IN VITRO PROTECTIVE EFFECT OF CELERGEN, A BIOACTIVE MARINE COMPOUND, ON INTERLEUKIN-6-RELATED INVASIVENESS OF PANCREATIC CANCER

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Abstract: The purpose of this study was to assess the effect of Celergen, a marine nutraceutical, against tumor cell invasion in human pancreatic cancer cell line (PSN-I). High invasive clone (HI) and low invasive clone (LI) were established from wild type PSN-I cell line after a repeated invasion assay test. The invasive ability of HI cells and the level of IL-6 in the conditioned medium of HI cells was significantly higher than that one of LI cells but both these parameters were significantly reduced by the addition of Celergen (p<0.01). Exogenous IL-6 administration induced a dose dependent enhancement of invasive ability in both cell populations. Moreover, IL-6 receptor expression was detected in 72% of HI cells whereas this occurred only in 37% of LI cells. When co-cultured with Celergen this parameter was significantly downregulated in both cellular subsets (p<0.05). The addition of conditioned medium derived from HI cells (HCM) and LI cells (LCM) enhanced the invasive ability in both cell populations without affecting cell proliferation. The effect of HCM on the invasive ability of HI cells was partially inhibited by the addition of Celergen (p<0.01). In summary, overexpression of IL-6 and its receptor may be one relevant factor
contributing to the highly invasive characteristic of the pancreatic cancer cell line we used while a significantly beneficial modulation was obtained by applying this novel marine nutraceutical. This advices to further explore the possibility of marine compounds regulation of IL-6 ligand/receptor and other possible invasive factor interaction in the therapy of this malignancy while further studies are awaited in this setting.

**Keywords:** pancreatic cancer, invasiveness, interleukin-6, inteleukin-6 receptor, Celergen, marine compound

**Short title:** Fish protein hydrolysate in pancreatic cancer invasiveness

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**INTRODUCTION**

Pancreatic cancer fourth leading cause of cancer-related death in men and women in the USA (1,) but the five-year survival rate is less than 5% despite surgery or combined surgery and adjuvant therapy (2). This brings to the consideration that the post-surgery prognosis is very unfavorable compared to that of other cancers of the digestive tract (3). This poor prognosis is mainly due to the tendency of pancreatic cancer cell to aggressively invade adjacent tissues and organs and
metastasize to the liver and lymph nodes even though the primary lesion is relatively small. Thus, aggressive invasion of pancreatic cancer cells from the primary lesion to adjacent tissue is usually expected and the survival for metastatic pancreatic cancer remains poor and less than 20% survive at the end of 1 year (2) and this causes an estimated 227,000 deaths per year (4).

To elucidate some of the mechanisms of distant metastasis in pancreatic cancer, we studied the extravasation of a human pancreatic cancer cell line PSN-1 whose high propensity of extravasation and release of vasodestruction factor has been shown in the past (5). Very recently it has been demonstrated that high IL-6 levels in patients with pancreatic cancer are poor prognostic factors leading to shortened overall and progression-free survival, a reduction in the tumour control rate while also predicting the efficacy of gemcitabine treatment (6). Furthermore, it was reported that serum IL-6 levels are highly elevated in pancreatic cancer patients compared to that in patients with cancers of other digestive organs (7). IL-6 is known as a multi-potent cytokine and it has been reported to promote proliferation of cancer cells in several tumors (8). Indeed, the group of Huang and colleagues have very recently reported that IL-6 is able to induce cten expression and factors known to promote cell migration, as well, the invasion ability of Caspan-2 cells (9, 10). Taken together, IL-6 is undoubtedly involved in the highly invasiveveness characteristic of pancreatic cancer; however, the relationship between IL-6 and invasive ability of pancreatic cancer cells has not been fully clarified. Therefore, we investigated the effect of IL-6 on the invasiveness of pancreatic cancer cells in vitro using a human pancreatic cancer cell line PSNI. On the other hand, we wanted to ascertain whether Celergen, a popular marine compound, could beneficially affect pancreatic cancer invasiveness. This is because this nutraceutical containing DNA, collagen elastin and protein extracts has been recently shown to exhibits strong skin fibroblasts protecting effect against mutagenic UV irradiation (11).

