Leukemia inhibitory factor functions as a growth factor in pancreas carcinoma cells: Involvement of regulation of LIF and its receptor expression

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Received September 8, 2006; Accepted October 30, 2006

Abstract. Leukemia inhibitory factor (LIF) is a pleiotrophic cytokine, which plays an important role in inducing cancer cachexia. We have previously reported that LIF promotes cell proliferation in some human carcinoma cells through c-fos, jun-B and cyclin-E expression. In the present study, we analyzed the regulation of LIF and its receptor (LIFR) expression in pancreatic carcinoma cells. Seven pancreatic carcinoma cells expressed constitutively LIF and its heterodimer receptor (LIFR and gp130) mRNA in RPMI-1640 medium without FBS. The amount of LIF immunoreactive protein was 132.5±52 pg/10^6 cells in culture supernatants without FBS. Pro-inflammatory cytokines, such as TNF-α, IL-1ß, IL-6, IL-8, or LIF, enhanced the expression of LIF mRNA in Hs-700T and Hs-766T cells. Addition of LIF significantly induced cell proliferation of Hs700T in 13 days LIF dose-dependently. However, anti-LIF IgG failed to suppress cell proliferation in Hs-700T cells. LIF acted as a paracrine growth factor in Hs-700T cells, which expressed low amount of LIF without stimuli. Cellular signal transductions by LIF was down-regulated by inhibitors of protein kinase C (PKC), protein tyrosine kinase (PTK), and Ca/Calmodulin. LIF induced phosphorylation of STAT3. Moreover, exogenous LIF upregulated the expression of LIFR mRNA. Antisense LIFR oligonucleotide significantly suppressed cell growth in the presence of LIF in Hs-700T cells. These results suggest that cytokine network might alter the expression and responsiveness to LIF in tumor microenvironment.

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Key words: leukemia inhibitory factor, leukemia inhibitory factor receptor, pancreatic cancer

Introduction

Pancreatic cancer shows high mortality rates in gastroenterogical neoplasms, and there are difficulties in early diagnosis and aggressive progression. The obstruction of pancreatic duct spreads out pancreatic proteases in advanced pancreas cancer, causing inflammation to the surrounding pancreas tissue locally and systemically. Cell biology of pancreas cancer consists of complex interactions of various growth factors and its receptors (1-5). The regulation of growth factors, including cytokines, produced by carcinoma cells and their relevance to tumor cell proliferation may have implications in the management of the disease.

LIF is a pleiotropic cytokine, which plays biological functions in cell proliferation and diffentiation. LIF regulates calcium and bone metabolism, induces acute phase proteins and causes cachexia in organisms with neoplastic disorder (6). Further, LIF was increased as a potent pro-inflammatory cytokine in sepsis (7,8), arthritis (9,10), injury (11) and dermatitis (12). Prior administration of high dose LIF protects from lethality in *E. coli*-induced septic shock (13). LPS induces 10-fold higher TNF-α and IL-6 serum level and reduces IL-10 production in LIF deficient mice (7). Thus, the detection of LIF expression in cachexia and inflammatory disease led us to hypothesize that cancer cells interact with inflammatory cytokines, such as TNF-α, IL-1ß, IL-6, IL-8 and LIF. Following the activation of LIF receptors (LIFR and gp130) might result in the promotion of growth in pancreas tumor microenvironment. We previously reported the effect of LIF on cell proliferation in various carcinoma cell lines. LIF promoted cell proliferation in pancreas and mammary carcinoma cells (2).

The aim of this study is to clarify the regulation of LIF as a growth factor in pancreas cancer cells. We reveal that inflammatory cytokines induce the expression of LIF, and addition of LIF would alter the responsiveness to LIF by upregulation of LIFR. Furthermore, reduction of LIFR mRNA by specific antisense oligonucleotide suppresses cells proliferation. Thus, we present data indicating that the expression of LIF and its receptor contributes to the progression of pancreas carcinogenesis. We also present evidence that inflammatory cytokines affect the expression of LIF with growth activity in cancer tissue microenvironment.

