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Journal of Nutritional Biochemistry 23 (2012) 452–457

Journal of Nutritional Biochemistry

Docosahexaenoic acid inhibits invasion of human RT112 urinary bladder and PT45 pancreatic carcinoma cells via down-modulation of granzyme B expression☆

Donatella D'Eliseo[a,b], Laura Manzi[a], Nicolò Merendino[a], Francesca Velotti[a,b,*]

[a]Department of Ecology and Sustainable Economic Development (DECOS), Tuscia University, Viterbo, Italy
[b]Centro Ricerca Sperimentale (CRS), Regina Elena Cancer Institute, Rome, Italy

Received 8 October 2010; received in revised form 11 January 2011; accepted 28 January 2011

Abstract

Fish oil-derived *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs) inhibit invasion of some tumor cell types in vitro and in vivo. The mechanisms underlying this activity are unclear. Here, we examined the capability of *n*-3 PUFA–docosahexaenoic acid (22:6*n*-3; DHA) to affect the invasiveness of human RT112 urinary bladder and PT45 pancreatic carcinoma cell lines in vitro and the mechanism underlying this activity; *n*-6 PUFA–arachidonic acid (20:4*n*-6; AA) served as control. We showed that, in contrast to AA, 25, 50 and 100μM DHA significantly inhibited in a dose-dependent manner the invasion through Matrigel of both RT112 and PT45 cells. Then, we analyzed whether the serine proteinase granzyme B (GrB), originally known as cytotoxic molecule of lymphocytes and recently also characterized for its extracellular functions such as invasion promotion of bladder cancer cells, might be involved in the invasion inhibitory activity exerted by DHA. We demonstrated that, accordingly to RT112 bladder cancer cells, PT45 cells expressed GrB and GrB promoted their invasion, since stealth RNA interference-mediated down-regulation of GrB dramatically suppressed PT45 cell invasion. Notably, we also showed that, in contrast to AA, 25, 50 and 100μM DHA induced a dose-dependent down-modulation of GrB expression in both RT112 and PT45 cells. In conclusion, DHA can reduce the invasive phenotype of bladder and pancreatic carcinoma cells, and we provide the first evidence for a possible causative role of GrB in DHA-induced inhibition of cancer cell invasion. The potential use of fish oil as adjuvant antibladder and antipancreatic cancer agent may be suggested.

Keywords: Docosahexaenoic acid (DHA); Granzyme B (GrB); Invasion; Extracellular matrix (ECM); Bladder cancer; Pancreatic cancer

1. Introduction

Epidemiological and laboratory studies have shown that dietary *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs), especially docosahexaenoic (22:6*n*-3, DHA) and eicosapentaenoic (20:5*n*-3, EPA) acids, present in fatty cold-water fish and fish oils are able to exert beneficial effects in inhibiting the incidence and the progression of a series of human diseases including cancer [1]. In contrast, *n*-6 PUFAs, such as arachidonic acid (20:4*n*-6; AA), have been shown to have no impact or an enhancing effect on cancer disease [1].

Different biological activities exerted by *n*-3 PUFAs, in particular by DHA, have been proposed to underlie the anticancer effect. Accumulating body of evidence show that DHA both inhibits proliferation and promotes apoptosis of a variety of cancer cells in vitro and in vivo [2]. These effects have been directly or indirectly linked to the anti-inflammatory and immunomodulatory activities exerted by DHA (such as the inhibition of proinflammatory eicosanoid and cytokine production) [3,4], as well as the induction of intracellular oxidative stress (such as the enhancement of lipid peroxidation) [2,5]. In this regard, we have previously shown that DHA is capable of inhibiting the growth and promoting apoptosis in pancreatic carcinoma cells through the induction of an active extrusion process of reduced glutathione (GSH), leading to GSH intracellular depletion and oxidative stress [2,6,7]. There is also evidence that *n*-3 PUFA intake inhibits tumor metastases in vivo in human and murine breast [8,9], colon [10] and lung [11] carcinomas. The metastatic process involves a complex series of events, first of all, the invasion of the surrounding tissues by tumor cells. In particular, DHA has been shown to be capable of reducing the in vitro invasive phenotype of human melanoma [12], breast [13,14] and renal [15] carcinoma cells, which implies that DHA could modify the tumor cell metastatic potential. The mechanisms by which DHA can directly affect the invasive phenotype of cancer cells remain unclear. A key event in tumor cell invasion is the extracellular matrix (ECM) remodeling by tumor cells through proteolysis of basement membrane. A certain number of proteinases have been shown to have ECM degradation capabilities, and their biological relevance in tumor progression is extensively investigated [16–18].

