Human pancreatic phospholipase A_2 stimulates the growth of human pancreatic cancer cell line

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Abstract **Phospholipase A_2 (PLA_2) from human pancreas, designated $hPLA_2$-I, functions as a digestive enzyme. Interestingly, the present study demonstrated that the mature form of $hPLA_2$-I stimulated the growth of a human pancreatic cancer cell line MIAPaCa-2, whereas the pro-form was ineffective. PLA_2s from *Laticauda semifasciata* fraction I, *Crotalus adamanteus* venom, *Streptomyces violaceoruber* and bee venom, showed no proliferative effect to the growth of MIAPaCa-2. The Scatchard plot analysis revealed that the MIAPaCa-2 cell had a specific binding site for the mature $hPLA_2$-I. The equilibrium binding constant (K_d) and the maximum binding capacity (B_{max}) were 2.6 nM and 0.4 fmol/10^6 cells, respectively. These results suggest that the mature $hPLA_2$-I, but not the pro-form, may function as a growth factor of pancreas carcinoma via the specific binding site.**

Key words: Pancreatic phospholipase A_2 (PLA_2); Pancreatic cancer cell line; Proliferation; Receptor for PLA_2

1. Introduction

The incidence of pancreatic carcinoma in Japan has risen sharply from 1.8 to 5.2 per 100,000 people during 1960–1985 [1]. In the United States, the ranking of the same carcinoma is the fourth of fifth leading cause of cancer death with an overall 1-year survival of only 12%. Judging from our observation that the outlook for patients after the diagnosis of pancreatic carcinoma has been poor, it is suggested that there are some mechanisms which play important roles in dramatically promoting the growth of pancreas carcinoma [2]. Gastrin, cholecystokinin, and epidermal growth factor, in fact, have recently been reported to stimulate the proliferation of pancreatic adenocarcinoma cells in tissue culture [3,4].

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the fatty acid at the *sn*-2 position in phospholipids to liberate free acids and lysophospholipids [5]. PLA_2s have been isolated from several mammalian sources as intracellular or extracellular enzymes [6]. The extracellular 14-kDA PLA_2s have been classified into three groups, according to characteristics in their primary structures [6]. The enzyme, included into group I, exists in the pancreatic juice, kidney, small intestine, stomach, and lung from various animal specimens [7]. In those organs, not only the function of PLA_2-I as a digestive enzyme but also any other functions have been suspected: it has been suggested that a receptor for PLA_2 is present on the surface of mammalian cells, as a result of the binding assay of the porcine PLA_2 to some mammalian cells except porcine [8–10]. Cobra venom PLA_2 also belongs to group I [6]. PLA_2 isolated from human rheumatoid arthritic synovial fluid, which belongs to group II together with PLA_2 from *Crotalus adamanteus* venom [11], has been reported to play an important role in inflammation. PLA_2 from bee venom, included in group III, plays a role in bee sting inflammation [11–14]. A new type of 14-kDa PLA_2 has been recently isolated from a filamentous bacteria *Streptomyces violaceoruber* [15].

The present study shows that $hPLA_2$-I, acting as a digestive enzyme, stimulates the growth of human pancreatic cancer cell line via specific binding to the PLA_2 receptor.

2. Materials and methods

2.1. Sources of PLA_2 and the cancer cell line

The $hPLA_2$-I was purified from pancreatic juice by modifying the method described previously [16]. The purified $hPLA_2$-I was electrophoretically transferred to a nitrocellulose membrane (Schleicher and Schuell, Germany) and detected immunologically using an anti-human PLA_2 mouse monoclonal antibody (Boehringer-Mannheim, Germany). The amino acid sequence of $hPLA_2$-I was determined by Edman degradation method using an autosequencer (PSQ-2, Shimadzu, Japan). Iodination of the mature $hPLA_2$-I was carried out by the methods using chloramine-T [17]. The specific activity of ^{125}I-labelled $hPLA_2$-I was about 2500 cpm/fmol. PLA_2s from *Crotalus adamanteus* venom (group II, product no. 790 in Sigma catalogue) and bee venom of *Apis mellifera* (group III, product no. 1264 in Sigma catalogue) were purchased from Sigma Chemical Co., Ltd. The microbial PLA_2, produced extracellulary by *Streptomyces violaceoruber*, was a kind gift from Asahi Chemical Industry Co., Ltd., Japan. PLA_2 from *Laticauda semifasciata* fraction I (group I) was a generous gift from Dr. C. Takasaki (Tohoku University, Japan) and Dr. K. Teshima (Hiroshima University, Japan). The pro-form of $hPLA_2$-I was a generous gift from Shionogi Pharmaceuticals Co., Ltd., Japan. MIAPaCa-2 was supplied by Japanese Cancer Research Resources Bank. MIAPaCa-2 cell line was grown in Dulbecco's modified Eagle's medium (D-MEM) with 10% foetal bovine serum (Gibco, USA) in humidified atmosphere of 5% CO_2 and 95% air at 37°C.

