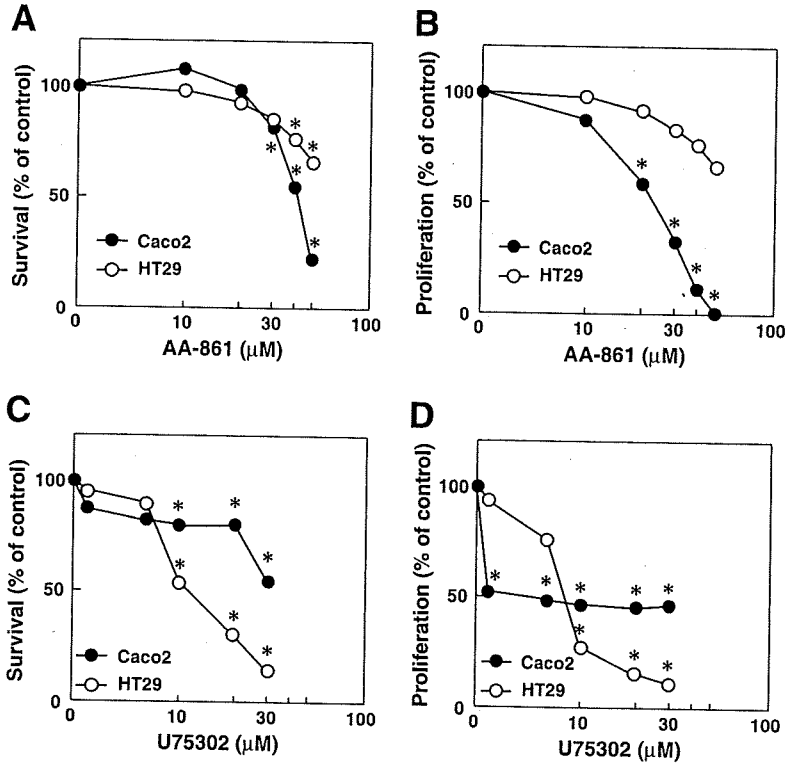

Fig. 3. Effects of AA-861 or U75302 on the survival and proliferation of colon cancer cells. A and B: Effect of AA-861 on human colon cancer cell survival (A) and proliferation (B). C and D: Effect of U75302 on human colon cancer cell survival (C) and proliferation (D). Cells were incubated with AA-861 or U75302 for 48 h at 37°C. The results are each expressed as a percentage of the control. Each data point is expressed the mean ± S.E.M. from 3 independent experiments. *$P<0.05$.

Inhibition of LTB_4-receptor expression by RNAi in colon cancer cell lines suppresses cell proliferation

To confirm the inhibitory effect of LTB_4-receptor signaling blockade on the cell proliferation, we applied siRNA corresponding to BLT1 genes. The gene-specific sequence of siRNA down-regulated BLT1 expression in both Caco2 (Fig. 5A) and HT29 (data not shown) cells. Compared with the negative control group, siRNA suppressed BLT1 expression by 77.15% at 20 nM in Caco2 cells. The decrease in BLT1 expression by siRNA caused the reproducible inhibition of Caco2 cell proliferation compared to the negative control (Fig. 5B).

Effect of the blockade of BLT1 on LTB_4-induced activation of ERK

To investigate the mechanisms of the inhibition of proliferation by blockade of LTB_4 signaling, we examined whether LTB_4 signaling is involved in the activation of the ERK pathway in colon cancer cells. LTB_4 itself induced phosphorylation of ERK 15 min after the treatment in both Caco2 (Fig. 6: A and B) and HT-29 cells (data not shown). The LTB_4-induced phosphorylation of ERK was reproducibly inhibited when cells were treated with the BLT1 antagonist U75302. These results indicate that ERK activation elicited by LTB_4 signaling via BLT1 may be involved in LTB_4-induced stimulation of colon cancer cell proliferation.

Discussion

Several studies reported that inhibition of the 5-LOX pathway suppresses the proliferation of various cancer cells (10 – 13). Since the major metabolite in the 5-LOX pathway is LTB_4, the involvement of LTB_4 in carcinogenesis in various organs has also been indicated (16, 17). In addition, overproduction of LTB_4 in human colon cancer tissue and rat esophageal adenocarcinomas (18, 19) and LTB_4-mediated proliferation of colon cancer cells were reported (20, 21). Thus, many fragmentary studies indicate the critical role of the LTB_4-signaling pathway on the proliferation of colon cancer cells, but stronger evidences, such as direct proof of LTB_4-receptor expression in colon cancer tissue and knock-down of the LTB_4 receptor itself by the siRNA approach, are required for definitive proof.

In the present study, we clearly demonstrated the

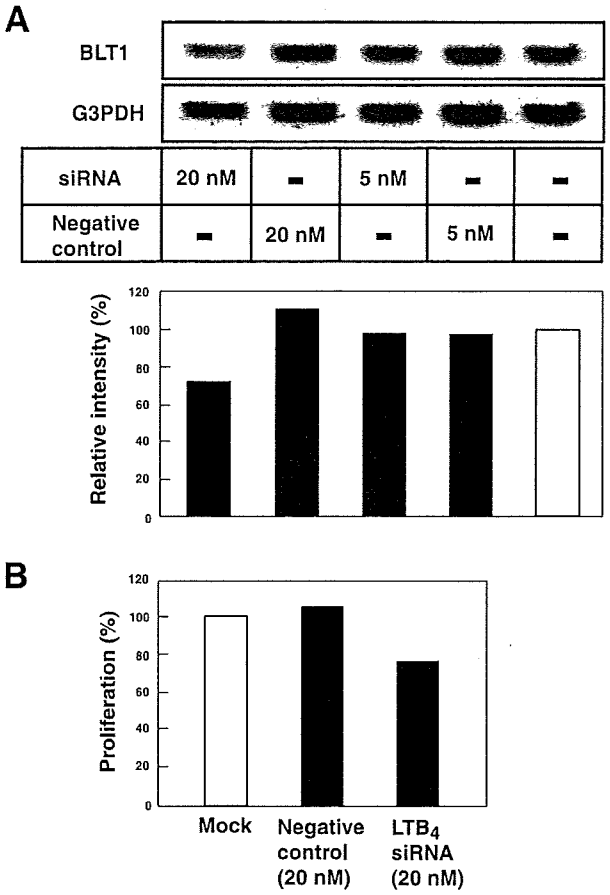

Fig. 5. Knockdown of LTB_4-receptor expression by siRNA in colon cancer cell lines (A) and inhibition of proliferation (B). The Stealth siRNA demonstrated successful knockdown of the LTB_4-receptor 1 (BLT1) expression in Caco2 cells at 20 nM and resulted in the inhibition of cell proliferation. The data are the mean from 3 independent experiments.

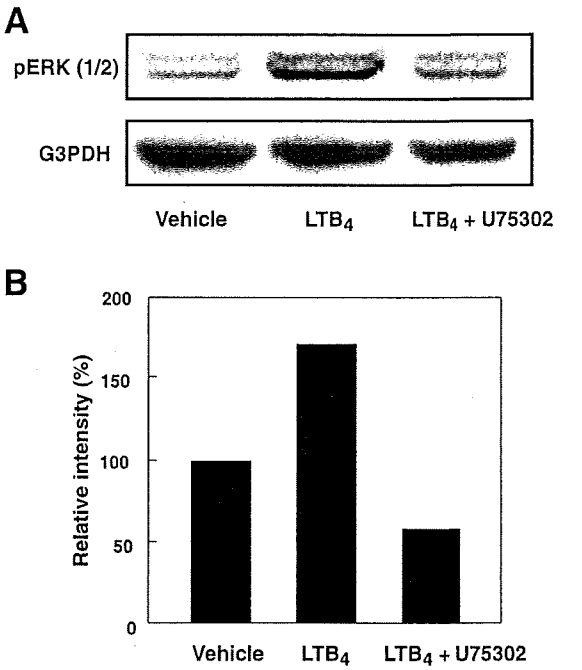

Fig. 6. Effect of U75302 on LTB_4-induced activation of ERK. A: Typical photographs of LTB_4-induced phosphorylation of ERK (pERK) 15 min after the treatment in Caco2 cells. B: Relative intensity of the results from panel A. The percentages compared to the vehicle control after the correction by G3PDH, an internal control, are expressed. The data are the mean from 3 to 5 independent experiments.

strong expression of BLT1 in the cancerous regions of human colon tissues, but not in the normal regions. BLT1 expression was also detected in the tissues from adenomas, but the staining intensity was much weaker than that in the cancer tissues. These results strongly indicate the involvement of the LTB_4-receptor signaling pathway in the colon cancer. BLT1 expression was also confirmed in the cultured human colon cancer cell lines Caco2 and HT-29, but not in non-malignant neural stem cells. It is well known that the LTB_4 receptor, BLT1, is almost exclusively expressed in peripheral leukocytes and to a much lesser extent in the thymus and spleen (5, 6). However, Western blot analysis revealed that strong expression of BLT1 protein was observed in established human colon cancer cell lines, Caco2 and HT-29. These findings and previous reports indicate that the LTB_4-receptor signaling pathway may be required for cancer cell growth and invasion.