The chymotrypsin-like serine proteinase granzyme B (GrB) is mainly known as a major component of cytoplasmic granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells [19–22]. It

☆ This work was supported by Ministero dell'Università e della Ricerca Scientifica e Tecnologica and Tuscia University, Viterbo, Italy.

* Corresponding author. Department of Ecology and Sustainable Economic Development (DECOS), Tuscia University, Largo dell'Università, Blocco C, 01100 Viterbo, Italy. Tel.: +39 0761 357035; fax: +39 0761 357134.
E-mail address: velotti@unitus.it (F. Velotti).

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doi:10.1016/j.jnutbio.2011.01.010

is released from cytotoxic lymphocytes by granule exocytosis, enters the target cell and induces apoptosis via proteolysis of key intracellular substrates [19–22]. Although GrB expression was originally thought to be restricted to lymphoid cells, it is now known that GrB can be also expressed by inflammatory nonlymphoid hematopoietic cells, as well as by some noncytotoxic nonhematopoietic cells [18,23–27]. Furthermore, an accumulating body of evidence shows that human GrB possesses, besides the above-mentioned intracellular apoptosis function, also extracellular functions such as inflammatory [26,27] and tissue remodeling [24,25] activities. We have recently demonstrated that GrB is expressed by bladder cancer cells in vitro and in vivo [18]. We have also shown that tumor-expressed GrB is capable of degrading ECM components such as vitronectin and promoting invasion of bladder cancer cells [18].

Recently, Kun *et al.* [28] have found that dietary *n*-3 PUFAs inhibit the expression of GrB in a rat model of small bowel transplant chronic rejection. We might assume that DHA can be responsible for the *n*-3 PUFA-mediated down-regulation of GrB and that, among the anti-inflammatory and immunomodulatory activities exerted by DHA, the down-modulation of GrB expression might also be included.

Taken together, these considerations prompted us to investigate whether DHA is capable of inhibiting the invasiveness of bladder and pancreatic carcinoma cells and whether the serine proteinase GrB can be implicated in the mechanism underlying the invasion inhibitory activity exerted by DHA.

We believe a better understanding of the mechanism underlying the anticancer activity exerted by *n*-3 PUFAs, such as DHA, should aid in the development of new therapeutic strategies involving the use of fish oil as anticancer supplement.

2. Material and methods

2.1. Chemicals and antibodies

DHA and AA were purchased from Sigma-Aldrich Chemical (St. Louis, MO). The protease inhibitor mixture "Complete" was acquired from Roche Diagnostic (Mannheim, Germany). The monoclonal antibodies (mAbs) for GrB were purchased from Chemicon (clone 2C5/F5; Prodotti Gianni, Milan, Italy) and Caltag (clone GB12; Walter Occhiena, Turin, Italy) and that for β-actin from Sigma-Aldrich (Ac-40). The polyclonal antibody anticalnexin CT was purchased from Stressgen Bioreagents (Enzo Life Sciences International, Plymouth Meeting, PA).

2.2. Cell culture and treatments

The human RT112 and RT4 urinary bladder cell lines were purchased from American Tissue Culture Collection (Rockville, MD), and the PT45 pancreatic carcinoma cell line was kindly provided by Professor A. Scarpa (University of Verona, Verona, Italy). YT-S is a GrB-positive human cytotoxic NK-leukemia cell line [18]. The cells were cultured in RPMI-1640 (Cambrex Biosciences, Verviers, Belgium) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin and 10 mg/ml streptomycin and incubated at 37°C in a humidified atmosphere containing 5% CO_2. For the experiments, cancer cells were seeded in a subconfluent condition at a density of 3×10^5 per well onto six-well cell culture plates and allowed to adhere for 24 h. Then, the medium was replaced with fresh medium supplemented with 12.5, 25, 50 and 100μM of DHA or AA dissolved in ethanol solution or with ethanol solution alone. At 24 h, cells were detached with trypsin and analyzed.