Materials and methods

Reagents. Human recombinant leukemia inhibitory factor (LIF), interleukin-1ß (IL-1ß), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8) and anti-human LIF goat IgG were purchased from R&D Systems (Minneapolis, MN). Goat IgG was from Zymed (San Francisco, CA). Anti-human signal transducer and activator of transcription 3 (STAT3) mouse IgG (F-2) and anti-human actin mouse IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against phosphotyrosine (4G10) was from Upstate Biotechnology (Lake Placid, NY). Protein G-sepharose (PGS) was from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). H7 and W7 were from Biomol (Plymouth Meeting, PA). 3-(4,5-dimethylthiazol-2-tl)-2,5-diphenyl-tetrazolium bromide (MTT), Herbimycin A and staurosporin were from Wako (Tokyo, Japan). [α-^{32}P]dCTP was from ICN (Costa Mesa, CA). Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA were from Clontech (Palo Alto, CA). Phosphate-buffered saline (PBS), RPMI-1640, fetal bovine serum (FBS) and TRIzol Reagent were from Life Technologies (Gaithersburg, MD).

Cell lines and cell culture. Carcinoma cell lines of the pancreas (BxPc-3, Hs-700T, Hs-766T, PANC-1, Capan-1, and Capan-2) were purchased from the American Type Cell Culture (ATCC). SUIT-2 was maintained in our laboratory. The cell lines were cultured in RPMI-1640, supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37˚C in a humidified 5% CO_2 to 95% air. The cells were starved overnight before isolation of mRNA.

Enzyme linked immunosorbent assay (ELISA). Pancreatic carcinoma cells were washed by PBS, and cells (1x10^5 cells/ml) were seeded in medium without FBS in triplicate using the 24-well microtitre plates. Then, plates were incubated for 72 h at 37˚C in a humidified 5% CO_2 incubator. After 72-h incubation, cell numbers were counted three times and culture supernatants were stored at -20˚C after centrifugation until analysis. LIF production from pancreatic carcinoma cells was determined by a commercially available ELISA kit (R&D Systems). This assay shows no measurable cross-reactivity with either human TNF-α, IL-1ß, IL-6, or IL-8. The lower limit of detection was 31.3 pg/ml.

Northern blot analysis. When carcinoma cells were harvested at 90% confluence, cells were washed with PBS. Cells were further incubated for 24 h in the serum-free medium until the experiment. The cells were stimulated with the reagents for indicated times. Total RNA of carcinoma cells was extracted by the guanidine thiocyanate-phenol-chloroform method and Northern blot analysis was performed as previously described (2). Membranes were hybridized with various [^{32}P]-labeled probes including LIF [0.8 kb EcoRI/HindIII fragment of pBR322(pLSV)-LIF] for Northern blot analysis. The following primers were used to obtain human LIFR cDNA by PCR from a human monocyte cDNA library. LIFR sense primer: 5'-AGTTACCACCTGGTCTTGCG-3', LIFR antisense primer: 5'-TGCTTGAGGCTGATACATCG-3'. G3PDH was purchased from Clontech (Palo Alto, CA, USA). The results were expressed as a ratio to G3PDH.

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR analysis was performed as described previously (2). The following primers was used for PCR. GP130 sense primer: 5'-GTGGAATGGACTACTCCAAGG-3', GP130 anti-sense primer: 5'-TCCTTCCCACCTTCATCTGT-3', G3PDH sense primer: 5-GAAATCCCATCACCATCTTCC-3, G3PDH antisense primer: 5-CCAGGGGTCTTACTCCTTGG-3. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR-assisted mRNA amplification was repeated twice for at least two separately prepared cDNA samples for each experiment. Data are representative in at least three different experiments.