Based on the above evidences, we hypothesized that LTB_4 and its signaling pathway may play an important role in the progression of human colon cancer via a direct stimulation of cancer cell proliferation. To clarify this hypothesis, we showed that LTB_4 itself increased the growth of both Caco2 and HT-29 cells. However, the stimulation of cell proliferation by LTB_4 itself was not so dramatic. One of the reasons for our results might be the instability of LTB_4 itself in culture conditions (5, 6) or the endogenous LTB_4 level might be already high enough to stimulate the proliferation. We, therefore, applied a 5-LOX inhibitor and BLT1 antagonist to suppress the LTB_4 level and block the receptor, respectively. Both of the 5-LOX inhibitor and BLT1 antagonist showed dramatic inhibition of cell survival and proliferation. These results clearly indicate that endogenously synthesized LTB_4 and its receptor pathway play the critical role in cell survival and proliferation of colon cancer, and the blockade of the signaling pathway causes the apoptotic cell death. In fact, nuclear condensation and Annexin V expression, markers of apoptosis, were observed when cells were treated with the 5-LOX inhibitor and BLT1 antagonist. Similar results have

been reported for the treatment with an LTB_4-receptor antagonist, LY293111, on apoptosis in human cancer cells (10, 22).

The K_i value of U75302 derived from binding assay is much lower than the IC_{50} derived from culture cell proliferation and survival. The binding assay data was obtained using tissue membrane prepared from guinea-pig lung; that is, the most sensitive material was used the in vitro binding assay. In contrast, we used cultured cells in our present study, and cell culture medium contains fetal bovine serum and other reagents. We think, therefore, it is not surprising to see the difference between the K_i value derived from the binding assay and the IC_{50} of cultured cell proliferation.

Although our data strongly suggested that the blockade of LTB_4-receptor signaling pathway suppressed cancer cell growth, definitive evidence is still required. Therefore, we determine whether knockdown of the LTB_4 receptor by the RNA interference approach can suppress the proliferation of colon cancer cells. Our results demonstrated that successful knockdown of the BLT1 by siRNA resulted in the inhibition of cell proliferation. These results, together with previous reports, strongly suggest that the LTB_4-receptor signaling pathway plays a critical role in the cell survival and proliferation of colon cancer cells.

However, it is still unclear how LTB_4-receptor signaling stimulates colon cancer cell proliferation. Several mechanisms for stimulation of cell proliferation by LTB_4-receptor signaling have recently been suggested. Induction of ERK phosphorylation by LTB_4 has been reported in human pancreatic cancer cells (10), and exogenous LTB_4 has been reported to stimulate ERK in rat fibroblasts (23). In the present study, we also demonstrated that LTB_4 induced phosphorylation of ERK in both Caco2 and HT-29 cells. In addition, LTB_4-induced phosphorylation of ERK was inhibited by the treatment with the BLT1 antagonist U75302. The MAPK/ERK signaling pathway is known to be important for growth in many cell types (24, 25). Activation of ERK occurs through phosphorylation of threonine and tyrosine residues, and activated ERK dimer can regulate targets in the cytosol and the nucleus where it phosphorylates a variety of transcription factors resulting in regulation of gene expressions (24, 25). Therefore, our results indicate that the stimulation of cell proliferation by LTB_4 may be mediated by the activation of the ERK pathway via BLT1.

In conclusion, strong expression of the LTB_4 receptor was observed in colon cancer tissues and cultured cancer cell lines. Blockade of the LTB_4-receptor-signaling pathway significantly decreased cell survival and proliferation of colon cancer cells via induction of apoptosis. Our findings have revealed the role of LTB_4 in the colon cancer cells and provide a new approach to prevent carcinogenesis in the colon.

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Forum Minireview

Life Style-Related Diseases of the Digestive System: Endocrine Disruptors Stimulate Lipid Accumulation in Target Cells Related to Metabolic Syndrome

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Abstract. Many reports indicated that endocrine disruptors (EDs) affect several hormonal functions in various living things. Here, we show the effect of EDs on lipid accumulation in target cells involved in the onset of metabolic syndrome. Treatment with nonylphenol and bisphenol A, typical EDs, stimulated the accumulation of triacylglycerol in differentiated adipocytes from 3T3-L1, preadipocytes, in time- and concentration-dependent manners. Up-regulation of gene expressions involved in lipid metabolism and metabolic syndrome were observed in adipocytes treated with EDs. Similarly, stimulatory effects of EDs were also observed on the human hepatoma cell line HuH-7. These observations indicate that exposure to EDs stimulates the lipid accumulation in target cells involved in the metabolic syndrome and may cause the dysfunction of those cells, resulting in induction of metabolic syndrome.

Keywords: life style-related disease, endocrine disruptor, metabolic syndrome, adipocyte, hepatocyte

Introduction

Diabetes mellitus, hyperlipidemia, hypertension, and atherosclerosis have recently been defined as typical life style-related diseases. The common background of these diseases is obesity. Recently, the incidence of obesity and associated metabolic syndrome has been dramatically increased. Although high caloric foods like those found in a typical Western style diet are believed to represent the root of such a dramatic increase, there may be other possible causes. It is plausible and provocative to relate the recent increased incidence of metabolic syndrome to a dramatic increase in the use of industrial chemicals over the past 50 years. Namely, exogenously obesity-inducible substances, "Environmental Obesogens", are now considered as the one of the potential causes of obesity and associated metabolic syndrome (1, 2). Especially, environmentally existing xenobiotic chemicals, such as endocrine disruptors (EDs), would be probable candidates.

EDs are a great concern throughout the world because they have adverse effects on human beings. However, most of previous reports about the adverse effects of EDs have been concerned with the reproductive system (3 – 6). We previously reported the inhibitory effect of EDs on neural stem cell proliferation and differentiation, indicating the possibility that EDs may influence various physiological systems as well as the reproductive system (7). Therefore, investigations for other potential adverse effects by EDs are required. In fact, high concentrations of EDs, such as nonylphenol or bisphenol A, exist in the environment. Nonylphenol is used in surfactants or

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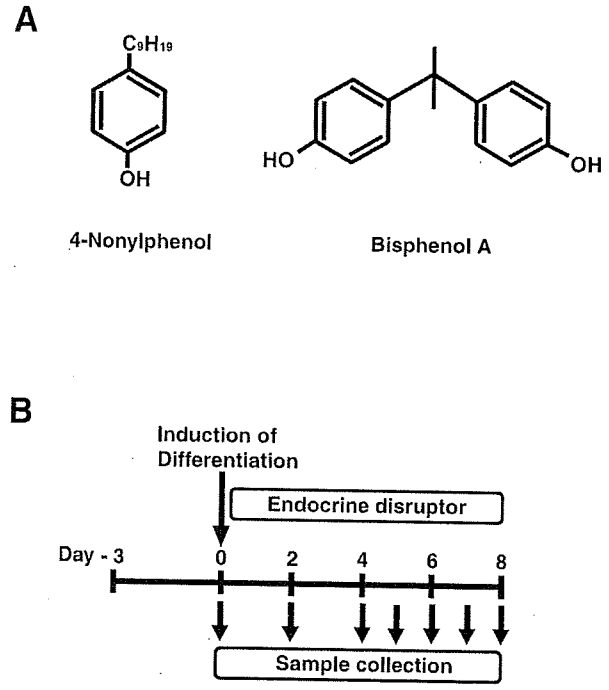

Fig. 1. Structures of endocrine disruptors and schematic protocol. A: Structures of endocrine disruptors (EDs) used in the present study (4-nonylphenol and bisphenol A). B: Schematic protocol of adipocyte differentiation. The 3T3L-1 preadipocytes were confluent during the culture for 3 days, and then induction of differentiation was started by the addition of insulin, dexamethazone, 3-isobutyl-1-methylxanthine, or rosiglitazone, a PPARγ agonist. Endocrine disruptors ($0.1-10\,\mu$M) were administered to the culture system at the start of induction (Day 0). Samples were collected at the points indicated.

plastic and petroleum processing, and bisphenol A is used in polycarbonate plastics or dental resin-based composites.

In this review, we summarize the effect of EDs on lipid accumulation in target cells, such as adipocytes and hepatocytes, related to the metabolic syndrome that induce diabetes, fatty liver, hyperlipidemia, and atherosclerosis. We selected nonylphenol and bisphenol A, two well-known EDs, , for the investigation because they are widely used and comparatively high concentrations of them were detected in the environment (Fig. 1A).

Nonylphenol and bisphenol A stimulate accumulation of triacylglycerol in adipocytes

The adipocytes in visceral fat are considered to be the most important cells involved in the onset of metabolic syndrome (8 – 10). We, therefore, applied the EDs nonylphenol and bisphenol A to 3T3-L1 preadipocytes to see their effects on the differentiation to mature adipocytes (Fig. 1B, Ref. 11). Treatment with nonylphenol and bisphenol A each significantly stimulated the accumulation of triacylglycerol, a marker of lipid accumulation, in mature adipocytes differentiated from 3T3-L1 preadipocytes (Fig. 2: A and B); the response to each ED was time- and concentration-dependent (data not shown). Interestingly, increase in cell size was observed in the nonylphenol-treated adipocytes, but not in the bisphenol A-treated ones. These results indicate the possibility that both nonylphenol and bisphenol A can promote the differentiation of preadipocytes to mature adipocytes.