2.3. Cell growth and viability assessment

Cell growth was assessed determining, in triplicate, the cell number with a Neubauer cell counter (Brand West Germany). Cell viability was assessed by the trypan blue dye exclusion assay.

2.4. Cell invasion assay

For cell invasion, BioCoat Matrigel Invasion Chambers (BD Biosciences, Lexington, KY) were used [18]. Matrigel, a basement membrane matrix, is a solubilized basement membrane preparation extracted from the Engelbreth–Holm–Swarm mouse sarcoma. Cells, in serum-free medium, were added to the upper well of invasion chambers in triplicate, allowed to adhere for 2 h and to migrate toward 10% fetal calf serum for 24 h at 37°C. Cells on the underside of the membrane were fixed, stained with the Diff-Quick staining kit (BD Biosciences) and counted using a light microscope.

2.5. Cell surface protein isolation

Cell surface proteins were biotinylated and isolated using the Pinpoint Cell Surface Protein Isolation Kit (Pierce, Rockford, IL), according to manufacturer's instructions.

2.6. Western blot analysis

Cells were lysed by a solution containing 50 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.5% Triton X-100, 0.5% Na deoxycolate, 0.1% SDS and the protease inhibitor mixture "Complete." Proteins (500μg for bladder and pancreatic cells; 25μg for YT-S cells) were separated by SDS-polyacrylamide gel, blotted into nitrocellulose (Schleicher & Shyell, Dassel, Germany) and incubated with anti-GrB 2C5/F5 or anti-β-actin Ac-40 mAb, or anticalnexin CT polyclonal antibody. The reaction was revealed by horseradish peroxidase-coupled secondary reagents (Pierce) and developed using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL).

2.7. Immunofluorescence and flow cytometric analysis

For total cellular GrB expression, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% saponin in PBS. Permeabilized and nonpermeabilized cells were incubated with anti-GrB-PE (clone GB12) mAb or the related isotype control antibody. Flow cytometry was performed with a FACSCalibur (BD Biosciences). At least 5×10^4 events were acquired and analyzed using CELLQuest software.

2.8. Cell transfection and RNA interference

Transient transfection was performed using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Stealth RNA interference (RNAi) (Invitrogen Life Technologies) is a modified small interfering RNA with enhanced stability and was performed as previously described [18]. Three 25-mer stealth RNAi duplexes targeting human GrB and control stealth RNAi were obtained by the BLOCK-iT RNAi design program (Invitrogen Life Technologies) for stealth RNAi duplexes 1: 5′-CCU ACA UGG CUU AUC UUA UGA UCU G-3′ and 5′-CAG AUC AUA AGA UAA GCC AUG UAG G-3′; for stealth RNAi duplexes 2: 5′-GCG AAU CUG ACU UAC GCC AUU AUU A-3′ and 5′-UAA UAA UGG CGU AAG UCA GAU UCG C-3′; and for stealth RNAi duplexes 3: 5′-GCC UGC ACC AAA GUC UCA AGC UUU G-3′ and 5′-CAA AGC UUG AGA CUU UGG UGC AGG C-3′. A pool of three different nonsilencing, nonoverlapping RNAi duplexes with matched GC content, served as control stealth RNAi (Invitrogen Life Technologies). Construct efficacy was tested by GrB- and control-stealth RNAi cell transfection and Western blot analysis. After transfection (24 h optimal time), cell invasion was assessed as mentioned earlier.

2.9. Statistical analysis

Each experiment was repeated at least three times, and the results of one representative experiments are shown. Experiments were performed in triplicates, and all data are expressed as means±S.D. Student's *t* test was used for all analyses. A statistical probability of $P<.05$ was considered significant.

3. Results

3.1. DHA inhibits invasion of bladder and pancreatic carcinoma cells

To investigate the effect of DHA on the invasion capability of the human RT112 bladder and PT45 pancreatic carcinoma cell lines, cells were treated with 12.5, 25, 50 and 100μM of DHA for 24 h and cell invasion was assessed. The *n*-6 PUFA–AA was used as a control for the *n*-3 PUFA–DHA. The concentrations of PUFAs used in these experiments were found to have no effects on cell proliferation, as verified by cell count and by the viability assay (data not shown). As shown in Fig. 1A,B, 25, 50 and 100μM of DHA significantly inhibited invasion of both cancer cell lines through Matrigel, the basement membrane component substrate. In fact, 25, 50 and 100μM of DHA induced invasion inhibition of 57%, 87% and 94% in RT122 cells (Fig. 1A), as well as of 47%, 50% and 81% in PT45 cells (Fig. 1B), respectively. In contrast, treatment with AA had no effect on the invasiveness of both cancer cell lines at any dose tested (Fig. 1A,B). These results show that DHA, used at concentrations that do not alter cell proliferation, is capable of reducing in a dose-dependent manner the invasive phenotype of both bladder and pancreatic cancer cells.