Immunoprecipitation and Western blotting. Carcinoma cells were lysed on ice for 20 min in a buffer containing 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 mM NaF, 1 mM ethylenediamine tetraacetic acid, 200 mM sodium orthovandate, 1 mg/ml leupeptin, 1 mM phenylmethyl-sulfonyl fluoride, 1 mg/ml aprotinin, and 1 mg/ml pepstatin A. The lysates were spun, and the supernatants were collected. The samples were incubated with 20 μl volume PGS for 1 h at 4˚C. After centrifugation, supernatants were collected, mixed with anti-human STAT3 mouse IgG, and incubated for 1 h. PGS (20 μl) was then added and incubated for another 1 h. IgG-coupled PGS were washed with the buffer containing 50 mM HEPES, 150 mM NaCl, 0.1 % Triton X-100, 10% glycerol buffer three times. Western blot analysis was performed as previously described (14). The membranes were incubated with monoclonal anti-human STAT3 IgG, or anti-phosphotyrosine IgG or anti-actin IgG, followed by sheep anti-mouse IgG coupled with horseradish peroxidase (Amersham). Peroxidase activity was visualized by the Enhanced Chemiluminescence Detection System (Amersham).

Cell proliferation assay. Carcinoma cells were washed by PBS, and suspended at 1x10^5 cells/ml in medium (RPMI-1640 + 2% FBS). Cells were transferred in triplicate to the 24- or 96-well microtitre plates containing diluted recombinant human LIF, LIFR sense or antisense oligonucleotide. Antisense oligonucleotide was designed with the same sequence as PCR analysis. Plates were then incubated for the indicated period. To evaluate the proliferation of pancreatic carcinoma cells, we counted the viable cells using trypan blue stain or MTT assay as described (15).

Statistical analysis. The significance of differences in numerical data was evaluated using the χ^2 test, or Student's t-test. The probability level of <0.05 was considered as the limit of significant difference.

Results

LIF and LIF receptor mRNA expression in human pancreatic carcinoma cells. We first investigated whether LIF and its receptor (LIFR and gp130) mRNA can be expressed constitutively in pancreatic carcinoma cells under FBS starvation. As shown in Fig. 1, all eight pancreatic carcinoma cells expressed LIF mRNA by Northern blot analysis. All pancreatic carcinoma cells also expressed LIFR and gp130 mRNA.

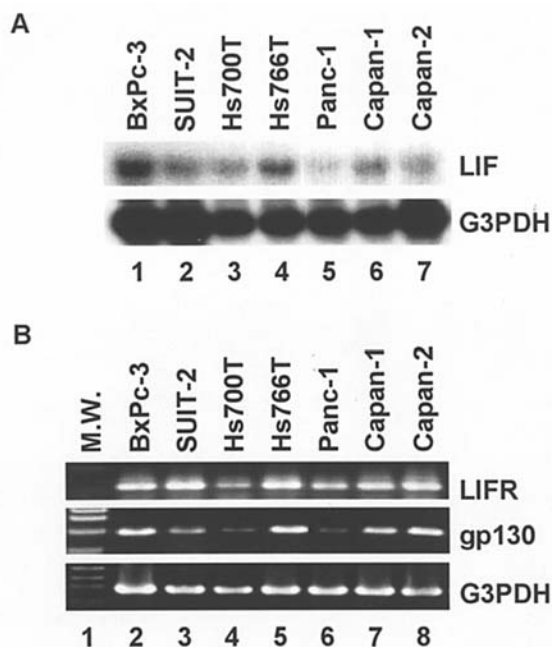

Figure 1. Pancreatic carcinoma cells constitutively expressed LIF and its receptor mRNA. (A) Carcinoma cells were cultured to 70-80% confluence in 25 cm^2 flask. They were then incubated with serum-starved medium for 24 h, and mRNA was isolated. Approximately 10 μg per lane total cellular RNA was used for Northern blot analysis. BxPc-3 (lane 1), SUIT-2 (lane 2), Hs-700T (lane 3), Hs-766T (lane 4), Panc-1 (lane 5), Capan-1 (lane 6), Capan-2 (lane 7). (B) Five μg of RNA was used for RT-PCR analysis. Molecular weight (lane 1), BxPc-3 (lane 2), SUIT-2 (lane 3), Hs-700T (lane 4), Hs-766T (lane 5), Panc-1 (lane 6), Capan-1 (lane 7), Capan-2 (lane 8).

Table I. LIF production in pancreatic carcinoma cells.