Nonylphenol and bisphenol A stimulate accumulation of triacylglycerol in hepatocytes

Hepatocytes in the liver are also considered to play important roles in metabolic syndrome because the liver controls the metabolism of various substances including lipids (9). Excessive lipid accumulation in hepatocytes leads to liver dysfunction, resulting in further aggravation of the systemic condition. Therefore, we applied nonylphenol and bisphenol A to HuH-7 cells, a human hepatocellular carcinoma cell line, to investigate their effects on lipid accumulation (12). Treatment with nonylphenol and bisphenol A caused time- and dose-dependent, significant increases in glycerol accumulation (data not shown). These results clearly indicate that nonylphenol and bisphenol A have the ability to stimulate the lipid accumulation not only in adipocytes but also in hepatocytes (Fig. 4).

Major alteration of gene expression in adipocytes treated with EDs

To investigate the alterations of genes treated with EDs, we performed exhaustive analysis of genes using a DNA microarray. Up-regulated gene expressions involved in lipid metabolism, adipocyte differentiation, and inflammation were observed by the treatment with nonylphenol (Fig. 3). Namely, the up-regulation of hormone-sensitive lipase, phospholipase A_2, and phospholipase C that are involved in lipid metabolism were observed by the treatment with nonylphenol. In addition, up-regulation of CD36, a receptor for oxidized- or acetylated-LDL, and TNF-α, an important adipocytokine to induce insulin resistance, were also observed (8 – 10). In contrast, up-regulation of the adiponectin gene was much smaller than those of phospholipase and TNF-α. Similar results were also observed when cells were treated with bisphenol A (data not shown). These results clearly indicate that EDs accelerate the differentiation of preadipocytes to mature adipocytes,

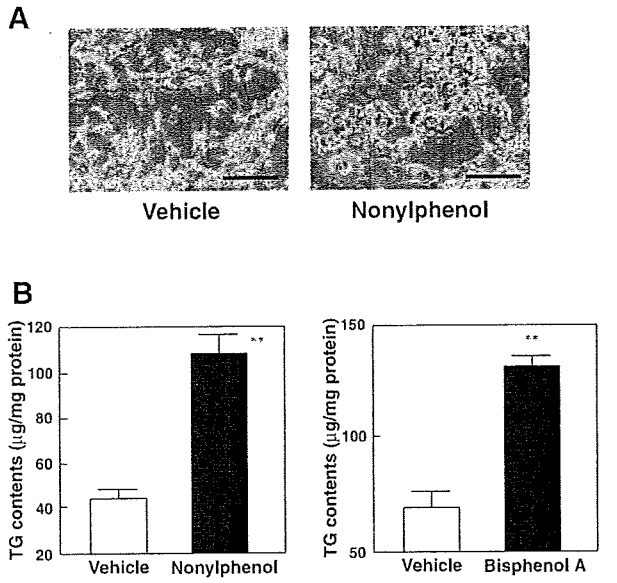

Fig. 2. Effects of EDs on lipid accumulation. A: Typical photographs of differentiated adipocytes from 3T3L-1 preadipocytes treated with nonylphenol (3 μM) or vehicle for 5 days. Cells were stained by the Oil Red O method to visualize lipid accumulation. Scale bar is 200 μm. B: Effects of nonylphenol and bisphenol A on lipid accumulation in differentiated adipocytes. The 3T3-L1 cells were treated with nonylphenol (10 μM, left panel) or bisphenol A (10 μM, right panel) for 5–6 days. Then, accumulation of triacylglycerol (TG), a marker of lipid accumulation, in mature adipocytes was measured and expressed as TG contents. Each column represents the mean ± S.E.M. from 5–8 independent experiments. $^{**}P<0.01$ *vs* vehicle control.

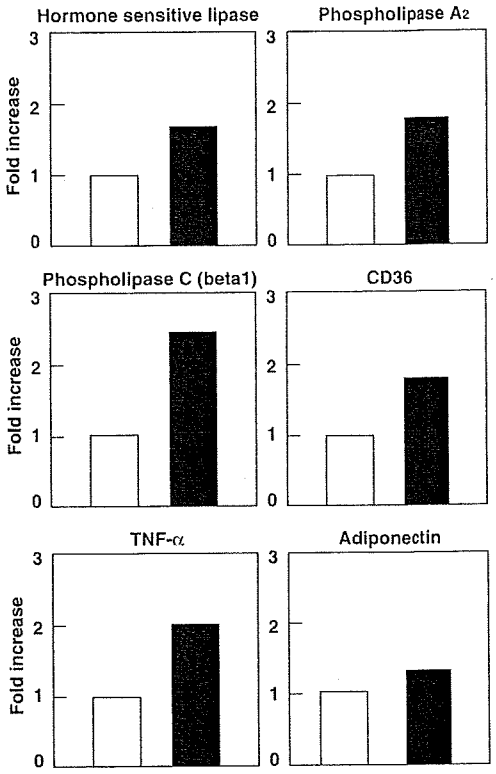

Fig. 3. Major alterations of gene expression in preadipocytes treated with nonylphenol on differentiated adipocytes. The 3T3L-1 preadipocytes were treated with nonylphenol (10 μM) for 4 days, and then total RNA was prepared and applied to DNA microarray analysis. Various genes were altered, and the major altered genes related to lipid metabolism and metabolic syndrome are shown. Fold increase of gene expression by nonylphenol treatment (closed column) compared to vehicle treatment (open column) is shown.

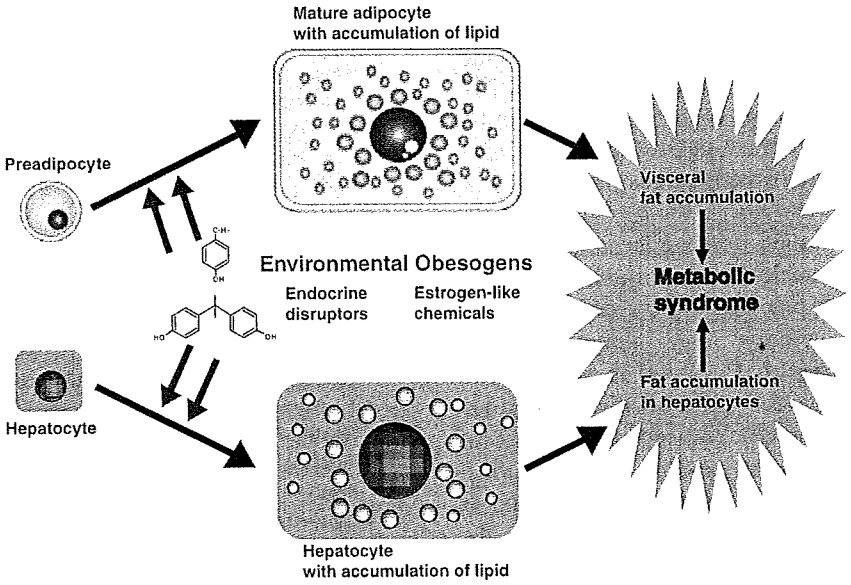

Fig. 4. Schematic illustration of possible involvement of EDs in lipid accumulation in adipocytes and hepatocytes.

resulting in the accumulation of lipids in the cell body. In addition, the direction of differentiation is toward enlargement of cell size, indicating large adipocytes rather than small adipocytes. Because adiponectin is mainly released from small adipocytes (8 – 10), these results suggest that the acceleration of adipocyte differentiation by EDs aggravate the condition of metabolic syndrome (Fig. 4).

Concluding remarks

So far, it is not fully understood whether environmental chemicals such as EDs promote the onset of metabolic syndrome. However, several recent reports, including our present observations, indicate that EDs have the potential to stimulate the lipid accumulation in target cells, such as adipocytes and hepatocytes, related to obesity and associated metabolic syndrome. These observations suggest that EDs are possible environmental obesogens, exogenously obesity-inducible xenobiotic chemicals. It has also been advocated that organotins are potent environmental obesogens because tributyltin promotes adipocyte differentiation via the peroxisome proliferator-activated receptor gamma (PPARγ) pathway (2, 13 – 15). Thus, the mechanism of adipocyte differentiation by organotins is comparatively well understood. In contrast, the mechanisms of the other EDs have not been fully investigated. Several reports indicated that most of the EDs such as nonylphenol and bisphenol A bind to estrogen receptors, resulting in their actions on the reproductive system (2 – 6). However, it is doubtful whether the effect on adipocyte differentiation by nonylphenol and bisphenol A may be mediated solely through the estrogen receptor signaling pathway because these observations, including ours, are in conflict with the general conclusions that estrogens are potentially anti-adipogenic (2, 16, 17). Therefore, other mechanisms could be involved in the adipocyte differentiation stimulated by nonylphenol and bisphenol A, and further investigations are required to clarify this point.