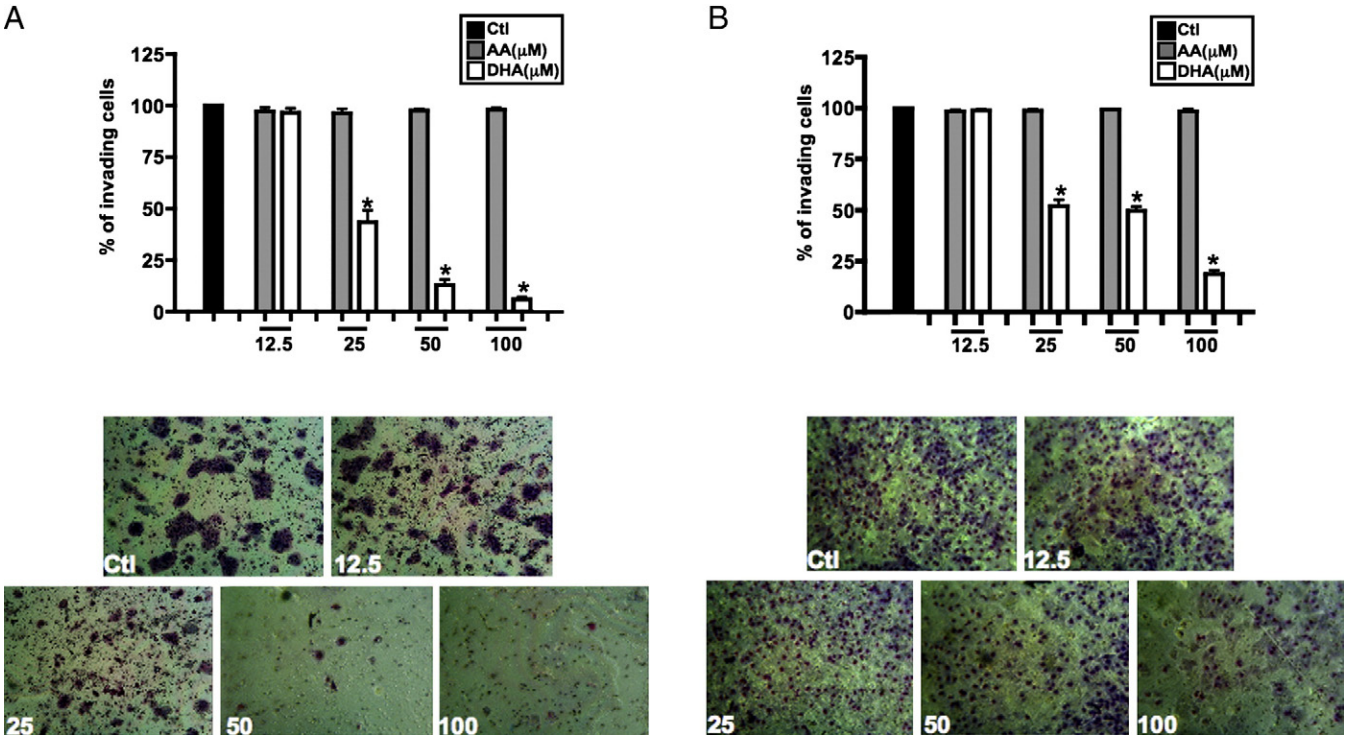

Fig. 1. Effect of DHA on the invasiveness of the human (A) RT112 bladder and (B) PT45 pancreatic cancer cell lines. Cancer cells were treated with 12.5, 25, 50 and 100μM DHA or AA dissolved in ethanol solution or with ethanol solution alone (Ctl) for 24 h, and cell invasion was assessed. Representative experiments (bars, S.D.) out of at least three. *P<.05.