Cell lines	LIF (pg/1x10^6 cells)
SUIT-2	354.4±96.8
BxPc-3	38.0±0.6
Hs-700T	77.7±3.3
Hs-766T	267.1±3.3
Panc-1	49.3±1.0
Capan-1	138.7±4.9
Capan-2	25.0±0

Data are presented as the means ± standard deviation (SD) of three independent experiments.

Expression of LIF protein in pancreatic carcinoma cells. To detect the expression of LIF protein in pancreatic carcinoma cells, we performed ELISA. All pancreatic carcinoma cells produced immunoreactive LIF in the culture supernatant after 72-h incubation without FBS by ELISA. LIF production per 1x10^6 cells ranged from 25.0 pg/ml to 354.4 pg/ml and their mean value among the producing cells was 135.7±25.2 pg/ml (Table I). Among the pancreatic carcinoma cells, high incidence was found in pancreatic carcinoma cells (7/7).

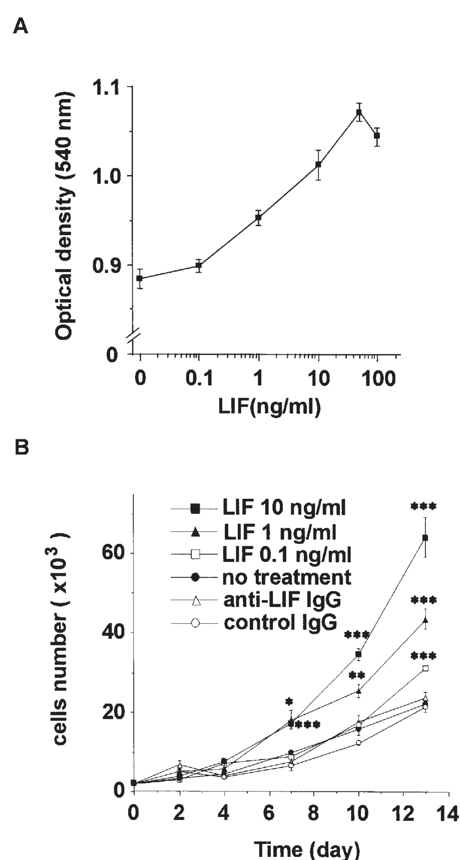

Figure 2. LIF promoted the proliferation of Hs-700T cells. Carcinoma cells (1x10^5 cells/ml) were exposed to indicated concentrations of LIF, anti-LIF IgG or control IgG containing medium (RPMI-1640 + 2% FCS) in triplicate using 24- or 96-well microtitre plates for the indicated period. Stimuli with medium were replaced each 3 days. (A) MTT assay was performed after the incubation for 48 h. (B) The number of viable cells were counted by trypan blue staining. Data are shown as mean ± SD of 3- or 4-wells. $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P<0.0005$.

LIF promotes cell proliferation of Hs-700T cells. To evaluate underlying mechanism of cell proliferation by LIF, we used Hs-700T cells. Carcinoma cells were stimulated by indicated concentrations of LIF for 48 h to determine its effect on cell growth. LIF significantly promoted cell growth at several dosages of LIF in Hs-700T cells by MTT assay, and the maximum effect was observed with a concentration of 10 ng/ml. A concentration of LIF as low as 1 ng/ml promoted cell proliferation (Fig. 2A). To analyze cell proliferation for more than 48 h, we counted the cells numbers each 3 days. Old culture supernatants were discarded and replaced with fresh medium containing LIF each 3 days. LIF dose-dependently promoted cell proliferation, as low as 0.1 ng/ml of LIF achieved significant effect in 13 days (Fig. 2B). Previously, we reported that various carcinoma cells produced biologically active LIF (15). To clarify whether endogenous LIF affect cell proliferation in an autocrine manner, we used neutralizing LIF antibody. Anti-LIF IgG did not affect cells growth in comparison with control IgG (Fig. 2B). Endogenous LIF had a minimum effect on cell proliferation without stimulus. These results indicated that high dose of exogenous LIF promoted cell growth in Hs-700T cells.