Another important issue is whether the target cells can actually be environmentally exposed to concentrations such as those used in previous reports and our study. Most of previous reports used nonylphenol or bisphenol A in the range of 1 – 30 μM in their in vitro studies. In contrast, we also observed the stimulation of lipid accumulation in the concentration range of 30 nM to 10 μM. It may be possible that in normal circumstances, the level of nonylphenol or bisphenol A in contaminated water or foods is still lower than the effective concentration on lipid accumulation in the present study. However, nonylphenol has been reported to appear in the aquatic environment, particularly in sediment, and can reach up to 3000 ppb in rivers and lakes (18). In addition, it has been reported that lipid-soluble environmental pollutants, including nonylphenol, accumulate to high concentrations in animal and human tissues (19). Furthermore, we detected the accumulation of nonylphenol and bisphenol A in liver, visceral fat, pancreas, and other target organs in mice orally administered with these compounds (unpublished data). Therefore, low-level chronic or high-level acute exposure to EDs might increase their blood and tissue concentrations in animals or humans to reach the levels high enough to elicit the effects on the target cells to stimulate lipid accumulation.

In conclusion, exposure to EDs stimulates the lipid accumulation in target cells involved in metabolic syndrome and may cause the dysfunction of those cells, resulting in induction of metabolic syndrome.

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Journal of Pharmacological Sciences

Forum Minireview

Life Style-Related Diseases of the Digestive System: Colorectal Cancer as a Life Style-Related Disease: from Carcinogenesis to Medical Treatment

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Abstract. Life style-related diseases are associated with an increased risk of colorectal cancer (CRC). Recently, an association has been demonstrated between obesity and CRC. CRC has been associated with markers of insulin or glucose control, and insulin resistance might be the unifying mechanism by which several risk factors affect colorectal carcinogenesis. We evaluated the association between the number of aberrant crypt foci (ACF) and obesity, insulin resistance, hyperlipidemia, and other factors of life style-related disease. As a result, age, body mass index (BMI), waist circumference, and visceral fat obesity were significantly associated with the number of ACF. These results suggest that visceral fat obesity is an important target for CRC prevention. Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the nuclear receptor superfamily and is highly expressed in CRC. PPARγ ligand administration for 1 to 8 months significantly reduced the number of ACF in human subjects. PPARγ ligand is a promising candidate as a chemopreventive agent. Further investigation is needed to elucidate these mechanisms.

Keywords: life style-related disease, colorectal cancer, chemoprevention, aberrant crypt foci (ACF), peroxisome proliferator-activated receptor gamma (PPARγ)

Introduction

A diet rich in fat and calories and low intake of vegetable, fruits, and fibers are referred to as a Western diet. Chronic conditions including obesity, diabetes, hyperlipedemia, hypertension, and cardiovascular disease have been shown to be associated with a Western diet, alcohol intake, and smoking. Indeed, obesity has been reported to be associated with an elevated risk of cardiovascular disease, diabetes, and mortality (1 – 4). Especially, visceral fat obesity is increasing and is becoming a significant social problem. Recently, these life styles have also been shown to be correlated with increase in colorectal cancer (CRC) risk.

CRC is a disease with a high mortality and morbidity rate, and currently, its prevalence has been increasing worldwide. On the other hand, CRC is potentially one of the most preventable malignancies (5, 6). Correction of the life styles mentioned above may have a major potential for CRC prevention. On the other hand, early detection of CRC or CRC precursor lesions may be promoted by screening of the population at high risk. In addition, chemoprevention, the use of medications to prevent disease, has now been extensively explored in CRC. Some of these interventions, such as supplemental fibers, calcium supplementation, aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs), and selective cyclooxygenase (COX)-2 inhibitors, have been shown to have a potential to reduce both CRC and colorectal adenomas. CRC is thought to progress through several morpho-

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logical stages, from the formation of adenomatous polyps to malignant conversion (7). Genetic alterations including mutations in the *APC, K-ras*, and *p53* genes have been reported to accompany the disease progression (8). The earliest identifiable lesion in this pathway may be the aberrant crypt foci (ACF). ACF are prepolyp abnormalities identified in single crypts by magnifying colonoscopy after the administration of methlyene blue dye. Therefore, ACF may be a surrogate marker of CRC, and analysis of the association between clinicopathological variables and ACF formation may be of great significance.

Diabetes, obesity, and CRC

Diabetes, obesity, hyperinsulinemia, and insulin resistance have been repeatedly shown to be associated with CRC (9 – 14). Diabetes was associated with an increased risk for CRC in cohort studies, in accordance with previous studies that demonstrated that diabetes is a moderate risk factor for CRC. Overweight, obesity, or high BMI has been consistently associated with increased risk for CRC incidence and mortality, at least in men and premenopausal women (15 – 18). The WHO definition of the life style-related disease allows the use of a body mass index (BMI) of at least 30 kg/m^2 instead of waist circumference or waist-to-hip ratio (19).

ACF as a biomarker of CRC

ACF, which represent clusters of aberrant colorectal crypts, were first discovered in mice treated with azoxymethane (20). ACF have been demonstrated to be precursor lesions of CRC, and with further investigations, ACF have been established as a biomarker of the risk of CRC in azoxymethane-treated mice and rats (21). In humans, the relationship between ACF and CRC is less clear. The number of ACF was measured using magnifying endoscopy, but the association between ACF and CRC was only partially evident. ACF, however, are also thought to be precursor lesions of colorectal adenoma and CRC in humans (22). Many factors are known to be associated with increased or decreased risk of CRC. Among them, only history of adenomas, age, and the use of NSAIDs have been examined in relation to the development of ACF. The relationships of other factors to the occurrence of ACF in the colon remain unknown.

In the rat model, the formation of ACF was enhanced by cancer promoters and suppressed by chemopreventive agents (23). ACF has also been reported in colonic mucosa in humans (24, 25). Patients with CRC had more ACF with *K-ras* mutations than those without CRC. These results suggest that ACF are not only morphologically but also genetically distinct lesions and are precursors of adenoma and CRC.

Table 1. Relationships between selected risk factors for colorectal cancer and the number of ACF in the colorectum

Risk factors	Correlation coefficient	*P*-value
Age (years)	0.256	0.0121*
BMI	0.263	0.0044*
Waist (cm)	0.370	0.0003*
Fasting blood sugar	0.021	0.7575
HOMA-IR	0.263	0.6174
Total cholesterol	0.263	0.4771
Triglyceride	0.263	0.2049
VFA (cm^2)	0.512	<0.0001*
SFA (cm^2)	0.108	0.2091

*P-values <0.05 were considered to denote statistical significance. HOMA-IR: homeostasis model assessment of insulin resistance.

ACF and visceral fat obesity

The association between the number of ACF and age, BMI, waist circumference, diabetes, serum lipid, visceral fat area (VFA), and subcutaneous fat area (SFA) were evaluated (Table 1). Our findings indicate that age, BMI, waist circumference, and VFA were significantly associated with the number of ACF. Especially, VFA was strongly associated with the number of ACF. Visceral fat tissue is known as an endocrine organ that secretes adipocytokines such as TNF-α, leptin, and adiponectin. These adipocytokines and/or the visceral fat itself may play an important role in colon carcinogenesis. The number of ACF increased with age. Genetic and epigenetic alterations accumulate with advancing of age; therefore, the increased risk of ACF formation with age may be mainly influenced by these genetic alterations.

Chemoprevention for CRC

Chemoprevention, the use of medications to prevent disease, has now been extensively explored in CRC. Supplemental fibers, calcium supplementation, aspirin, NSAIDs, and selective COX-2 inhibitors have a potential to reduce both CRC and colorectal adenomas. Higher doses and longer durations of use of NSAIDs and COX-2 inhibitors seem to be associated with greater protection from CRC and adenoma. However, these agents are associated with significant cardiovascular events and/or gastrointestinal damage. The balance of benefits to risk does not favor chemoprevention by these

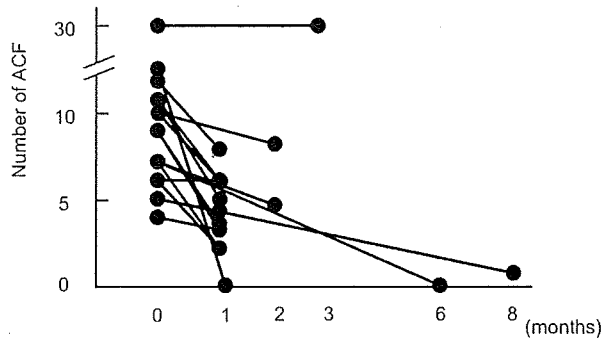

Fig. 1. The numbers of ACF were prospectively examined in 14 subjects before and after 1 – 8 months of pioglitazone treatment. After PPARγ ligand treatment, the number of ACF significantly decreased.

agents in average-risk individuals.

ACF and PPARγ

PPARγ is mainly expressed in adipose tissue and plays a central role in adipocyte differentiation and insulin sensitivity. Activating synthetic ligands for PPARγ, such as pioglitazone, are commonly used to treat diabetes. PPARγ is also overexpressed in many tumors. This suggests that modulation of PPARγ expression or function might have impact on tumor cell survival. Chemopreventive effects of PPARγ ligand on the formation of the human ACF were evaluated. Fourteen patients were examined for ACF by magnifying colonoscopy before and after 1 to 8 months of pioglitazone treatment (Fig. 1). After PPARγ ligand treatment, the number of ACF was significantly decreased. These results suggest that a PPARγ ligand is a promising candidate as a chemopreventive agent for CRC.