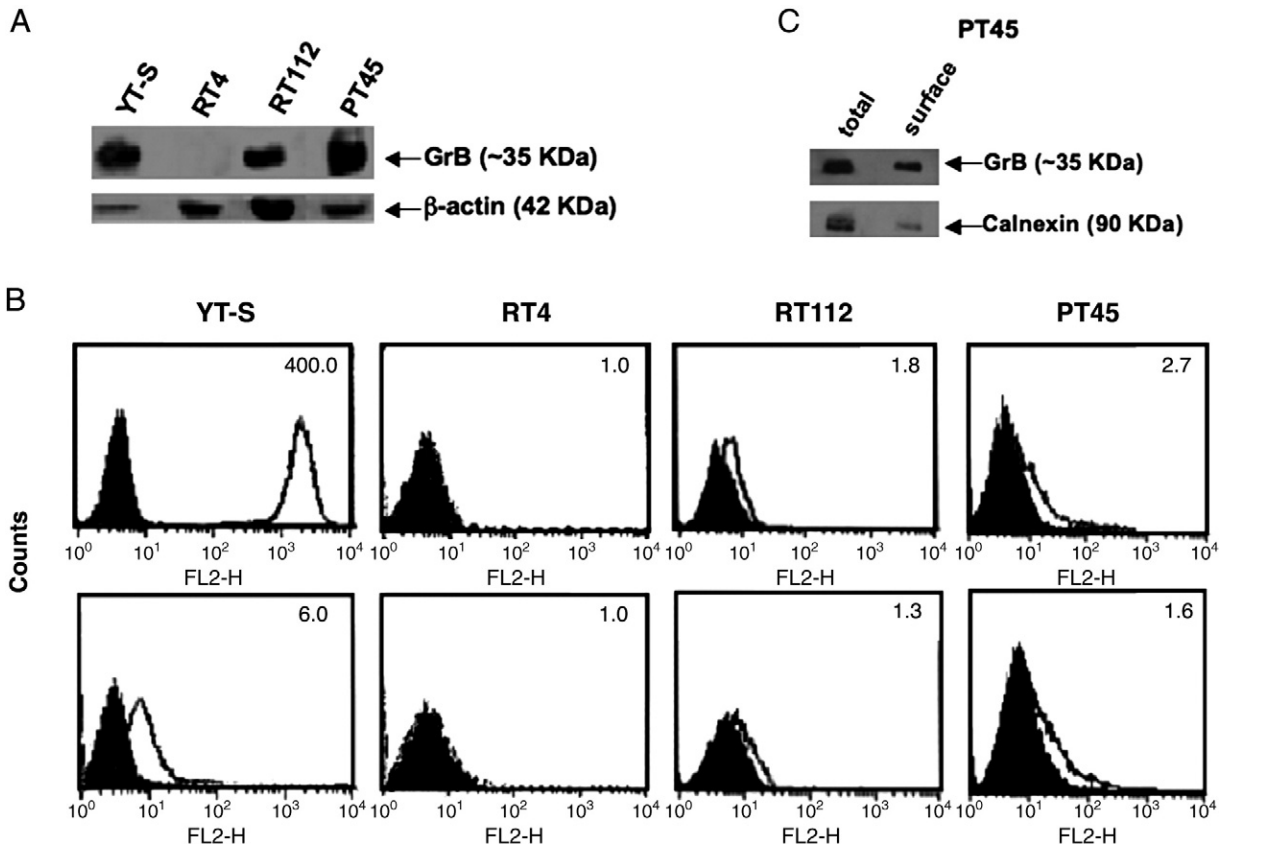

Fig. 2. GrB expression in the PT45 pancreatic cancer cell line. (A) GrB protein was analyzed by Western blot using the anti-GrB 2C5/F5 mAb in total cell lysates; β-actin was used as loading control. (B) Immunofluorescence and flow cytometric analysis on permeabilized (upper) and nonpermeabilized (lower) cells, using the anti-GrB-PE GB12 mAb or the related isotype control antibody; numbers indicate mean fluorescence intensity (MFI) ratio, calculated as the ratio between MFI of positive cells and MFI of controls. YT-S and RT112 cells were used as positive controls and RT4 as negative control. (C) Western blot using the anti-GrB 2C5/F5 mAbin total cell lysate (total) and in surface proteins (surface) isolated by the biotinylation technique; calnexin was used as intracellular protein control. Representative experiments out of three.