MATERIAL AND METHODS
Test compound.

Celergen was obtained by Swisscap company (100mg composition: DNA Extract from fish milt 46mg, fish collagen hydrolysate plus fish elastin 35mg, whole fish protein hydrolysate 6mg, lutein-coenzyme Q10-selenium 11mg). As a pre-requisite to proceed with the study, samples were blindly sent to an officially GLP registered toxicology laboratory which found no traceable amounts of heavy metals including organic and inorganic arsenic, having set a threshold of > 5ppm (Redox Lab, Monza, Italy, report n. 2013001054/LAB).

Culture of pancreatic cancer cells

Wild-type PSN-1 (WT), a cell line established from human pancreatic cancer, cells was cultured in RPMI l640 medium (Gibco BRL, Grand Island, N.Y., USA) supplemented with 10 % heat-inactivated fetal calf serum (FCS), 100µg/ml of streptomycin and 100 IU/ml of penicillin G (Gibco BRL). Cancer cells were cultured in a humidified atmosphere containing 5 % carbon dioxide at 37°C.

Invasiveness assay

Invasiveness ability of cancer cells in vitro was assessed by the method of Albini et al with some minor modifications (12). Briefly, Transwell booth (Comstar, Cambridge, MA, U.S.A.) with an 8µm pore size polycarbonate membrane filter and coated the upper surface of each filter with 5µg of Matrigel (reconstituted basement membrane; Lexington, MA, U.S.A.) were used. The booth (upper chamber) was placed in a 24-well culture plate (lower chamber). Cancer cells were resuspended to a final concentration of 5 X 10^5 cells/ml in RPMI l640 medium with 0.1 % FCS. One hundred micro liters of cell suspension was added to the upper chamber. The lower chamber contained 600µl of RPMI l640 medium with 0.1 % FCS. The plate was incubated for 6 hr at 37°C. After incubation, cancer cells on the upper surface of the membrane were wiped
off with cotton swabs. The filter was fixed with methanol and stained with hematoxylin and eosin. Cancer cells that had passed through the filter coated with Matrigel to the surface of the lower membrane were counted under a microscope at X 200 magnification. Incubation tests were performed in triplicate. To estimate the effect of IL-6 on cell invasion graded concentrations of IL-6 (Gibco BRL, Grand Island, N.Y, USA.) were added to the upper or lower chambers. After incubation for 6 hr at 37°C, the cells that had passed through the membrane were counted as described above.

**Separation of high and low invasive clones subsets**

To study the distinctiveness of the invading tumor cells, we divided PSN-1 cells via the chemoinvasion assay in the presence of IL-6 (100ng/ml) in RPMI 1640 with 10% FCS. PSN1 cells were seeded at 1 X 10^5 cells/100µl/well into the upper chamber. IL-6 was added at a concentration of 100 ng/ml to the lower chamber. The cells that filtered through the Matrigel-coated 8µm pore-size membrane during the first 4 hr were collected (HI cells) whereas the cell population remaining in the upper chamber for 24 hr were also collected (LI cells). This procedure was performed five times and made possible to established two distinct clones subsets (HI and LI).

**Preparation of cancer cell-derived conditioned medium and invasion ability test**

Cancer cells were seeded into a 100 mm plastic dish and incubated in RPMI 1640 medium with 10% FCS to a semi confluent monolayer state. After removal of the supernatant, cells were washed three times with PBS. Then, the cells were incubated in 10ml of RPMI 1640 medium with 0.5% bovine serum albumin (BSA; Sigma Chemical Company, St.Louis, MO, USA) for 24 hr. The supernatant was collected from PSN-1, LI and HI cells. Supernatants were centrifuged at 1,000 r.p.m. for 30 min, dialyzed against RPMI 1640 medium without FCS for 24 hr, filtered through a
micro filter (pore size 0.22µm; Millipore, Bedford, MA, USA) and stored at - 20°C until use as CM derived from PSN-I subsets, i.e. HI (HCM) and LI (LCM). To examine the effects of Celergen compound on the invasion ability of HI cells with HCM, we prepared Celergen(5µM)-enriched HCM. Protein agarose conjugates (Calbiochem, Cambridge, MA, U.S.A.) were pre-blocked with both BSA and HCM to reduce non-specific binding of immunoglobulin present in HCM. Celergen compound was added to the conjugates and incubated for 4 hr at 4°C. Conjugates were washed three times with PBS, and HCM was added. After incubation for 24 hr at 4°C, the supernatant was retrieved. The supernatant was dialyzed against RPMI 1640 medium, passed through a micro filter and stored at -20°C until use. The concentration of IL-6 in Celergen (50µM) treated HCM was determined by ELISA methods.