Conclusions

Our results suggest that visceral fat obesity may be a risk factor for CRC and visceral fat may play an important role in colorectal carcinogenesis at an earlier stage in the adenoma-carcinoma sequence (25). Adipocytokines secreted by visceral fat tissue and/or the visceral fat itself may play an important role in colon carcinogenesis. PPARγ ligand is a promising candidate for CRC chemoprevention.

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Forum Minireview

Life Style-Related Diseases of the Digestive System: from Molecular Mechanisms to Therapeutic Strategies: Preface

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Diabetes, obesity, atherosclerosis, hypertension, hyperlipidemia, myocardial infarction, stroke, hepatitis, fatty liver, colorectal cancer, pulmonary cancer, for example, were defined as life style-related diseases by the Japanese Ministry of Health, Labor and Welfare in 1996. Individual life style factors such as food, exercise, smoking, drinking, and stress are closely related to the onset of diseases. Recently, the number of patients with life style-related diseases has been markedly increasing year by year. Therefore, drastic steps including prevention and drug therapy of the diseases must be taken to prevent further increases in the number of patients with these diseases and to bring relief to those who have already developed these diseases. This is particularly urgent because the percentage of life style-related diseases of the digestive system, including the liver, intestine, and pancreas, has remarkably increased.

The purposes of the reviews presented here are to introduce the recent advances in elucidating the molecular mechanisms for these diseases and discuss the application of this information to develop an appropriate drug therapy for life style-related diseases of the digestive system. The first article presented by Takahashi and colleagues (doi: 10.1254/jphs.FM0070022) provides clear evidence showing that the metabolic syndrome is an important risk factor for colorectal cancer. The interesting article clarifies the relationship between aberrant crypt foci, pre-cancerous stage, and visceral fat, body mass, and serum lipid and suggests to us the application of peroxisome proliferator-activated receptor gamma (PPARγ) ligand to prevent the aberrant crypt foci formation in human subjects. In the second article (doi: 10.1254/jphs.FM0070034), Wada and colleagues show the possibility that endocrine disruptors stimulate lipid accumulation in target cells and organs related to metabolic syndrome. We are exposed to various endocrine disruptors derived from synthetic detergents, plastics, oils, and so on, and therefore our bodies accumulate endocrine disruptors in adipose tissue, liver, and pancreas. Thus, these endocrine disruptors in our environment may be one of the risk factors for metabolic syndrome.

Hepatitis C virus (HCV) infection and the following hepatitis and cirrhosis are major liver diseases, and the patients have increased on a world-wide scale. The third article presented by Saito and colleagues (doi: 10.1254/jphs.FM0070040) shows a new and unique model of hepatitis C virion production. Their data suggest that it may be possible to develop vaccines and therapeutic drugs for hepatitis C. In addition, Ikeda summarizes the screening for anti-HCV reagents using his unique cell culture system (doi: 10.1254/jphs.FM0070050). He shows the utility of combination therapy of statin with interferon. This exciting article provides us with a new therapeutic strategy of drugs for hepatitis C.

Nonalcoholic steatohepatitis is now recognized as one of the most common causes of liver disease. However, the molecular mechanisms of the onset of nonalcoholic steatohepatitis (NASH) are unclear. Yoneda and colleagues, based on their DNA microarray analysis,

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demonstrate the target genes involved in the onset of NASH (doi: 10.1254/jphs.FM0070063). They also show the therapeutic utility of a PPARγ ligand to attenuate liver dysfunction.

This forum minireview is based on the symposium on "Life style-related diseases of the digestive system: from molecular mechanisms to therapeutic strategies" at the 80th Annual Meeting of The Japanese Pharmacological Society held on March 14, 2007, Nagoya. All the authors hope that this forum minireview helps the readers to understand the basic molecular mechanisms for these diseases and the application of this knowledge to develop appropriate drug therapies for these diseases.

Interleukin-4 Cytotoxin Therapy Synergizes with Gemcitabine in a Mouse Model of Pancreatic Ductal Adenocarcinoma

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Abstract

Targeting cell surface receptors with cytotoxins or immunotoxins provides a unique opportunity for tumor therapy. Here, we show the efficacy of the combination therapy of gemcitabine with an interleukin-4 (IL-4) cytotoxin composed of IL-4 and truncated *Pseudomonas* exotoxin in animal models of pancreatic ductal adenocarcinoma (PDA). We have observed that 42 of 70 (60%) tumor samples from patients with PDA express moderate- to high-density surface IL-4 receptor (IL-4R), whereas normal pancreatic samples express no or low-density IL-4R. IL-4 cytotoxin was specifically and highly cytotoxic [50% protein synthesis inhibition (IC_{50}) ranging from >0.1 to 13 ng/mL] to six of eight pancreatic cancer cell lines, whereas no cytotoxicity (IC_{50} >1,000 ng/mL) was observed in normal human pancreatic duct epithelium cells, fibroblasts, and human umbilical vein endothelial cells (HUVEC). We also showed that IL-4 cytotoxin in combination with gemcitabine exhibited synergistic antitumor activity *in vitro*. To confirm synergistic antitumor activity *in vivo* and monitor precise real-time disease progression, we used a novel metastatic and orthotopic mouse model using green fluorescent protein–transfected cancer cells and whole-body imaging system. The combination of both agents caused complete eradication of tumors in 40% of nude mice with small established PDA tumors. In addition, combined treatment significantly prolonged the survival of nude mice bearing day 14 advanced distant metastatic PDA tumors. Similar results were observed in mice xenografted with PDA obtained from a patient undergoing surgical resection. These results indicate that IL-4 cytotoxin combined with gemcitabine may provide effective therapy for the treatment of patients with PDA. [Cancer Res 2007;67(20):9903–12]

Introduction

Pancreatic ductal adenocarcinoma (PDA) is one of the most lethal human malignancies (32,000 deaths per year). Because of its aggressive growth and rapid metastasis to lymph nodes and liver, only 10% to 15% of patients are found to be resectable at diagnosis (1). Currently, the most common strategy for the treatment of advanced pancreatic cancer is treatment with gemcitabine, although the median survival time continues to be <6 months for these patients (2, 3). Recently, several types of inhibitors targeting the epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, and nuclear factor-κB (NF-κB) have shown their effectiveness in pancreatic cancer in murine models (4–7). In clinical trials, EGF receptor tyrosine kinase inhibitor (Erlotinib, Tarceva) plus gemcitabine enhances 1-year survival for patients with advanced pancreatic cancer (8). However, the difference in median survival between Erlotinib plus gemcitabine group and gemcitabine alone group is <1 month. An effective new approach is needed for management of patients with this disease.

Targeting cell surface receptor with cytotoxins or immunotoxins provides a unique opportunity for tumor therapy. Targeted toxins offer the advantage of enhanced specificity and direct toxicity for tumor cells that overexpress the receptor, thus limiting the potential toxicity to normal tissues (9). Several clinical trials using IL-4 cytotoxin, IL-13 cytotoxin, and recombinant immunotoxin BL22 have shown survival benefits in patients with glioblastoma multiforme, chronic lymphocytic leukemia, and hairy cell leukemia (10–13).

Interleukin-4 (IL-4) is an important Th2-derived cytokine, which is involved in mediating antitumor immune-modulating activities (14). IL-4 has been shown to have a modest but direct inhibitory effect on the growth of several tumor cells *in vitro* and *in vivo* (15, 16). Based on these properties, IL-4 was tested in the clinic as a treatment for hematopoietic and solid malignancies, but it showed limited antitumor activity (17). To improve this limited activity, we targeted IL-4 receptor (IL-4R) because a variety of human tumor cells, including pancreatic cancer, express high-affinity receptors for IL-4 (18–22). We have created a circular permuted IL-4, which was fused to a mutated form of *Pseudomonas* exotoxin [the fusion protein termed IL4(38-37)-PE38KDEL or IL-4 cytotoxin; ref. 23]. After binding to IL-4R, IL-4 cytotoxin internalizes, translocates, and then ribosylates elongation factor 2 to prevent the initiation of protein synthesis, leading to cell death (24). The IL-4 cytotoxin is highly and specifically cytotoxic to several types of tumor cells *in vitro* and has remarkable antitumor activity in animal xenograft models of a variety of human tumors (18–22). The safety and tolerability of IL-4 cytotoxin was shown in phase 1 clinical trials in patients with advanced solid tumors (25). The efficacy of IL-4 cytotoxin was also shown by long-term survival of patients with recurrent malignant glioma (10, 11, 26).

In this study, we examined expression of IL-4R in samples derived from PDA and the efficacy of IL-4 cytotoxin, gemcitabine, and combination of both in primary and metastatic tumor models. To imitate aggressive clinical situation and to monitor precise real-time disease progression, we used a novel metastatic and orthotopic advanced pancreatic cancer model using retroviral green fluorescent protein (GFP)–transfected pancreatic cancer cell

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line and whole-body imaging system (27). Together, our study shows that IL-4 cytotoxin synergizes with gemcitabine, significantly inhibiting the growth of primary and metastatic tumor lesions, prolonging the survival time, and completely eradicating tumors in 40% of mice in an early pancreatic cancer model.