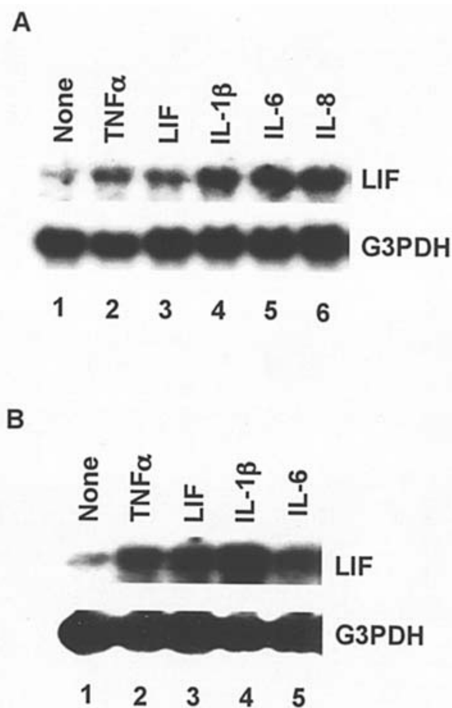

Figure 3. Regulation of LIF mRNA expression in pancreatic carcinoma cells. Carcinoma cells were incubated in medium RPMI-1640 without FBS for 24 h. Then, cells were incubation with or without TNF-α (10 ng/ml), LIF (10 ng/ml), IL-1ß (10 ng/ml), IL-6 (10 ng/ml) or IL-8 (10 ng/ml) for 8 h. None (lane 1), TNFα (lane 2), LIF (lane 3), IL-1ß (lane 4), IL-6 (lane 5), IL-8 (lane 6). The expression of LIF and G3PDH mRNA were analyzed by Northern blotting. Approximately 10 μg per lane total cellular RNA was used. (A) Hs-700T cells. (B) Hs-766T cells.

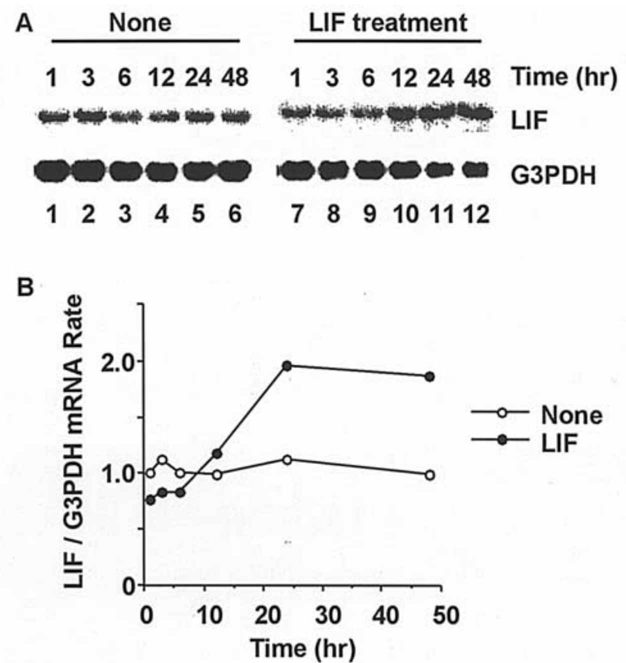

Figure 4. Effect of LIF on endogenous LIF mRNA expression in Hs-700T cells. (A) Carcinoma cells were serum-starved and then treated with (lanes 7-12) or without (lanes 1-6) 10 ng/ml of LIF for various lengths of time. Total cellular RNA (10 μg) was extracted and analyzed by Northern blotting; 1 h (lanes 1 and 7), 3 h (lanes 2 and 8), 6 h (lanes 3 and 9), 12 h (lanes 4 and 10), 24 h (lanes 5 and 11), 48 h (lanes 6 and 12). (B) Autoradiographic densities of each mRNA band were quantitated using a Bio-Image Analyzer. The results were standardized against the levels of G3PDH, and are presented as relative density. The level of expression detected in untreated cells for 1 h equals 1.

Regulation of LIF mRNA expression by cytokines. We investigated the effect of TNF-α, IL-1ß, LIF, IL-6 and IL-8 on LIF mRNA expression in Hs700T and Hs-766T cells. They expressed a low amount of LIF mRNA without stimulus (Fig. 3, lane 1). Addition of TNF-α, IL-1ß, LIF, IL-6 or IL-8 further augmented the expression levels of LIF mRNA (Fig. 3, lanes 2-6).