2.2. Cell proliferation assay

The cell proliferation was assayed by two methods. Method A is a method which assays the growth of cells by measuring the absorbance at 450 nm. The MIAPaCa-2 (2.5×10^4) were plated into 0.6 cm^2 in 96-well plates, in D-MEM containing 10% foetal bovine serum (FBS). After incubation for 24 h, the medium was removed, and then the same medium containing 0.1% FBS was added to each well. The cells were grown for 24 or 48 h in D-MEM containing 0.1% FBS medium, or the same medium containing PLA_2s from human, bee venom, *Streptomyces violaceoruber*, *Crotalus adamanteus* venom, and *Laticauda semifasciata* fraction I at the given concentrations (10, 25 and 50 nM), respectively. The cells were grown in humidified atmosphere of 5% CO_2 and 95% air

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Abbreviations: h, human; PLA_2, phospholipase A_2; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

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at 37°C. After incubation for 24 h or 48 h, the cell proliferation assay was carried out using a sulphonated tetrazolium salt that produced a highly water-soluble formazan dye (Cell Counting Kit; Dojindo Laboratories Ltd., Japan) [18]. The absorbances of the aliquot supernatant of MIAPaCa-2 cells were measured to assay the cell growth at 450 nm using an automatic plate analyzer (Tokyo Sokki, Japan). Method B is as follows: the MIAPaCa-2 cells (5×10^5) were plated onto 4 cm^2 tissue culture wells in D-MEM containing 10% FBS. The cells cultured by the method described above were detached with 0.25% trypsin and 0.1% EDTA, and counted in triplicate by using a hemocytometer.

2.3. Binding assay

The MIAPaCa-2 cells were grown in 12-well plates. The confluent cells were washed with phosphate-buffered saline (PBS) and incubated with 1 ml of the binding medium (Hank's medium, pH 7.6, supplemented with 0.1% bovine serum albumin) containing various concentrations of ^{125}I-labelled mature $hPLA_2$-I for 2 h at 4°C. After the cells were washed with PBS, the radioactivity bound to the cells was measured. The specific binding was calculated from the differences between in the absence and the presence of unlabelled mature $hPLA_2$-I (500 nM).

2.4. Statistical analysis

Statistical analysis was done using the Student's *t*-test. A probability of $P < 0.05$ was considered statistically significant.