Materials and Methods

Cell culture, reagents, and tissue specimens. Cell lines were obtained from the American Type Culture Collection and Sciencell. Human pancreatic duct epithelium (HPDE) cells were cultured routinely in keratinocyte serum-free medium supplemented with bovine pituitary extract and epidermal growth factor (Life Technologies; ref. 28). IL-4 cytotoxin [IL4(38-37)-PE38KDEL] was produced as described previously (23). Fifteen paraffin-embedded tissue sections and tissue arrays containing 70 tumor specimens were obtained from Cooperative Human Tissue Network and U.S. Biomáx, respectively. Gemcitabine was procured through the pharmacy of the clinical center (NIH).

Immunohistochemistry and flow cytometry. Immunohistochemistry was done as described previously (24). Deparafinized tissue sections were incubated with anti-human IL-4Rα polyclonal antibody (Santa Cruz Biotechnology) or isotype control (IgG). The results were scored on the basis of the density of staining 0%, 0% to 10%, 11% to 50%, 51% to 100% as negative, weak, moderate, and strong, respectively. Tissue sections for IL-4R were evaluated by Dr. Satoru Takahashi who is a pathologist at Nagoya City University in Japan.

Expression of IL-4Rα on pancreatic cancer cell lines and HPDE cells was assessed by flow cytometry using phycoerythrin-conjugated anti–IL-4Rα monoclonal antibody as previously described (29). Staining with isotype-matched IgG served as control.

Protein synthesis inhibition assay and assessment of synergism or antagonism. The *in vitro* cytotoxic activity of IL-4 cytotoxin, gemcitabine, and their combination was measured by the inhibition of protein synthesis (18).

Drug interaction between IL-4 cytotoxin and gemcitabine was assessed at a concentration ratio of 1:1, using the combination index (CI), where CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively (30). On the basis of the isobologram analysis for mutually exclusive effects, the CI value was calculated as follows:

$$\mathrm{CI} = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2}$$

where $(D_x)_1$ and $(D_x)_2$ are the concentrations of IL-4 cytotoxin and gemcitabine, respectively, required to inhibit cell growth by 50%, and $(D)_1$ and $(D)_2$ are the drug concentrations in combination treatments that also inhibit cell growth by 50% (isoeffective compared with the single drugs).

Semiquantitative and real-time TaqMan reverse transcription-PCR. Semiquantitative reverse transcription-PCR (RT-PCR) was done as described previously (31). Quantification of IL-4Rα mRNA expression levels in pancreatic cancer cell lines was determined by real-time RT-PCR using a set of IL-4Rα–specific TaqMan probe (5′-FAM, 3′-MGB) and primers (Applied Biosystems; ref. 24). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase or β-actin before the fold change in gene expression was calculated.

Retroviral transduction and selection of high-GFP–expressing MIA-PaCa-2 pancreatic cancer cells. MIA-PaCa-2 cells expressing GFP were established using a 1:1 precipitated mixture of retroviral supernatants of the HEK293 cells and RPMI 1640 (Life Technologies, Inc.), as described previously (32).

Animals. Severe combined immunodeficient (SCID) mice and nude *nu/nu* mice between age 5 and 6 weeks were maintained in a barrier facility on HEPA-filtered racks. All animal studies were conducted under an approved protocol in accordance with the principles and procedures outlined in the NIH Guideline for the Care and Use of Laboratory Animals.

Whole-body imaging. The tumor-bearing mice were periodically examined in a fluorescence light box illuminated by fiberoptic light at 440/20 nm wavelength (Lightools Research, Inc.). Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology) on a Hamamatsu C5810 3-chip cooled color charge coupled device camera (Hamamatsu Photonics Systems). Real-time determination of tumor burden was done by quantifying fluorescent surface area as described previously (32).

Surgical orthotopic implantation of MIA-PaCa-2-GFP tumors. MIA-PaCa-2-GFP cells were injected s.c. into the right dorsal flank of nude mice. Pancreatic tumors, grown s.c. in nude mice, were cut with scissors and minced into ~3 × 3 × 3-mm pieces. For orthotopic surgery, the pancreas was carefully exposed, and tumor chunks were transplanted on the middle of the pancreas with a 6-0 Dexon surgical suture (Davis-Geck, Inc.). The pancreas was then returned to the peritoneal cavity, the abdominal wall, and the skin was closed with 6-0 Dexon sutures.

Experimental design and treatment. For early pancreatic cancer model, primary tumor lesions were detected by external whole-body imaging on day 4 after transplantation. Once the tumors were visualized, mice were randomized into four groups of 10 each. Treatment was initiated on day 5. For advanced pancreatic cancer model, primary and metastasis tumor lesions were detected by external whole-body imaging on day 14 posttransplantation of tumor chunk and randomized into four groups of 10 mice each. Treatment was initiated on day 15.

Primary and orthotopic pancreatic cancer model using a clinical sample. Primary pancreatic cancer specimens were obtained from a patient undergoing radical pancreatectomy at National Cancer Institute under institutional review board–approved protocol. Viable tumor tissue from specimen was cut into small pieces (3 × 3 × 3 mm) and implanted in the pancreas of 5- to 6-week-old male SCID mice. Primary xenografts were propagated continuously in SCID mice for *in vivo* testing. Clinical sample–bearing mice were also treated after day 31 by the same protocol as described above.

Statistical analysis. The mean tumor volume in therapeutic and control groups was analyzed by ANOVA. Survival curves were generated by Kaplan-Meier method and compared by using the log-rank test.

Result

Expression of IL-4R in PDA tissues. Tissue sections from 15 normal pancreas and 70 PDA specimens were analyzed by immunohistochemical analysis for the expression of IL-4Rα (data not shown). As shown in Fig. 1*A*, tumor specimens showed weak to strong staining for IL-4Rα in PDAs. Only weak staining was observed in tumor-infiltrating stromal fibroblasts and endothelial cells. When the proportion of IL-4Rα–positive cancer cells was counted, 23 of 70 primary tumors classified into strong expression group, 19 into moderate expression group, 11 into weak expression group, and 17 into the negative expression group. Thus, 42 of 70 (60%) PDA samples expressed moderate to high-density IL-4Rα. In contrast, only 2 of 15 normal pancreas samples showed weak staining for IL-4Rα in normal acinar and ductal cells (Fig. 1*B*).

Pancreatic cancer cell lines expressing IL-4R are sensitive to IL-4 cytotoxin. We examined the expression of IL-4Rα mRNA by RT-PCR and real-time RT-PCR in eight pancreatic cancer and one normal HPDE cell lines. Six of eight cancer cell lines showed varied density of IL-4Rα mRNA expression, whereas HPAF-II, PK-1, and HPDE cell lines showed no expression (data not shown). Real-time RT-PCR analysis confirmed conventional RT-PCR results and showed that MIA-PaCa-2 and SW1990 cell lines expresses highest level of IL-4Rα mRNA, followed by Capan-1, ASPC-1, Panc-1, and HS766T cell lines (Fig. 1*C*). Flow cytometric analysis confirmed mRNA expression data and showed that IL-4Rα is expressed on the cell surface of three pancreatic cancer cell lines but not in normal HPDE cells (Fig. 1*D*).