To estimate the effect of HI-conditioned medium (HCM) and LI-conditioned medium (LCM) on invasion ability of HI and LI cells, cells were suspended to a final concentration of 1 X 10^6 cells/ml in RPMI 1640 medium without FCS. One hundred micro liter of cancer cell suspension was added to the upper chamber. HCM or LCM was added at a concentration of 50% in RPMI 1640 medium without FCS in the lower chamber. After incubation for 12 hr at 37°C, the cells that had passed through the membrane were counted as described before. RPMI 1640 medium without FCS was used as the negative control.

Measurement of IL-6 in the conditioned medium and effect of Celergen

Measurement of IL-6 in the conditioned medium (CM) was assayed by IL-6 enzyme-linked immunosorbent assay (Elisa) Kit (Genzyme, Cambridge, MA, USA). One hundred micro liters of CM was added into the wells and incubated at 37°C for 30 min. After recovery of contents, test wells were washed for five times. Fifty micromoles of Celergen were added to each well and the plate was incubated at 37°C for 30 min. After retrieval of contents and washing for five times, 100µl/well of avidin reagent was added into each test well. After incubation at 37°C for 15 min,
wells were washed five times with washing reagent. One hundred microliters of substrate reagent were added to each well and the plate was incubated at room temperature for 10 min. Then, one hundred microliters of blocking solution was added and the absorbance assessed at 450 nm.

**Flow cytometric analysis for IL-6 receptor and effect of Celergen**

Flow cytometric analysis was performed to study the expression of IL-6 receptor on LI and HI cells. Cells were prepared as single cell suspension and then washed two times with PBS to remove residual IL-6 in the culture medium. Cells were then resuspended in PBS with 1% BSA with 0.1% sodium azide at a final concentration of 1 X 10^6 cells/ml. Celergen (50µM) was added to the cancer cell suspension and the cells were incubated for 30 min at 4°C. After incubation, cells were washed two times and fluorescence isothiocyanate (FITC)-labeled antimouse IgG antibody (Sigma Chemical Company, St. Louis, MO, USA) was added. After further incubation for 30 min at 4°C, cells were washed twice with PBS and analyzed using a flow cytometer. PBS was used as a negative control against Celergen (50µM) compound.

**Statistical analysis**

Statistical analysis was undertaken using SPSS 13.0 Software. All assays were conducted 3 times and found to be reproducible. Data were expressed as mean ± SD. Statistical correlation of data between groups was checked for significance by Student's t test. A two-tailed p-value of <0.05 was considered statistically significant.
RESULTS

Assessment of IL-6-related invasiveness of PSN-1 cells

The number of PSN-1 cells filtering through the membrane in the absence of IL-6 was 32.3 ± 12.3 /field in the invasion assay system (fig. 1). When IL-6 was added to the lower chamber at concentration of 1, 10, and 100 µg/ml, the number of invading cells was 47.1 ± 13.2, 58.1 ± 17.6 and 81.2 ± 15.4 /field, respectively where exogenous IL-6 showed to significantly induce cell migration in a dose-dependent manner (p < 0.01). On the contrary, when IL-6 was added only to the upper chamber, the number of cells passing through the membrane didn’t show any significant change (data not shown).