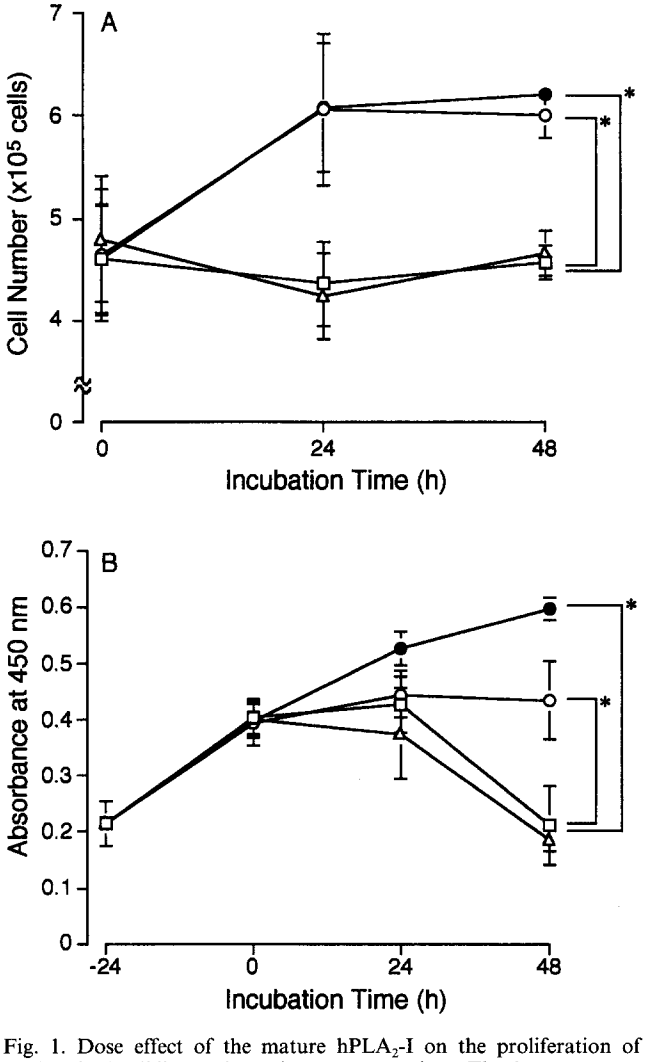

Fig. 1. Dose effect of the mature $hPLA_2$-I on the proliferation of MIAPaCa-2 cell line at the various concentrations. The data are representative of three experiments and each point and bar is the mean and the standard deviation, respectively. (A) the cell numbers counted by a hemocytometer; (B) the absorbances at 450 nm measured by the Cell Counting Kit. □, 0 nM; △, 10 nM; ○, 25 nM; ●, 50 nM. *$P < 0.05$.

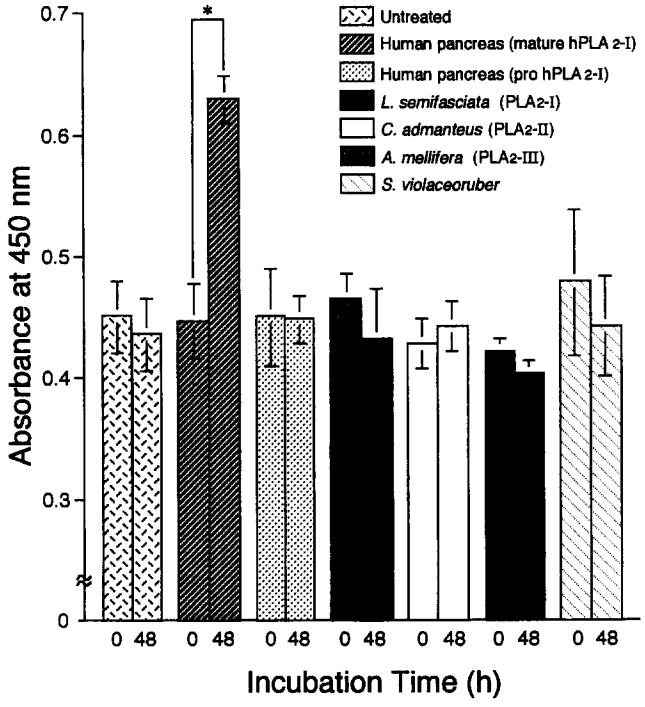

Fig. 2. Dose effects of pro and mature forms of $hPLA_2$-I, and PLA_2s from various sources on the proliferation of MIAPaCa-2 cell line. Each PLA_2 was used at the final concentrations of 50 nM. The data are representative of three experiments and each column and bar is the mean and the standard deviation, respectively. *$P < 0.05$.

3. Results and discussion

3.1. Purification of $hPLA_2$-I from human pancreatic juice

$hPLA_2$-I is mainly secreted from the pancreas as an inactive zymogen and converted into the active form by trypsin. The mature form has been thought to function as a digestive enzyme [19]. In the present study, we purified to homogeneity the 14-kDa protein from the pancreatic juice by chromatography on Octyl-Sepharose and CM-Sepharose column. The Western-blot analysis confirmed that the 14-kDa protein is $hPLA_2$-I. The N-terminal 20 amino acids sequence of the isolated $hPLA_2$-I was identical with that of the mature $hPLA_2$-I reported previously [20].