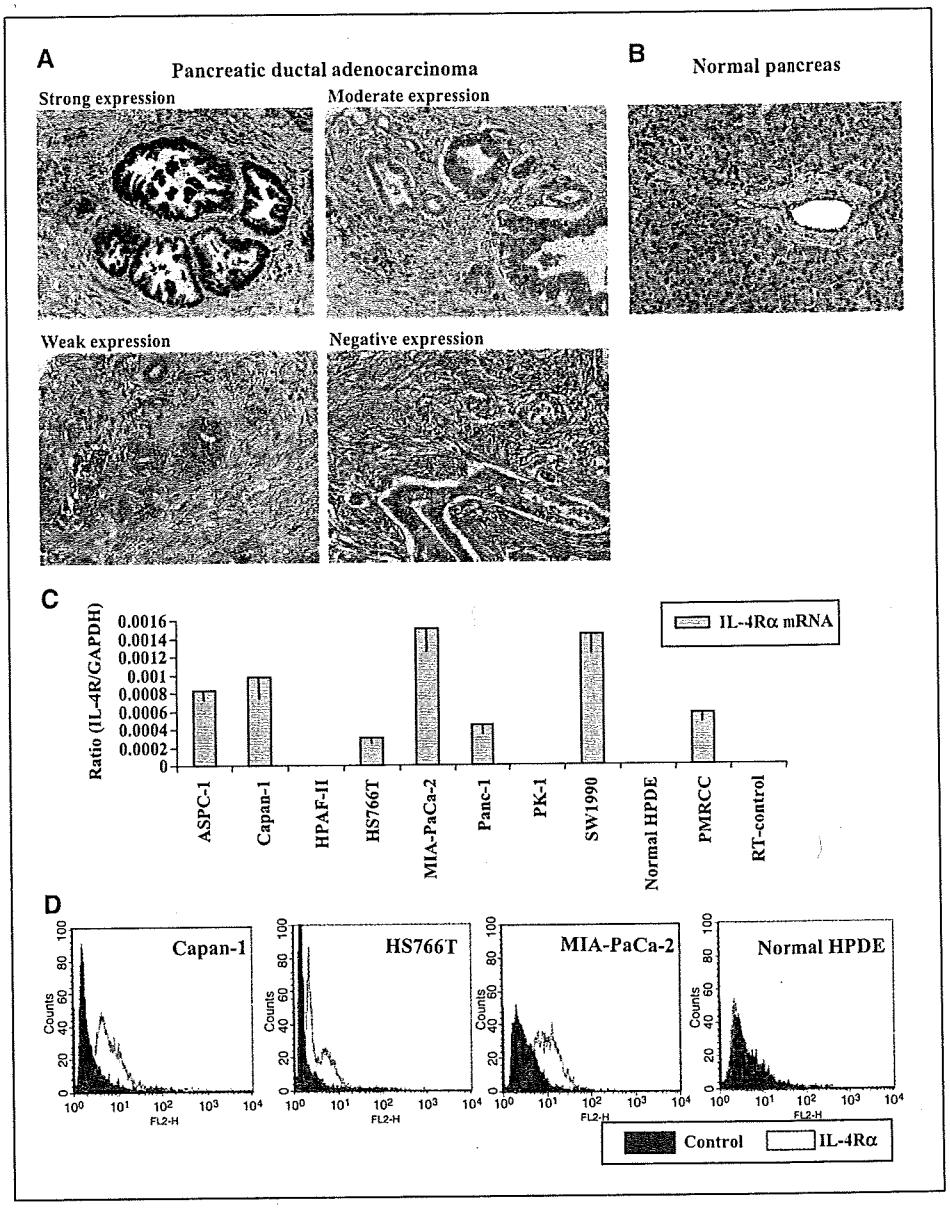

Figure 1. *In situ* expression of IL-4R in normal pancreas and PDA sections, mRNA, and surface expression of IL-4R in pancreatic cancer cell lines and normal HPDE. *A*, surgically resected tumor tissues were stained with anti–human IL-4Rα polyclonal antibody. Representative results from 70 tumor specimens. Magnification, ×200. *B*, representative result from 15 normal pancreas specimens stained with anti–human IL-4Rα polyclonal antibody. Magnification, ×200. *C*, RNA was isolated from 5 × 10^6 various pancreatic cancer cell lines and gene expression was quantified as described in Materials and Methods. Renal cell carcinoma (*PMRCC*) was used as a positive control. *Columns*, means of triplicate determinations; *bars*, SE. *D*, expression of IL-4Rα on pancreatic cancer cell lines and HPDE cells was assessed by flow cytometry using phycoerythrin-conjugated anti–IL-4Rα monoclonal antibody (*open curve*). Staining with isotype-matched IgG served as control (*shaded curve*).

Next, we determined the sensitivity of pancreatic cancer cell lines to IL-4 cytotoxin by protein synthesis inhibition assay, which has been shown to be directly proportional to cell death (19). IL-4 cytotoxin inhibited protein synthesis of pancreatic cancer cell lines in a concentration-dependent manner. MIA-PaCa-2 and SW1990 cell lines were extremely sensitive to the cytotoxin (IC_{50} 0.08 and 0.36 ng/mL, respectively), followed by Capan-1 (IC_{50} 7 ng/mL) and HS766T (IC_{50} 13 ng/mL; Fig. 2*A*). IC_{50} in Panc-1 and ASPC-1 cell lines was <10 ng/mL (data not shown). Consistent with the lack of IL-4Rα mRNA expression, HPAF-II and PK-1 cell lines were not sensitive to IL-4 cytotoxin ($IC_{50} \geq$ 1,000 ng/mL; data not shown). The cytotoxic activity of IL-4 cytotoxin was neutralized by incubation with an excess of IL-4, suggesting specific cytotoxicity through binding of IL-4 cytotoxin to IL-4R (data not shown). We also examined the cytotoxicity of IL-4 cytotoxin in fibroblast, HUVEC, and HPDE cell lines, because some of the specimens revealed weak expression of IL-4Rα in nontumor cells. However, IL-4 cytotoxin was not found to be cytotoxic to these cells ($IC_{50} \geq$ 1,000 ng/mL; Fig. 2*B*). The IL-4 cytotoxin cytotoxic activity correlated with extent of IL-4Rα expression. For example, MIA-PaCa-2 cells showed lowest IC_{50} and highest density IL-4R expression as determined by flow cytometric and real-time PCR analyses whereas PK-1 cell line showed highest IC_{50} as this cell line showed undetectable level of mRNA expression. We also used another cytotoxin IL-13 *Pseudomonas* exotoxin, an IL-13 receptor-specific fusion protein (12), to assess the cytotoxicity to pancreatic cancer cell line. However, IL-13 cytotoxin was not cytotoxic to HPAF-II cells ($IC_{50} \geq$1,000 ng/mL; data not shown).

Synergistic cytotoxicity of IL-4 cytotoxin and gemcitabine in pancreatic cancer cell lines. Gemcitabine alone mediated a dose-dependent inhibition of protein synthesis with IC_{50} of 22 nmol/L in MIA-PaCa-2 cells, 3.2 nmol/L in Capan-1 cells, 1,000 nmol/L in SW1990 cells, and 14 nmol/L in HS766T cells (Table 1). When it was combined with IL-4 cytotoxin, the protein synthesis inhibition in

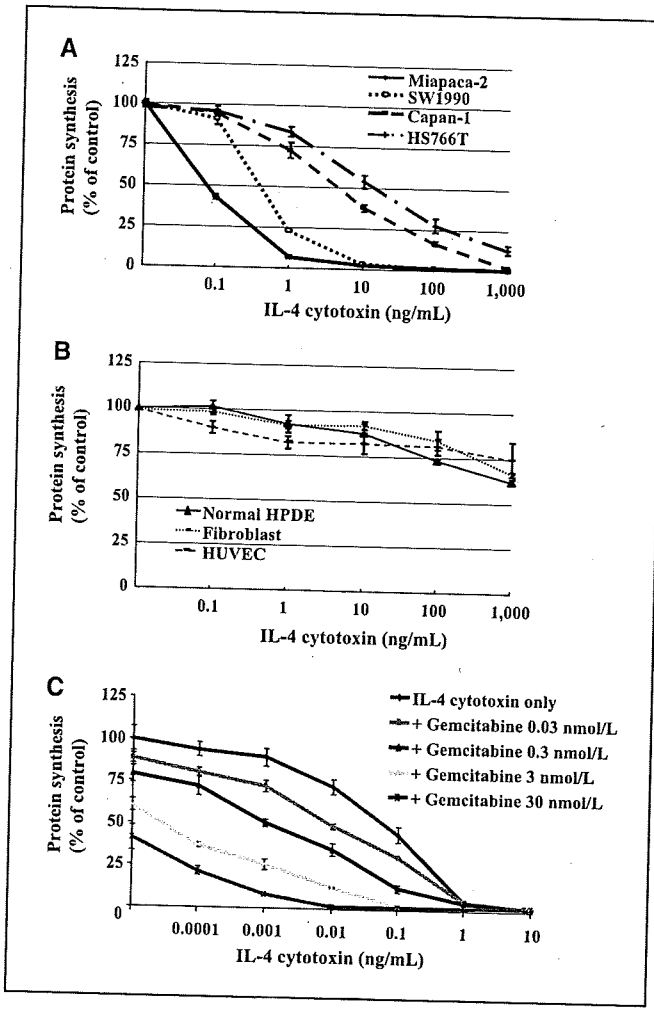

Figure 2. Cytotoxic activity of IL-4 cytotoxin in pancreatic cancer cell lines (*A*), and in normal HPDE, fibroblast, and HUVEC (*B*). Cells (1×10^4) were incubated with various concentrations of IL-4 cytotoxin (0–1,000 ng/mL). *Bars,* SD of quadruplicate determinations. The assay was repeated twice. *C,* cytotoxic activity of simultaneous exposure of IL-4 cytotoxin and gemcitabine against MIA-PaCa-2 cell line. *In vitro* cytotoxic activity of IL-4 cytotoxin (0–10 ng/mL) with or without various concentrations of gemcitabine (0.03–30 nmol/L) was assessed by protein synthesis inhibition assay.

MIA-PaCa-2 cells was greatly enhanced: IC_{50} of IL-4 cytotoxin became 0.012, 0.001, and 0.00004 ng/mL by adding 0.03, 0.3, and 3 nmol/L gemcitabine, respectively (Fig. 2*C*). These same phenomena were also observed in SW1990 and Capan-1 cells, but not in HS766T cells (data not shown). The combination index at IC_{50} and IC_{75} (concentration of drug causing 75% inhibition of protein synthesis) in MIA-PaCa-2, SW1990, and Capan-1 cells was <1 at all concentrations of gemcitabine (Table 1).