Effect of LIF on endogenous LIF mRNA expression in Hs-700T cells. Addition of exogenous LIF upregulated endogenous LIF mRNA in Hs-700T dose-dependently (2). To confirm endogenous LIF mRNA induction, we additionally investigated kinetics of its expression in Hs-700T cells. Cells were stimulated with 10 ng/ml of LIF for the indicated period, because a concentration of 10 ng/ml had a maximum effect of cell growth (Fig 2). Untreated cells exhibited no drastic change (Fig. 4, lanes 1-6). Exogenous LIF increased the level of endogenous LIF mRNA after the stimulation of LIF for 24-48 h in comparison with untreated cells (Fig. 4, lanes 11 and 12).

Cellular signal transduction by LIF. To understand underlying mechanisms of LIF mRNA induction, we investigated cellular signal transduction by LIF. The treatment of exogenous LIF induced about 2-fold endogenous LIF mRNA (Fig. 5A and B, lane 2). Inhibitors of protein kinase C (H7, staurosporin), tyrosine kinase (Herbimycin A), or calmodulin (W7) reduced LIF mRNA expression by the stimulation of LIF (Fig. 5A and B, lanes 2-6). Signal transduction by LIF was dependent of a Ca/calmodulin pathway significantly. Futhermore, we analyze whether LIF activates JAK-STAT pathway by immunoprecipitation and Western blotting. Carcinoma cells had no autophophorylation of STAT3 after serum starvation. The levels of phosphorylation of STAT3 increased at 20 min maximally and appeared to decrease after 40 min of the stimulation of LIF (Fig. 5C). These results suggested that the activation by LIF might consist of different cellular signal pathways in Hs-700T cells.

LIF upregulates LIFR mRNA expression and antisense oligonucleotide of LIFR suppresses cell proliferation. We also evaluated the expression of LIFR by Northern blotting. Hs-700T cells were serum-starved and then stimulated with various LIF concentrations for 8 h. As shown in Fig. 6A, addition of LIF induced endogenous LIFR mRNA expression most at 0.1 ng/ml LIF. Exogenous LIF augmented its receptor on the cell membranes and reinforced the affinity to ligands. To control LIFR expression by exogenous LIF, we investigated whether LIFR antisense oligonucleotide (AS) regulated cell proliferation in the presence of LIF. LIFR AS significantly suppressed cell proliferation in comparison with its sense oligonucleotide (S) after the incubation with LIF for 48 h (Fig. 6B). LIFR downregulation by LIFR AS caused the alteration of the responsiveness to LIF.