3.2. Pancreatic carcinoma cells express GrB and stealth RNAi-mediated down-regulation of GrB suppresses pancreatic cancer cell invasion

We have recently shown that bladder cancer tissues and a panel of bladder cancer cell lines, including RT112, express GrB and that tumor-expressed GrB promotes bladder cancer cell invasion [18]. Here, we investigated whether GrB is also expressed in the PT45 pancreatic carcinoma cell line and whether GrB is involved in its invasion process.

GrB expression was investigated by Western blot analysis on total cell lysates, using the anti-GrB 2C5/F5 mAb [18]. The GrB-positive NK-like cytotoxic YT-S and bladder cancer RT112 cells were used as positive controls, while the GrB-negative noninvasive bladder cancer RT4 cell line as negative control. As shown in Fig. 2A, a band at ~35 kDa corresponding to GrB and also present in YT-S and RT112 cells was detected in PT45 pancreatic cancer cells. In contrast, as previously shown [18], RT4 cells were negative. To investigate the possible extracellular function of GrB, the expression of GrB was analyzed both in the culture supernatant and to the surface of RT112 and PT45 cancer cell lines. YT-S and RT4 cells were used as positive and negative controls, respectively. Using an ELISA for GrB, we found that, in contrast to cytotoxic YT-S lymphocytes, no GrB could be detected in the culture supernatants of bladder and pancreatic cancer cells (data not shown). However, performing immunofluorescence on both permeabilized and nonpermeabilized cells, a surface staining for GrB was observed in both RT112 and PT45 cell lines, as well as in YT-S cells (Fig. 2B). As expected, RT4 cells resulted negative for both total and surface GrB (Fig. 2B). To confirm the identity of cell surface GrB, Western blot was performed on surface proteins isolated from PT45 cells by the biotinylation technique. As shown in Fig. 2C, a migration band at ~35 kDa corresponding to GrB appeared in the surface protein preparation and also in the related total cell lysate. We also assessed that the surface protein preparation was almost completely devoid of intracellular proteins by incubation of the stripped membrane with anticalnexin CT polyclonal antibody (Fig. 2C). Altogether, these results can be explained by the fact that, since we have previously shown (by GrB-ELISA on total cell lysates) that noncytotoxic tumor cell lines contained from ~390 to ~30 times less GrB than cytotoxic YT-S lymphocytes [18], GrB protein levels released in the supernatant by the tumor cells might be not high enough to be detected by the ELISA. Indeed, the fact that GrB is partially expressed to the cell surface membrane might reflect either a step of GrB extracellular release or a cell surface GrB localization. In any case, GrB to the cell surface membrane supports its potential extracellular function.

To verify the role of GrB in the invasion of PT45 cells, stealth RNAi-mediated GrB down-regulation was performed in cancer cells and their invasion capability was tested. RT112 cells were used as control. Fig. 3A showed that, similar to RT112, transfection of PT45 cells with GrB-targeted stealth RNAi (GrB-RNAi), but not with control stealth RNAi (Ctl-RNAi), noticeably reduced GrB protein levels. The analysis of the invasion capability of stealth RNAi-transfected cancer cells showed, accordingly to RT112 cells, a dramatic suppression (65%) of invasion in the PT45 cell line transfected with GrB-RNAi compared to Ctl-RNAi-transfected cells (Fig. 3B). These results indicate a role for GrB in promoting the invasion not only of bladder but also of pancreatic cancer cells.

3.3. DHA inhibits GrB expression in bladder and pancreatic carcinoma cells

To analyze whether GrB might be implicated in the inhibition of invasion induced by DHA, we analyzed whether DHA could modulate the expression of GrB in cancer cells. To this purpose, RT112 and PT45 cells were treated with 12.5, 25, 50 and 100μM of DHA for 24 h and GrB expression was assessed by Western blotting. AA was used as a control for DHA. As shown in Fig. 4A,B, 25, 50 and 100μM of DHA inhibited in a dose-dependent manner GrB expression in both RT112 and PT45 cells, respectively. In contrast, treatment with AA had no effect on both cancer cell lines at any dose tested (Fig. 4A, B). Moreover, the comparison of these results (Fig. 4A, B) with those concerning the invasiveness of bladder and pancreatic cancer cell lines following treatments with DHA and AA (Fig. 1A and B) showed that the expression of GrB paralleled the invasion capability of both cancer cell lines.