***In vivo* whole-body optical imaging of PDA.** We developed pancreatic cancer models to investigate antitumor effects of IL-4 cytotoxin and showed its correlation with imaging studies *in vivo*. Pancreatic cancer cells were transfected with GFP. Our transfection technique using retroviral vector revealed consistent bright GFP fluorescence of MIA-PaCa-2 cells. There was no significant difference in morphology, growth rate, and sensitivity to IL-4 cytotoxin between parent and GFP-transfected cells (data not shown). GFP-transfected MIA-PaCa-2 tumor chunks were orthotopically transplanted to pancreas of nude mice. These tumor pieces were derived from MIA-PaCa-2-GFP cells transplanted s.c. As shown in Figs. 3*A* and 4*A*, GFP fluorescence enabled real-time and sequential whole-body imaging of tumors. Noninvasive quantitative measurements of external visible fluorescent area enabled the construction of *in vivo* tumor growth curves, which seem to correlate with visible tumor growth (Figs. 3*B* and 4*B*).

Complete eradication of tumors by combination of IL-4 cytotoxin and gemcitabine in an early tumor model. Small primary tumor lesions on day 4 after transplantation were observed in all mice by the real-time whole-body imaging (average fluorescent area 26.17 ± 4.19 mm^2). Imaging studies on days 14 and 24 confirmed the significant primary tumor growth and metastasis in nontreatment group (Fig. 3*A*). In contrast, gemcitabine or IL-4 cytotoxin treatment group showed a reduction in the rate of tumor growth, compared with nontreatment group (Fig. 3*A* and *B*). The IL-4 cytotoxin treatment group showed no tumor lesions in 6 of 10 mice on day 14, although tumor recurred by day 34. Remarkably, the combination treatment group revealed significant suppression of tumor growth of primary tumor lesions. Tumor lesions were undetectable in all 10 mice on day 14. By day 44, 6 of 10 mice showed local recurrence and distant metastasis as shown in the bottom row of Fig. 3*A*. The rest of the four mice showed complete eradication of tumor and mice remained tumor-free through day 94 when the experiment was terminated.

Synergistic increase in survival of mice treated with a combination of IL-4 cytotoxin and gemcitabine in an early tumor model. As shown in Fig. 3*C*, median survival time of animals was 27 days in nontreatment group, whereas it was significantly increased to 54, 64, and 92 days in gemcitabine group ($P < 0.0001$), IL-4 cytotoxin group ($P < 0.0001$), and their combination group ($P < 0.0001$) compared with nontreatment group, respectively. Compared with gemcitabine group, significant prolonged survival time was observed in IL-4 cytotoxin group ($P = 0.017$) and the combination group ($P < 0.0001$). Increase in significant survival advantage correlated with tumor area as detected by GFP fluorescence. Prolonged survival time in the combination group was 341% compared with the nontreatment group. In addition, we did not observe any organ toxicity in heart, liver, lung, kidney, and spleen of IL-4 cytotoxin–injected mice evaluated by histologic examination (data not shown).

Real-time imaging of tumor growth of the primary and metastasis lesion in an advanced *in vivo* model. As ~85% patients with PDA are diagnosed at an advanced stage at initial diagnosis, an advanced PDA *in vivo* model needs to be established to imitate the clinical situation and to monitor the disease and treatment effect (33). Fluorescence imaging on day 14 posttransplantation confirmed the tumor growth of primary lesions in all mice and also detected the metastasis lesions to liver, lymph nodes, and peritoneal locations in 40 of 62 mice. Six mice showed metastatic lesions to liver or lymph nodes around hepatoduodenum ligament, 8 showed metastasis lesions corresponding to peritoneal locations, and 26 with both metastasis lesions. We did not include mice with the GFP spot at spleen as a metastasis group. Forty mice with confirmed primary and metastasis tumor lesions on day 14 posttransplantation were divided into four groups and treated as described in Materials and Methods (average fluorescent area 94.67 ± 8.31 mm^2). The real-time whole-body imaging of tumor growth confirmed the significant primary tumor growth and metastatic spread on days 14, 21, and 28 after transplantation of tumor in nontreatment control group (Fig. 4*A*). Gemcitabine and IL-4 cytotoxin treatment group showed a reduction in the rate of

tumor growth compared with the nontreatment group (Fig. 4*B*). Especially, the combination treatment group revealed significant suppression of tumor growth at primary and metastasis tumor lesions. The reduction in tumor size on day 28 was 39.8% in the gemcitabine group ($P < 0.001$), 71.2% in the IL-4 cytotoxin group ($P < 0.001$), and 79.6% in the combination group ($P < 0.001$) compared with the no treatment group.

Combination of IL-4 cytotoxin and gemcitabine prolongs the survival of mice with advanced orthotopic pancreatic tumor. We examined the efficacy of IL-4 cytotoxin on survival of animals in an advanced PDA model. As shown in Fig. 4*C*, median survival time of animals was 28 days in nontreatment group, whereas it was significantly increased to 34, 43, and 52 days in gemcitabine group ($P = 0.0089$), IL-4 cytotoxin group ($P < 0.0001$), and their combination group ($P < 0.0001$), respectively. Compared with gemcitabine group, significant prolonged survival time was also observed in the IL-4 cytotoxin group ($P = 0.0047$) and the combination group ($P = 0.0002$). Prolonged survival time in the combination group was 186% compared with the nontreatment group. Increase in significant prolongation of survival correlated with tumor area as detected by whole-body imaging.

Expression of IL-4R in a clinical sample and development of orthotopic xenograft tumor model. We obtained a tumor tissue sample that was surgically resected at Surgery Branch at NIH and pathologically diagnosed as moderately differentiated adenocarcinoma (data not shown). This tumor section showed strong staining for IL-4Rα in the ductal adenocarcinoma cells and faint staining of fibroblasts (Fig. 5*A*). We also established tumor and fibroblast cells cultured from this sample to examine the antitumor activity of IL-4 cytotoxin. The cancer cells expressing IL-4R were highly sensitive to IL-4 cytotoxin (IC_{50} 0.32 ng/mL), whereas fibroblast cells were not sensitive ($IC_{50} \geq 1{,}000$ ng/mL; Fig. 5*B*).

IL-4 cytotoxin, gemcitabine, and their combination significantly prolonged survival of mice transplanted with a clinical pancreatic cancer sample. The clinical sample was orthotopically transplanted on the pancreas of SCID mice and when tumors grew, they were harvested and then orthotopically propagated in the next set of SCID mice. All mice showed growth of primary tumor and metastasis to lymph nodes in peritoneum, hepatoduodenum ligament, and para-aortic areas. Seventy-five percent of these mice showed the metastasis lesion to liver when mice were sacrificed 30 days after tumor implantation (Fig. 5*C*). To assess the effect of IL-4 cytotoxin in an advanced metastasis model, a third set of SCID mice were orthotopically implanted with tumor pieces obtained from the second set of mice. These mice, when advanced disease developed, were divided into four groups on day 31 and treated as described in Materials and Methods. As shown in Fig. 5*D*, median survival time of animals was 62 days in the nontreatment group, whereas it was significantly increased to 86, 102, and 134 days in the gemcitabine group ($P = 0.0081$), IL-4 cytotoxin group ($P = 0.0006$), and combination group ($P < 0.0001$), respectively. Compared with gemcitabine, significant prolonged survival time

Table 1. Cytotoxicity of IL-4 cytotoxin, gemcitabine, and their combination in pancreatic cancer cell lines

Cancer cell line	Drug	IC_{50}*	IC_{75}†
MIA-PaCa-2	IL-4 cytotoxin	0.065 ng/mL	0.32 ng/mL
	Gemcitabine	22 nmol/L	280 nmol/L
Capan-1	IL-4 cytotoxin	3.5 ng/mL	22 ng/mL
	Gemcitabine	3.2 nmol/L	9 nmol/L
SW1990	IL-4 cytotoxin	0.36 ng/mL	1 ng/mL
	Gemcitabine	1,000 nmol/L	3,000 nmol/L

Cancer cell line	Drug combination	CI‡	
		IC_{50}	IC_{75}
MIA-PaCa-2	IL-4 cytotoxin + gemcitabine 0.03 nmol/L	0.153	0.563
	IL-4 cytotoxin + gemcitabine 0.3 nmol/L	0.0336	0.094
	IL-4 cytotoxin + gemcitabine 3 nmol/L	0.137	0.0138
Capan-1	IL-4 cytotoxin + gemcitabine 0.003 nmol/L	0.287	0.34
	IL-4 cytotoxin + gemcitabine 0.03 nmol/L	0.026	0.0713
	IL-4 cytotoxin + gemcitabine 0.3 nmol/L	0.096	0.0603
	IL-4 cytotoxin + gemcitabine 3 nmol/L	0.938	0.401
SW1990	IL-4 cytotoxin + gemcitabine 0.003 nmol/L	0.5	0.65
	IL-4 cytotoxin + gemcitabine 0.03 nmol/L	0.27	0.5
	IL-4 cytotoxin + gemcitabine 0.3 nmol/L	0.021	0.3
	IL-4 cytotoxin + gemcitabine 3 nmol/L	0.014	0.19
	IL-4 cytotoxin + gemcitabine 30 nmol/L	0.03	0.11
	IL-4 cytotoxin + gemcitabine 300 nmol/L	0.3	0.31

NOTE: CI < 1, CI = 1, and CI > 1 indicate synergistic, additive, and antagonistic effects, respectively.
*Fifty percent protein synthesis inhibition.
†Seventy-five percent protein synthesis inhibition.
‡CI values were calculated using the formula described in Materials and Methods.