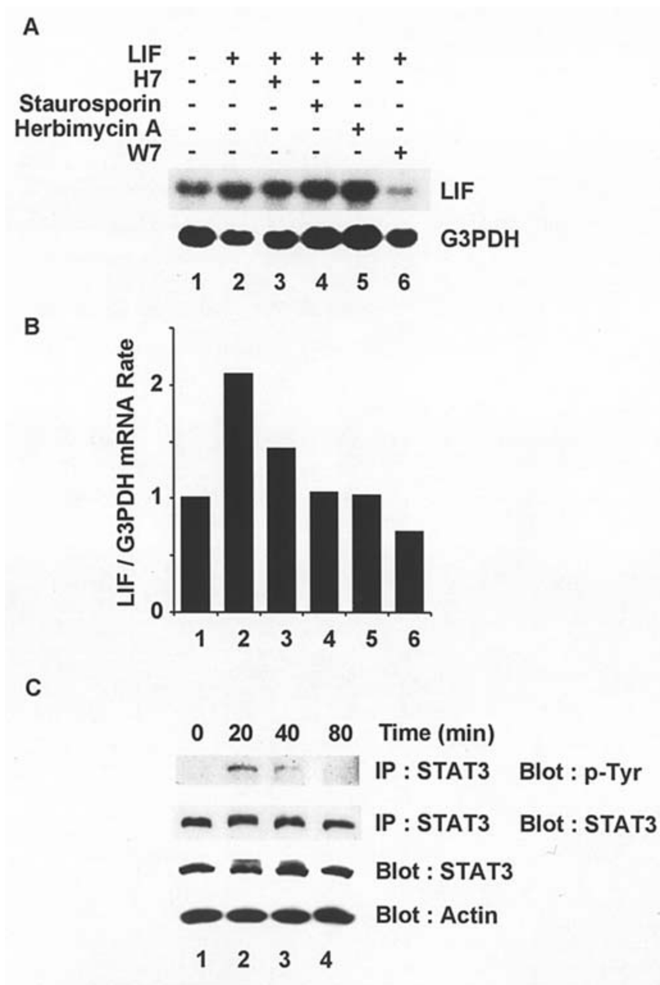

Figure 5. Cellular signal transduction by LIF in Hs-700T cells. (A) Hs700T cells were serum-starved and then treated with LIF (10 ng/ml) in combination with the protein kinase C inhibitor Staurosporin (50 nM), H7 (50 μM), tyrosine kinase inhibitor, Herbimycin A (4 μM), or Ca/Calmodulin inhibitor W7 (50 μM). Total cellular RNA (10 μg) was extracted and analyzed by Northern blotting. None (lane 1), LIF (lane 2), LIF and H7 (lane 3), LIF and Staurosporin (lane 4), LIF and Herbimycin (lane 5), LIF and W7 (lane 6). (B) Autoradiographic densities of each mRNA band were quantitated using a Bio-Image Analyzer. The results were standardized against the levels of G3PDH, and are presented as relative density. The level of expression detected in untreated cells equals 1. (C) Cell lysates were prepared and analyzed by Western blotting. The membranes were blotted by anti-STAT3 IgG and anti-actin IgG. Then, immunoprecipitation was performed with anti-STAT3 IgG. Immunoprecipitated samples were subjected to SDS-PAGE and Western blotting. The membranes were blotted by anti-pTyr and anti-STAT3 IgG; 0 min (lane 1), 20 min (lane 2), 40 min (lane 3), 80 min (lane 4).

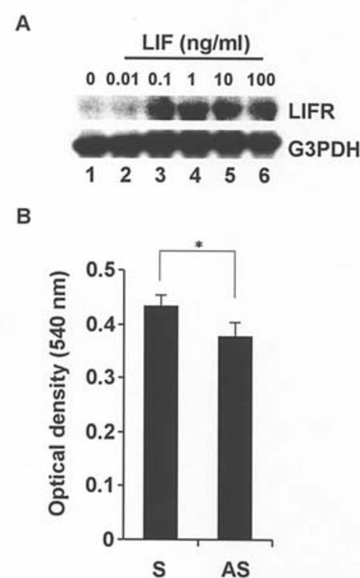

Figure 6. Regulation of LIFR mRNA expression by exogenous LIF and antisense LIFR oligonucleotide in Hs-700T cells. (A) Carcinoma cells were serum-starved and then treated with indicated concentrations of human recombinant LIF for 8 h. Total cellular RNA was extracted and the expression of LIFR mRNA was analyzed by Northern blotting. None (lane 1), 0.01 ng/ml (lane 2), 0.1 ng/ml (lane 3), 1 ng/ml (lane 4), 10 ng/ml (lane 5), 100 ng/ml (lane 6). (B) Carcinoma cells ($1x10^5$ cells/ml) were exposed to LIF (10 ng/ml) with sense or antisense oligonucleotide (1 μM) for LIFR in triplicate using a 96-well microtitre plate for 48 h. Then, MTT assay was performed as described in Materials and methods. Data are shown as mean ± SD of 3-4-wells. *P=0.0372.

