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Dexamethasone-induced differentiation of pancreatic AR42J cell involves p21$^{waf1/cip1}$ and MAP kinase pathway

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Abbreviations: Dig, digoxigenin; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; PCR, polymerase chain reaction; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one; SDS, sodium dodecyl sulfate

Abstract

Dexamethasone converts pluripotent pancreatic AR42J cells into exocrine cells expressing digestive enzymes. In order to address molecular mechanism of this differentiation, we have investigated the role of mitogen-activated protein (MAP) kinase pathway and gene expressions of p21$^{waf1/cip1}$ and nuclear oncogenes (*c-fos* and *c-myc*) during AR42J cell differentiation. Dexamethasone markedly increased the intracellular and secreted amylase contents as well as its mRNA level. However, cell growth and DNA content were significantly decreased. With these phenotypic changes, AR42J cells induced transient mRNA expression of p21$^{waf1/cip1}$ gene, which reached maximal level by 6 h and then declined gradually toward basal state. In contrast to p21$^{waf1/cip1}$, *c-fos* gene expression was transiently inhibited by 6 h and then recovered to basal level by 24 h. Increased *c-myc* expression detected after 3 h, peaked by 12 h, and remained elevated during the rest of observation. Dexamethasone inhibited epidermal growth factor-induced phosphorylation of extracellular signal regulated kinase. Inhibition of MAP kinase pathway by PD98059 resulted in further elevation of the dexamethasone-induced amylase mRNA and p21$^{waf1/cip1}$ gene expression. These results suggest that p21$^{waf1/cip1}$ and nuclear oncogenes are involved in dexamethasone-induced differentiation and inhibition of MAP kinase pathway accelerates the conversion of undifferentiated AR42J cells into amylase-secreting exocrine cells.

Keywords: amylase; AR42J cell differentiation; dexamethasone; MAP kinase; oncogene; p21$^{waf1/cip1}$

Introduction

The pancreas is an organ containing two distinct populations of cells, the exocrine cells that secrete enzymes into digestive tract, and the endocrine cells that secrete hormones into the bloodstream. Recent studies have revealed that both the exocrine and endocrine cells develop from the epithelial cells of primitive pancreatic ducts during embryogenesis (Teitelman, 1991; Debas, 1997). However, the molecular mechanisms controlling the differentiation of these cell types from common putative stem cells is still largely unknown. The rat pancreatic acinar carcinoma cell line, AR42J cells provide useful model system for this type of study, since these cells have the feature of pluripotency of the common stem cells of the pancreas. It has been demonstrated that dexamethasone converts them into matured exocrine cells expressing digestive enzymes (Logsdon *et al*., 1985; Rajasekaran *et al*., 1993), whereas activin A converts them into insulin-producing neuroendocrine cells (Ohnishi *et al*., 1995). The dexamethasone-induced phenotypic changes of AR42J cells include increases in secretory organelles (Rajasekaran *et al*., 1993), tissue specific gene expression (Estival *et al*., 1991) and receptors for cholecystokinin (Rosewicz *et al*., 1994). Moreover, glucocorticoids inhibit the growth of animal cells including AR42J cells (Guthrie *et al*., 1991), another hallmark of cellular differentiation. However, the signaling mechanisms underlying glucocorticoid-induced AR42J cell differentiation still remain largely unknown.

p21$^{waf1/cip1}$ is the first cyclin-dependent kinase (Cdk) inhibitor to be identified and it is known to be induced by cell growth arrest, differentiation, p53, transforming growth factor and cellular senescence (Gartel *et al*., 1996; Oh *et al*., 2002). p21$^{waf1/cip1}$ directly interacts

with Cdk2-, Cdk4-, and Cdk6-containing complexes, blocks kinase activity, and thus arrests cell cycle at G1 phase. It has previously reported that glucocorticoids stimulate $p21^{waf1/cip1}$ gene expression by targeting multiple transcriptional elements within a steroid responsive region of the $p21^{waf1/cip1}$ promoter in rat hepatoma cells (Cha *et al*., 1998) and $p21^{waf1/cip1}$ is thought to be involved in differentiation of specific tissue types (Halvey *et al*., 1995; Parker *et al*., 1995). In the other hand, the induction of early responsive nuclear oncogenes expression has been reported in many cell lines to be associated with cell growth and differentiation by regulating the expression of specific genes (Curran *et al*., 1988; Karin *et al*., 1997).