Effect of Celergen on the invasiveness behavior of the two distinct clones subsets from PSN-1,

IL-6 content of conditioned medium and IL-6 receptor expression

The number of cells passing through the membrane in HI cells (47.4 ± 11.2 /field) was significantly higher than in LI cells (19 ± 3.4 /field) (p<0.05) while the latter values were comparable to the whole PSN-1 penetrating cells (fig. 2). The co-culture with Celergen (50µM) partly but significantly decreased such phenomenon (p<0.05 vs untreated HI cells).

The concentration of IL-6 in the conditioned medium was 22342 ± 479 ng/ml in HI cells, which was around 13-fold greater than in LI cells (1815 ± 124 ng/ml) (fig. 3). The expression of IL-6 receptor on HI and LI cells was examined by flow cytometric analysis and it appeared to be in 72 ± 11 % of HI cells and 37 ± 16 % of LI cells (fig. 4). When the test was repeated after the addition of 50µM of Celergen, it significantly decreased to 42 ± 8% and in 19 ± 9% in HI and LI subsets, respectively (p<0.05).
Effect of IL-6 on HI and LI cells

On addition of 100 ng/ml of IL-6 to HI and LI cells, the number of invading cells increased significantly (from 57.9 ± 15.8 /field to 123.2 ± 24.6 /field in HI cells (p < 0.001) and 29.7 ± 3.9 /field to 44.8 ± 7.2 /field in LI cells (p < 0.001). When IL-6 was added to HI cells, the invasive ability of HI cells was enhanced in dose-dependent manner (data not shown, p < 0.01). This enhancement was higher in HI cells than LI cells (fig. 5). On the contrary, there was no significant difference in growth rates between HI and LI cells after 48 hr incubation with or without IL-6 administration (data not shown).

Effect of HCM and Celergen-enriched HCM on HI and LI cells invasiveness

After administration of HCM to culture dishes the number of invading cells significantly increased from 1.9 ± 1.1 /field to 21.9 ± 9.7 /field in HI cells (p < 0.005) and from 0.7 ± 0.5 /field to 29.7 ± 11.4 /field (p <0.005) in LI cells (fig. 6). However, these effect was partially mitigated on addition of Celergen with increase to 13.2 ± 11.3 /field in HI cells and to 6.1 ± 4.2 /field (p < 0.05 vs untreated HCM) in LI cells. A significant promoting effect of LCM on LI was shown (p<0.05) although to a lesser extent than with HCM. However, LCM promoting effect on HI was comparable to what occurred with HCM (fig. 6) invasive ability was far less than what observed with HCM (data not shown).

