteworthy that galectin-3 has been shown to be able to interact with cancer-associated TF on MUC4 and increase cancer cell adhesion to vascular endothelial cell, just like galectin-3-MUC1 interaction (23). It is therefore possible that the presence of PNA in the circulation of cancer patients, like circulating galectin-3, may interact with TF antigen on cancer-associated MUC4 and contribute to metastasis.

It should also be mentioned that lengthy presence of PNA in the circulation after peanut ingestion by cancer patients may, like circulating galectin-3 (24), interact with the blood vascular endothelium and induce changes in the endothelium that might contribute to metastasis. Thence, the appearance of PNA in the bloodstream after peanut consumption by cancer patients mimics the actions of endogenous TF-binding human galectin-3 and promotes disseminating tumour cell metastatic spread. As metastasis accounts for the majority of cancer-associated fatality, regular consumption of peanut by cancer patients would therefore be expected to have an adverse consequence on cancer survival. Although there is large body of epidemiological evidence linking diet and cancer causation (1), little is known of the influence of diet on progression of cancer once established. Intake of a Western dietary pattern (high intakes of meat, fat, refined grains and sugar desserts) (2), and a higher dietary glycemic load after cancer diagnosis, has been reported to be associated with increased risk of recurrence and mortality among patients with stage III colon cancer (3), although mechanisms are unclear. This study indicates a likely adverse impact on risk of metastasis, particularly if they recognize oncogenic carbohydrate ligands.

**Supplementary material**

Supplementary Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/

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