In this study, we examined whether $p21^{waf1/cip1}$ and nuclear oncogenes serve as intracellular signals mediating glucocorticoid-induced differentiation of the AR42J cells. Possible role of MAP kinase pathway in the regulation of this differentiation was also investigated.

Materials and Methods

Materials

AR42J cell line (ATCC CRL 1492), derived from an azaserine-induced pancreatic rat tumor, was purchased from the ATCC (Rockville). Fetal bovine serum (FBS) and TRIzol reagent were from GibcoBRL (Frederick). Dulbecco's modified essential medium (DMEM), epidermal growth factor (EGF) and dexamethasone were from Sigma (St. Louis). PD98059 was from RBI (Natick). Reagents used for digoxigenin (Dig)-nonradioactive Northern blotting were from Boehringer Mannheim (Mannheim, Germany). Dig-labelled antisense oligonucleotide for the exon (1,427-1,457) sequences of rat amylase mRNA was synthesized from Operon (Alameda). Taq DNA polymerase and AMV reverse transcriptase XL were from Promega (Madison). Oligo-primers for PCR amplification were synthesized from Bioneer (Daejeon, Korea).

Cell culture and Western blot analysis

AR42J cells were cultured in DMEM containing 25 mM HEPES/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% FBS and antibiotics (100 g/ml streptomycin, 100 units/ml penicillin) at 37°C under a humidified condition of 95% air and 5% CO_2. Cells were routinely plated at 5×10^5 cells onto 6 wells culture dish. After overnight attachment of the cells, the culture medium was replaced by fresh medium with or without dexamethasone. After incubation, cells were detached with trypsin-EDTA, stained with trypan blue and counted using a hemocytometer. The amount of α-amylase released or retained by cells was determined in an aliquot of medium or cell lysate as previously described (Rick and Stegbauer, 1974). α-Amylase activity was expressed as moles of maltose formed/min. Total DNA was extracted from cultured cells using TRIzol reagent according to the protocol of the manufacturer. To assess the effect of dexamethasone on EGF-induced extracellular signal regulated kinases (ERKs) activation, Western blot analysis was carried out using an antibody against phospho-ERK1/2 (Cell Signaling, Beverly) as described previously (Kwon *et al*., 2000)

RNA isolation and cDNA synthesis

Total RNA was extracted from AR42J cells cultured with or without dexamethasone and PD98059 at different time points using TRIzol reagent in accordance with the manufacturer's protocol. After determination of the RNA contents, samples were adjusted to 100 ng/ l RNA and RNA was submitted to reverse transcription using 500 U AMV reverse transcriptase XL. Reverse transcription was carried out at 42°C for 60 min. cDNA synthesis was terminated by heating at 99°C for 5 min.

Northern blot analysis

The quality of isolated RNA preparations was verified by running aliquots on agarose-formaldehyde gel and controlling the relative amounts of 18S and 28S rRNAs. Amylase mRNA was analyzed by Dig-nonradioactive Northern blotting in accordance with the manufacturer's protocol. Approximately 10 g of total RNA was size-fractioned on an 1% agarose gel containing 0.66 M formaldehyde and blotted to Hybond-N^+ membrane in 20× saline-sodium citrate buffer (SSC). And then, the membrane was cross-linked with UV light. The prehybridization was performed for 1 h at 59°C in 5× SSC (0.15 M NaCl, 0.015 M sodium citrate), 50% (v/v) deionized formamide, 50 mM sodium phosphate, 0.1% (w/v) sodium lauryl sarcosine, 7% SDS, and 2% (w/v) blocking reagent. The hybridization at 59°C for 3 h was carried out with the Dig-labeled antisense oligonucleotide for rat amylase. After hybridization, the blot was washed twice for 15 min in 1× SSC containing 0.1% (w/v) SDS and twice for 15 min in 0.1× SSC containing 0.1% (w/v) SDS. After exposing the blot to a blocking solution for 1 h, the anti-Dig antibody was added to the solution and incubated for 30 min. The blot was washed twice for 15 min in 0.1 M maleic acid, pH 7.5, 0.15 M NaCl, and incubated with 1:100 diluted CSPD in 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 25 mM $MgCl_2$. After incubation for 10 min at 37°C, the labeled bolts were exposed to X-ray film.