3.2. Cell proliferation assay

Through three experiments, we confirmed that the mature type of exogenous $hPLA_2$-I, purified from pancreatic juice, stimulated the MIAPaCa-2 cell proliferation when added at the concentrations of 25 or 50 nM, respectively ($P < 0.05$) (Fig. 1A,B) The dose of $hPLA_2$-I at the high concentration of over 100 nM, however, gave rise to reduction of the cell viability (data not shown). The treatment with the pro-form of $hPLA_2$-I (50 nM) was ineffective to the cell proliferation of MIAPaCa-2. Other PLA_2s (50 nM) from *Laticauda semifasciata* fraction I, *Streptomyces violaceoruber, Crotalus adamanteus* venom and bee venom did not stimulate the cell growth of MIAPaCa-2 (Fig. 2). These results suggest that the PLA_2-induced MIAPaCa-2 proliferation is specific for the mature form of $hPLA_2$-I at the optimal dose concentration.

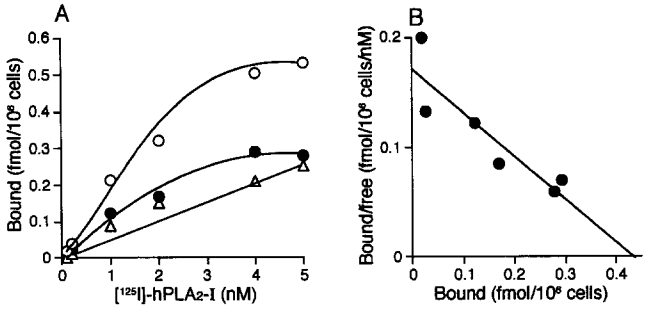

Fig. 3. Typical equilibrium binding assay of the mature $hPLA_2$-I to MIAPaCa-2 cells. (A), saturation curves for the equilibrium binding of the mature $hPLA_2$-I to MIAPaCa-2 cells. Specific binding (●) was calculated by subtracing the nonspecific binding (△) from the total binding (○). (B), Scatchard plot analysis on the specific ^{125}I-labelled $hPLA_2$-I binding data in (A).

3.3. Binding assay

We measured whether the mature ^{125}I-labelled $hPLA_2$-I is bound to the MIAPaCa-2 cell (Fig. 3A). The Scatchard plots demonstrated a single class of binding site for the mature $hPLA_2$-I, with an equilibrium binding constant (K_d) value of 2.6 nM and a maximum binding capacity (B_{max}) of 0.4 fmol/10^6 cells (Fig. 3B). Although the K_d value to the MIAPaCa-2 cell was similar to that for the porcine PLA_2-I bound to mouse embryonic fibroblast (Swiss 3T3) cell [8] or rat embryonic thoracic aorta smooth muscle cell [21], the B_{max} value in the present study was lower than that for the porcine PLA_2-I for Swiss 3T3 cells (8.34 fmol/10^6 cells), or rat embryonic thoracic aorta smooth muscle cells (35 fmol/10^6 cells) [8,21]. Judging from the B_{max} value (0.4 fmol/10^6 cells), it may be reasonable that the higher dose (25, 50 nM) of $hPLA_2$-I is necessary to stimulate the growth of MIAPaCa-2 cells.

It has been reported that cancer cells may overexpress some receptors which are activated by specific growth factors, leading to excessive activation of growth signaling pathways and cell growth [22]. Human pancreatic carcinoma cells may overexpress the receptors for epidermal growth factor (EGF) [23], or cholecystokinin (CCK) [24]. Cultured human pancreatic cancers overexpress EGF, transforming growth factor-alpha (TGF-α), and the receptors for EGF. The concept of a functional EGF autocrine cycle in human pancreatic carcinoma indicates that both EGF and TGF-α may lead to excessive activation of the EGF receptor within the tumour [25]. Surprisingly, we demonstrated that a human pancreatic cancer cell line, MIAPaCa-2, had a single class binding site for the mature $hPLA_2$-I, and that the mature $hPLA_2$-I might function, not only as a digestive enzyme of pancreas, but also as a growth factor of pancreas carcinoma. Pancreatic neoplasms are usually ductal adenocarcinoma [25], and the ducts are encased and/or obstructed by the carcinoma. Previous studies have reported that some cases of pancreas carcinoma increase the PLA_2 level in serum, because of pancreatitis associated with pancreas carcinoma [26]. In addition, since the mature type of $hPLA_2$-I has been found to be originally contained in the human pancreatic juice, together with the pro-form [27], a functional $hPLA_2$-I paracrine loop might be present in human pancreas carcinoma. Further studies will be needed to investigate a functional $hPLA_2$-I autocrine loop, and to understand the signal pathways for cell proliferation in MIAPaCa-2.