Discussion

LIF is a pleiotrophic cytokine, which can modulate inflammation, immune responses, and connective tissue metabolism, and act as a pathogenic mediator in different disease (9). There is evidence that the physiological aspects of tumor-bearing host such as cachexia and paraneoplastic syndrome are under the influence of multiple actions of inflammatory cytokines derived from carcinoma cells, including TNF-α, IL-1ß, and IL-6. LIF also regulated lipid metabolism, and developed paraneoplastic syndrome and cancer cachexia (6). Thus, LIF producing cells could be involved in induction of these manifestations in cancer-bearing patients. We previously examined biological active LIF protein and mRNA expression in a variety of carcinoma cells (2,15). LIF promoted cell proliferation in tumor cell lines, such as skin (16), medulloblastoma (17), breast, kidney, and prostate (18). LIF acts as a paracrine or an autocrine growth factor for some solid carcinoma cells. LIF could be a maker of cell transformation in endometrial tissues (19). The presence of LIFR correlated with the growth promotion of cancer cells (20). The expression of LIF and its receptor contributes to the progression of carcinogenesis in cancer tissue microenvironment. We previously reported that exogenous LIF augmented endogenous LIF mRNA in some human carcinoma cells with increased cell proliferation through *c-fos*, *jun-B* and cyclin-E expression (2).

In this study, we revealed that induction of LIF mRNA by additive LIF was related to various cellular signaling pathways, especially Calcium/Calmodulin system in Hs-700T cells. LIF promoter was activated by phorbol ester (TPA) and inomycin in endometrial carcinoma cells (21). Metabolism of cellular calcium might be essential in cellular signal transduction by LIF. Inflammatory cytokines, such as TNF-α, IL-1ß, IL-6, IL-8, or LIF, induced endogenous LIF mRNA in two pancreatic carcinoma cell lines. LIF promoter was constutively activated under serum free conditions, and further stimulated by TNF-α or IL-1ß in murine bone marrow stromal cells (22). Thus, LIF can induce or be induced by other cytokines. LIF may influence carcinoma cells and host immunity in a part of the cytokine network.

We also demonstrated that LIF induced phosphorylation of STAT3 in Hs-700T cells. Consistently, LIF activates STAT3 and enhances invasiveness by alteration of protease expression in choriocarcinoma cells (23). LIF has activated transcription factors, such as TRE3 and TFEB, which are activators of E-cadherin in renal carcinoma cells (24). LIF affects the promotion of carcinogenesis in a diverse range of carcinomas through activation of various transcription factors.

Human LIF was initially isolated to induce differentiation and suppress growth in murine myeloid leukemic cells (25). LIF or raf induced IFI16 that was the mediator of growth inhibition in medullary thyroid carcinoma cells (26). In our previous study, LIF induced apoptosis through expression ICE and *c-myc* in gastric and gallbladder carcinoma cells (2). It was considered that different signal transduction and induction of genes contributed to opposite functions.

LIF acted as an autocrine growth factor in medulloblastoma and anti-LIF antibody and antisense LIF oligonucleotide suppressed cell proliferation (17). This could be a new therapeutic approach for the promotion of cancer cells by endogenous LIF. In the present study, anti-LIF antibody failed to suppress cell proliferation in the absence of LIF in Hs-700T cells, however, exogenous LIF promoted cell proliferation. Hs-700T cells produce low amount of LIF, which is not enough to promote growth. Regulation of LIF production may suppress cell proliferation in carcinoma cells which abundantly produced LIF. We also demonstrate that all examined pancreas carcinoma cells have receptors of LIF and addition of LIF induced LIFR mRNA in Hs-700T. Exogenous LIF results in enhancing response to LIF. These results enable us to regulate the signal transduction through LIFR. Consequently, blockade of LIFR antisense oligonucleotide could suppress cell proliferation by the stimulation of LIF. LIF was produced by a wide variety of cells, such as leukocytes, fibroblasts and endothelial cells. This might be important to control LIF surround tumor microenvironments. Pancreatic carcinoma cells promoted cell proliferation by LIF in paracrine or endocrine manner. These results may be of potential value in immunotherapy for pancreatic carcinoma.

In summary, regulation of LIF in pancreatic carcinoma cells leads to a new field of investigation involving a LIF dependent signaling pathway that is activated on interaction of the receptor. Further study will be necessary to understand the molecular mechanisms regulating LIF and the activation of LIFR.

Acknowledgments

We are grateful to Drs H. Egami and S. Mita for their encouragement and invaluable comments throughout this study.

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