Reverse transcription-polymerase chain reaction (RT-PCR)

The synthesized cDNAs (5 g) were submitted to PCR using Taq-polymerase and oligonucleotide primers. Amplification of $p21^{waf1/cip1}$, *c-fos* and *c-myc* was carried out using the following primer sets: $p21^{waf1/cip1}$, 5' AGTATGCCGTCGTCTGTTCG 3' (sense) and 5' GAGTGCAAGACAGCGACAAG 3' (antisense); *c-fos*, 5' CTGCAACATCCCCAATGACC 3' (sense) and 5' AGGTCCACATCTGGCACAGA 3' (antisense); *c-myc*, 5' AACTTACAATCTGCGAGCCA 3' (sense) and 5' AGCAGCTCGAATTTCTTCCAGATAT 3' (antisense). PCR was performed using a Perkin-Elmer thermocycler (model 9600) for 35 cycles. Amplification programs of denaturation, primer annealing and primer extension were utilized as follows, respectively: $p21^{waf1/cip1}$ (94°C, 1 min; 52°C, 1.5 min; 72°C, 1.5 min), *c-fos* (94°C, 1 min; 51°C, 1.5 min; 72°C, 1.5 min) and *c-myc* (94°C, 1 min; 58°C, 1 min; 72°C, 2 min). For control, glyceraldehyde-3-phosphate dehydrogenase was used. The PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining.

Results and Discussion

Treatment of AR42J cells with dexamethasone led to an inhibition of cell growth as determined by cell counting and DNA content. AR42 cells treated with 50 nM dexamethasone for 48 h resulted in lowering