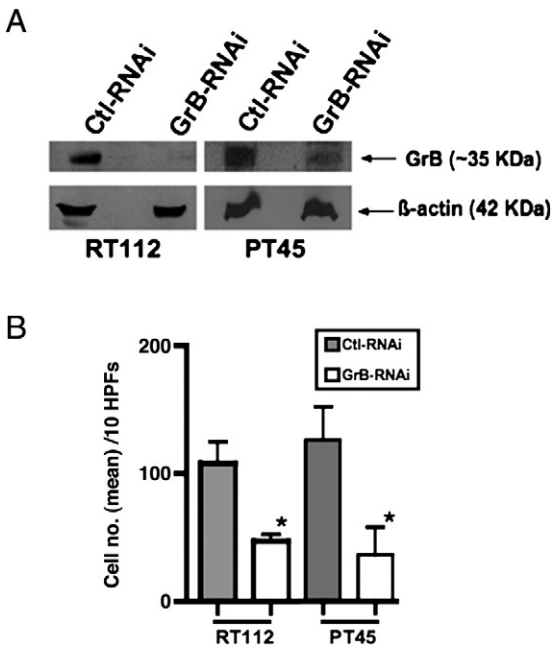

Fig. 3. Effect of loss of GrB function on the PT45 pancreatic cancer cell invasiveness. Loss of GrB function was performed by stealth RNAi-mediated GrB down-regulation. (A) Western blot analysis, using the anti-GrB 2C5/F5 mAb, in cell lysates transfected with GrB-targeted stealth RNAi (GrB-RNAi) or control stealth RNAi (Ctl-RNAi); representative experiment (bars, S.D.) out of three (B) invasion assay using cells transfected with GrB-targeted stealth RNAi (GrB-RNAi) or control stealth RNAi (Ctl-RNAi); representative experiment (bars, S.D.) out of four. The RT112 bladder cancer cell line served as control. *$P<.05$.

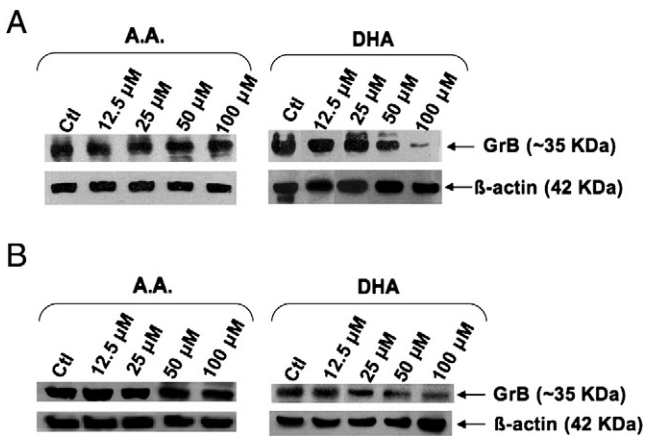

Fig. 4. Effect of DHA on the expression of GrB in the human (A) RT112 bladder and (B) PT45 pancreatic cancer cell lines. Cancer cells were treated with 12.5, 25, 50 and 100μM DHA or AA dissolved in ethanol solution or with ethanol solution alone (Ctl) for 24 h, and GrB protein was analyzed by Western blot using the anti-GrB 2C5/F5 mAb in cell lysates; β-actin was used as loading control. Representative experiments out of three.

4. Discussion

Tumor invasion and metastasis are the most life-threatening aspects of different types of tumors, including bladder and pancreatic tumors, and are responsible for the high mortality rates among cancer patients. Indeed, although the overall incidence of urinary bladder and pancreatic cancers is approximately 22–24 and 8–10 cases, respectively, per 100,000 person per year, both tumors represent the fourth leading cause of cancer related death [29,30]. These data largely depend on the invasive and metastatic phenotype of cancer cells composing the tumor at the time of diagnosis. In fact, for bladder urothelial carcinomas (UCs) (usual type of bladder cancers), at the time of diagnosis, more than 70% of patients have a papillary non-invasive UCs, which progress to invasive disease only infrequently. Thus, most of the mortality occurs in the other 20%–30% of patients who present with invasive UCs, characterized by a high metastatic rate [29]. On the other hand, for pancreatic cancer (notoriously difficult to diagnose in its early stages), at the time of diagnosis, 52% of patients have distant disease and 26% have regional spread; thus, the mortality rate for all patients is very high [30]. Therefore, the investigation on the mechanisms underlying the invasive and metastastic phenotypes of cancer cells as well as on the possible therapeutic agents affecting these phenotypes is crucial to decrease the mortality rate for cancer disease.