DISCUSSION
Pancreatic cancer is ranked as the fourth leading cause of cancer-related deaths in the United States, and despite intensive basic and clinical research over the last few years, the survival benefit for the majority of patients with pancreatic cancer is still disappointing. Indeed, this is one of the most virulent malignancies, with an overall 5-year survival rate of only 3-5% and a median survival time after diagnosis of less than 6 months. This highly lethal disease is usually diagnosed in an advance stage, when there are few or no effective therapies (4). Even among patients undergoing a potentially curative resection, the long-term outcome remains unsatisfactory because of early recurrence and metastatic disease (3). Several studies investigating cancer cell invasion are indeed focusing as to clarify whether the most relevant detrimental factor is chemotactic or chemokinetic (13-15). In this study the invasion assay using the Boyden booth showed that the number of invading PSN-l cells was not affected by the IL-6 concentration in the upper chamber that directly acts on the cells, but it was dependent on IL-6 concentration in the lower chamber thus suggesting that IL-6 may have chemotactic activity on PSN-l cells. Since IL-6 production in the pancreas is markedly higher than in other organs (7), cancer cells may individually invade the normal pancreatic parenchyma and this is in agreement with the clinical observation of pancreatic cancer cells detected in the interstitium relatively distant from main tumor without forming a colony. Therefore, it was important to investigate which clone of PSN-l cells responded to the chemotactic effect of IL-6. In the present study, two sub-clones based on a difference in invasive ability were established in the presence of IL-6 by the chemoinvasion assay. The biological behavior of these two sub-clones were investigated by examining IL-6 production and IL-6 receptor expression. HI cells exhibited higher expression of IL-6 and its receptor than LI cells. Furthermore, the invasive ability was enhanced by IL-6 in a dose-dependent manner in all clones thus proving that this cytokine is closely involved in the invasive ability of PSN-l cells. It was of interest to note that the addition of Celergen to culture batch significantly decreased the invasiveness of HI cells and this may at least be partly explained by the associated reduction of IL-6 concentration in HCM while LCM had negligible baseline concentration of this cytokine.
Moreover, in both clones the addition of Celergen enabled a significant decrease of IL-receptor expression and this may have further contributed to its beneficial role against cell invasiveness.
This finding is worth of interest since the invasive ability of HI cells which show overexpression of IL-6 receptor was not significantly changed by differences in IL6 concentration. Thus, it may depend on the number of IL-6 receptors on HI cells saturated by IL-6 contained in LCM and the majority of IL-6 in HCM may remain as an unbounded form.
Nakase et al. has shown that the serum IL-6 level is markedly elevated after pancreatic surgery regardless of whether the malignancy of the tumor compared to serum IL-6 levels after other organ surgery (16). This finding suggested that cancer cells are very likely to be induced to invade by the overexpressed IL-6 around the primary lesion if IL-6 is chemotactically involved in pancreatic cancer invasion.
Marine drugs are receiving an increasing attention in this field and, for instance, in chemically induced pancreatic carcinogenesis models, fish oils has been shown to be capable of reducing the incidence and progression of pancreatic cancers (17) and this finding has very recently received a partly confirmatory report from an human trial where treatment with gemcitabine plus i.v. n-3FA reduced the concentration of angiogenetic and proinflammatory markers (18).
Understandably, other factors are involved in the invasive ability of these clones and indeed not only HCM but also LCM that contains less than one tenth of IL-6 than HCM significantly enhanced HI cells invasion at a comparable extent. But also when applied to LI cells(fig. 6), p<.005 vs HCM) As a matter of fact, there is a well-established link between pro-inflammatory circulating cytokines, growth and motility factors and the development of cancer. One possibility to explain this phenomenon is the production of soluble IL-6 Receptor (sIL-6R). It was reported that increased IL-6 production after stimulation by IL-1 or TNFa result in complex formation with sIL-6R with growth enhancement in Kaposi’s sarcoma (19). This IL-6/sIL-6R complex can bind to gap130, which is a relevant signal transduction receptor component in conjunction with IL-6 family, without IL-6R and can induce intracellular signal. Aberrant overexpression of IL6/ sIL-6R complex in HCM may induce strong invasive activity in LI cells.
Therefore, the present data suggest that Celergen, a marine bioceutical, may exert a beneficial regulation of IL-6 ligand/receptor interaction which may warrant further studies, given the presence of other cancer-promoting cofactors whose role needs to be tested as yet.

A final note has to be drawn on the issue of the potentially deleterious effect of seafood and seafood-derived compounds regarding their arsenic content, as suggested by the albeit isolated clinical report of Chua et al. (20). However, besides the untraceable amounts of arsenic (total, inorganic and organic) reported in our sampled compound, a similarly seafood compound bashing in another isolated case report study (21) has heralded severe criticisms and dismissed as seriously flawed by subsequent authoritative reviews (22-24). As a matter of fact, the Food Chemicals Codex has set a limit of 3 ppm inorganic arsenic but clarifying that there is no limit for total or organic arsenic compounds (25). Indeed, the toxicologic property of organic arsenic compounds are profoundly dissimilar from those of inorganic arsenic, issue which was totally neglected by Chua et al (20) rendering their report inconsistent and misleading. Inorganic arsenic is significantly more toxic than the pentavalent organic arsenic compounds, arsenosugars and arsenobetaine, being harmless substances normally found in seafood (26, 27). For instance, FDA guidance (28) points out a level of concern for total arsenic in crustaceans as 86 ppm, a concentration which almost 7 times higher than the amount found in the marine product Celergen by Chua et al. (20).

While our study needs further research and, our general conclusion is that marine compounds, if properly regulated in their farming, collection and processing maintain a promising potential as a safe source of novel drugs.

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