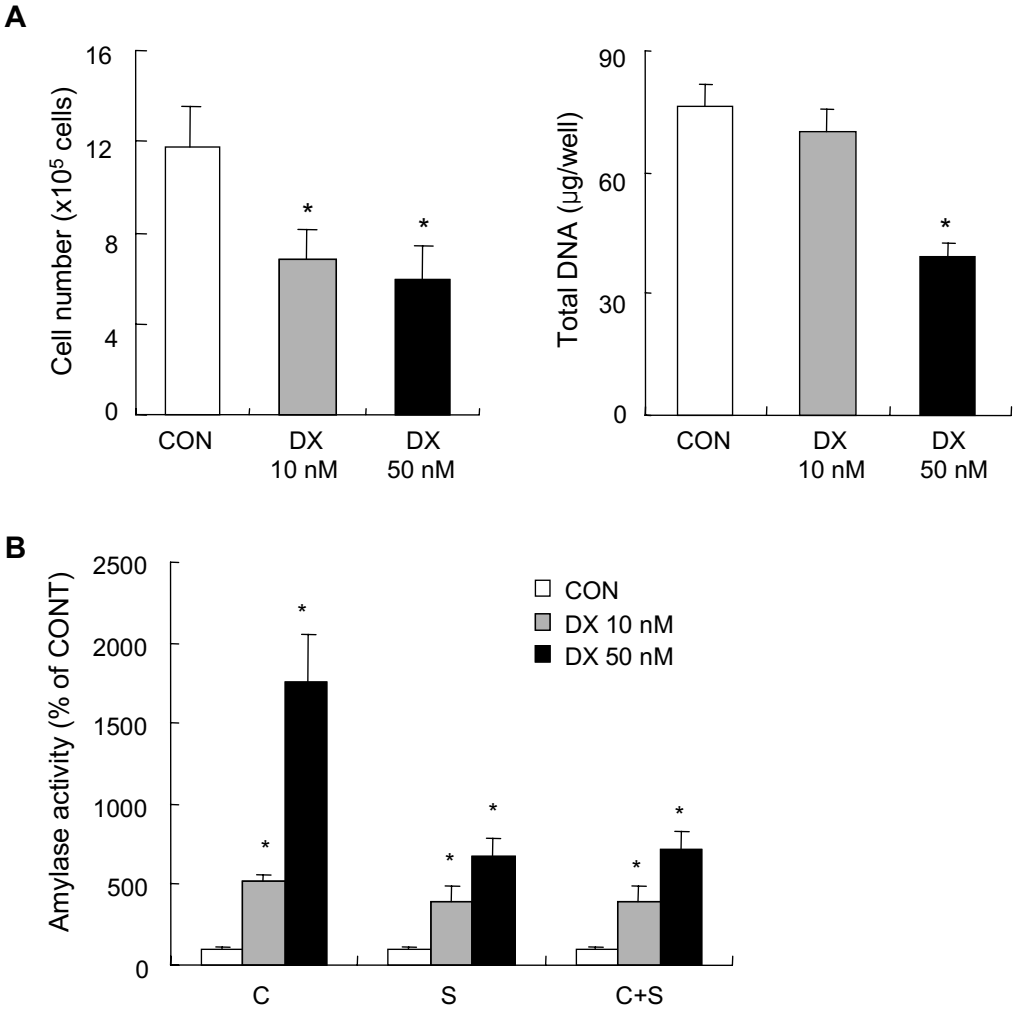

Figure 1. Effects of dexamethasone on cell growth (A) and amylase activity (B) in AR42J cells. Cells grown in 6 well culture dishes in the absence (CON) or presence (Dx) of dexamethasone for 48 h were trypsinized and counted using a hemocytometer. DNA was extracted from cultured cells using TRIzol reagent. The amount of amylase secreted (S) or retained by cells (C) was determined in an aliquot of medium or cell lysate. Amylase activity was expressed as moles of maltose formed/min. Values are represented as means±SD of four separate experiments done in triplicate. Asterisks denote statistical significance at $P < 0.01$.

of both of cell number and total DNA content about half of those of untreated cells (Figure 1A). The RNA and protein content, when normalized to DNA, were practically unchanged (results not shown). On the contrary, dexamethasone had a profound effect on the production and release of amylase, a major secretory product of exocrine pancreatic acinar cells. An approximate 17 fold increase in intracellular amylase activity and an approximate 7 fold increase in the secreted amylase activity was observed after treatment of 50 nM dexamethasone (Figure 1B). In order to determine whether the dexamethasone-induced amylase activity resulted from change in the level of its mRNA, Northern blot analysis was performed by probing identical amount of total RNA with an antisense oligonucleotide for rat amylase mRNA. As shown in Figure 2A, amylase mRNA expression level was notably increased by dexamethasone treatment in a dose- and time-dependent manner. These results indicated that dexamethasone inhibits growth and induces differentiation of AR42J cell toward an amylase-secreting exocrine phenotype. Our observation was similar to those of previously reported by others for dexamethasone-induced parameters of differentiation (Logsdon *et al*., 1985; Estival *et al*., 1991; Rajasekaran *et al*., 1993). We examined the effect of MAP kinase pathway on amylase mRNA expression induced by dexamethasone. Pretreatment of 20 M PD98059, an inhibitor of the MAP kinase kinase (MEK), resulted in further increment of dexamethasone-induced amylase mRNA expression (Figure 2B). This result indicates that MAP kinase pathway exerts inhibitory influence on dexamethasone-induced amylase mRNA expression in AR42J cells. As shown in Figure 2C, dexamethasone markedly inhibited EGF-induced phosphorylation of ERKs in AR42J cells. Furthermore, phosphorylation of ERKs was perfectly inhibited by co-treatment of dexamethasone with PD98059. Recently, glucocorticoids have been known to have inhibitory actions on activities of ERK and p38 MAP kinases *via* rapid induction of activated MAP kinase phosphatase-1 in other cultured cells (Kassel *et al*., 2001; Engelbrecht *et al*., 2003). Therefore, it is likely that dexamethasone and PD98059 synergically inhibit MAP kinase pathway *via* increased dephosphorylation and decreased phosphorylation of MAP kinase, respectively. Further inhibition of MAP kinase pathway by PD98059 may facilitate the amylase mRNA expression in dexamethasone-treated pancreatic AR42J cells. In contrast to our observation, PD98059 has been known to inhibit activin A/hepatic growth factor-induced insulin expression in AR42J cells (Furukawa *et al*., 1999). Thus, it is suggested that activation of MAP kinase pathway stimulates differentiation of AR42J cells toward neuroendocrine phenotype, whereas, simultaneously inhibits conversion into another phenotype, amylase-secreting exocrine cells.