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References

[1] Hirayama, T. (1989) Jpn. J. Clin. Oncol. 19, 208–215.
[2] Warshaw, A.L. and Fernandez-Del Castillo, C. (1992) N. Engl. J. Med. 326, 455–465.
[3] Johnson, L.R. (1981) Cancer 47, 1640–1645.
[4] Edwards, B.F., Redding, T.W. and Schally, A.V. (1989) Int. J. Pancreatol. 5, 191–201.
[5] Arita, H., Nakano, T. and Hanasaki, K. (1989) Prog. Lipid. Res. 28, 273–301.
[6] Denis, E.A. (1994) J. Biol. Chem. 269, 13057–13060.
[7] Matsuda, Y., Ogawa, M., Shibata, T., Nakaguchi, K., Nishijiama, J., Wakasugi, C. and Mori, T. (1987) Res. Commun. Chem. Pathol. Pharmacol. 58, 281.
[8] Arita, H., Hanasaki, K., Nakano, T., Oka, S., Teraoka, H. and Matsumoto, K. (1991) J. Biol. Chem. 266, 19139–19141.
[9] Hanasaki, K. and Arita, H. (1992) Biochim. Biophys. Acta 1127, 233–241.
[10] Hanasaki, K. and Arita, H. (1992) J. Biol. Chem. 267, 6414–6420.
[11] Heinrikson, R.L., Kruegar, E.T. and Keim, P.S. (1979) J. Biol. Chem. 252, 4913–4921.
[12] Hanahan, D.J. (1986) Annu. Rev. Biochem. 55, 483–509.
[13] Samuelsson, B., Dahlen, S.E., Lingren, J.A., Rouzer, C.A. and Serhan, C.N. (1987) Science 237, 1171–1176.
[14] Davidson, F.F. and Dennis, E.A. (1990) J. Mol. Evol. 31, 228–238.
[15] Sugiyama, M., Yasa, K., Kumagai, T., Kinoshita, E., Matsuno, H., Nishimura, M. and Suzuki, K. (1995) Jpn. J. Clin. Pathol. 43, 765–771.
[16] Nishijima, J., Okamoto, M., Ogawa, M., Kosaki, G. and Yamano, T. (1983) J. Biochem. 94, 137–147.
[17] Hopp, T.P. and Woods, K.R. (1981) Proc. Natl. Acad. Sci. USA 78, 3824–3828.
[18] Ishiyama, M., Shiga, M., Sasamoto, K., Mizoguchi, M. and He, P. (1993) Chem. Pharm. Bull. 41, 1118–1122.
[19] de Haas, G.H., Postema, N.M., Nieuwenhuizen, W. and van Deenen, L.L.M. (1968) Biochim. Biophys. Acta 159, 118–129.
[20] Ohara, O., Tamaki, M., Nakamura, E., Tsuruta, Y., Shin, M., Teraoka, H. and Okamoto, M. (1986) J. Biochem. 99, 733–739.
[21] Kanemasa, T., Hanasaki, K. and Arita, H. (1992) Biochim. Biophys. Acta 1125, 210–214.
[22] Aaronson, S.A. (1991) Science 254, 1146–1153.
[23] Korc, M., Meltzer, P. and Trent, J. (1986) Proc. Natl. Acad. Sci. USA 83, 5141–5144.
[24] Ian, R., Swift, B.S. and Jill, P.S. (1994) Dig. Dis. Sci. 35, 1007–1013.
[25] Morohoshi, T., Held, G. and Kloppel, G. (1983) Histopathology 7, 645–661.
[26] Kitagawa, M., Hayakawa, T., Kondo, T., Shibata, T., Sakai, Y., Sobajima, H., Ishiguro, H. and Nakase, Y. (1991) Gastroenterol. Jpn. 26, 62–68.
[27] Mizumoto, R., Isaji, S. and Tabata, M. (1990) Nippon Rinsho. 48, 69–74.