In this study, we show that DHA, at concentrations that do not affect cell proliferation and achievable in vivo [31], inhibits the invasion of RT112 and PT45 cell lines through Matrigel reconstituted basement membrane, thus affecting the metastatic potential of both bladder and pancreatic cancer cells. Therefore, we can add two new types of carcinomas to the list of tumors sensible to the anticancer activity exerted by DHA. Our data are in agreement with Blanckaert *et al.* [14], who have shown that the invasive phenotype of the human breast carcinoma cell line MDA-MB-231 was markedly decreased following cell incubation with 100μM of DHA for 24 h, whereas they could not observed any effect when cells were treated with 20μM of DHA whatever the incubation time.

The invasion process involves a complex series of events regulated by a variety of molecules, including proteinases, which enable cancer cells to degrade the various components of ECM, thus invading the surrounding tissues [16,17]. We have recently shown that bladder cancer cells express the serine proteinase GrB in vitro and in vivo and that tumor-expressed GrB promotes invasion of bladder cancer cell lines in vitro [18]. The role of GrB in bladder cancer cell invasion has been also supported by the finding of a significant association between GrB expression in vivo and tumor epithelial–mesenchymal transition (a key event in invasion of several carcinomas) [18,32], as well as the pathological tumor spreading [18]. In the present work, we showed that, besides bladder cancer cells, the PT45 pancreatic cell line also expresses GrB. In addition, accordingly to bladder cancer cells, GrB loss of function (by stealth RNA interference-mediated down-regulation of GrB) significantly suppressed pancreatic cancer cell invasion, indicating that GrB promotes the invasion of PT45 pancreatic cancer cells. These data suggest that GrB could be implicated in the invasion of more than one cancer cell type.

Finally, in agreement with Kun *et al.* [28], who have shown that dietary *n*-3 PUFAs inhibit GrB expression in rats, we demonstrated that DHA induced down-modulation of GrB expression in both RT112 and PT45 cancer cells. We also observed that this modulation paralleled cancer cell invasion inhibition by DHA. However, the fact that DHA's effect on GrB expression is low at 25μM (Fig. 4), whereas at the same DHA concentration, the effect on cell invasion is significant (Fig. 1), might suggest that other molecules besides GrB can be implicated in DHA-mediated inhibition of invasiveness. Taken together, we provide the first evidence for the involvement of GrB in the inhibition of cancer cell invasion induced by DHA, indicating that, among the anti-inflammatory and immunomodulatory activities exerted by DHA, the negative regulation of GrB expression can also be included. Our findings lay down a novel and solid foundation for future studies aiming to investigate the molecular mechanisms that underlie the modulation of GrB expression by DHA.

We can speculate on other possible important consequences derived from the negative regulation of GrB expression by DHA. Extracellular GrB has been previously shown to induce perforin-independent death of primary vascular smooth muscle and endothelial cells through anoikis [33]; this activity might allow tumor cells to infiltrate lymphatic and blood vessels, thus promoting tumor metastasis. Moreover, matrix proteins (*e.g.*, vitronectin, fibronectin) have been previously shown to provide a co-stimulatory signal for CTL activation [25]; therefore, ECM protein cleavage by tumor-expressed GrB might down-modulate the antitumor CTL response. Furthermore, GrB-mediated ECM degradation has been proposed to result in the release of biologically active proteolytic fragments able to recruit inflammatory cells [25]; the induction of inflammation by tumor-expressed GrB might potentiate tumor development. Therefore, the inhibition of the above-mentioned GrB-mediated activities by DHA might also be implicated in the anti-inflammatory, immunomodulatory and anticancer effects induced by DHA.

In summary, we showed that DHA is capable of reducing the invasive phenotype of both bladder and pancreatic cancer cells, and we provide the first evidence for a possible causative role of GrB in DHA-induced inhibition of cancer cell invasion. Our data provide new insights into the mechanisms implicated in the anticancer activity exerted by DHA and suggest a potential use of fish oil as an adjuvant antibladder and antipancreatic cancer agent.

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