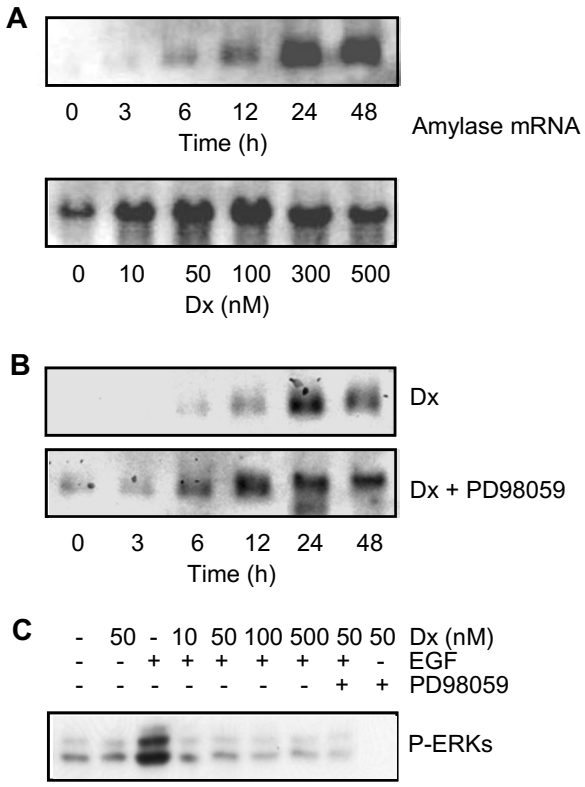

Figure 2. Effect of dexamethasone on expression levels of amylase mRNA in AR42J cells (A). 50 nM of dexamethasone (Dx) was added to the culture medium for 3-48 h, and 10-500 nM dexamethasone was added to the culture medium for 48 h. Total RNA was extracted from AR42J cells using TRIzol reagent and mRNA expression of amylase was determined by Dig-nonradioactive Northern blotting as described in Materials and Methods. Effect of PD98059 on dexamethasone-induced amylase mRNA expression in AR42J cells (B). After the cells were pretreated with 20 M PD98059 for 30 min, 50 nM dexamethasone was added to the culture medium for 3-48 h. Effect of dexamethasone on EGF-induced ERKs activation (C). Cells were pretreated with the indicated amounts of dexamethasone and 20 M PD98059 for 30 min and 20 nM EGF was added to the culture medium for 3 h. The ERKs activation was determined by immunoblotting of cellular lysates with an antibody that specifically recognizes the dually phosphorylated forms of ERK1/2 (P-ERKs).

To determine the involvement of $p21^{waf1/cip1}$ gene expression during dexamethasone-induced differentiation, RNA was isolated and RNA expression of the $p21^{waf1/cip1}$ gene was analyzed by RT-PCR. Addition of 50 nM dexamethasone to culture medium causes a transient induction of $p21^{waf1/cip1}$ gene expression, which was reached maximal by 6 h and thereafter declined slowly toward basal level at 48 h (Figure 3). The $p21^{waf1/cip1}$ protein has been shown to be a potent inhibitor of Cdk-2 and Cdk-4 kinase activity *in vitro* (Gartel *et al*., 1996). Binding of the Cdk inhibitors prevents the Cdk-mediated phosphorylation of the Rb protein, which functions to sequester the E2F-1 trans-

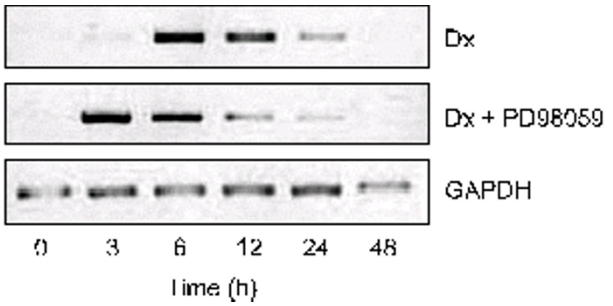

Figure 3. Time course of RNA expression of the p21$^{waf1/cip1}$ gene in dexamethasone-treated AR42J cells. 50 nM of dexamethasone (Dx) was added to the culture medium for 3-48 h in the absence or presence of 20 M PD98059. Tatal RNA was extracted from cultured AR42J cells at different time points, was amplified by RT-PCR, and PCR product was separated in 1.5% agarose gel. This is a representative of at least three independent experiments.

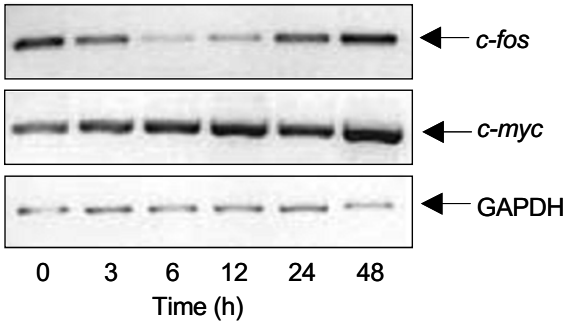

Figure 4. Time course of RNA expression of the *c-fos* and *c-myc* oncogenes in dexamethasone-treated AR42J cells. 50 nM of dexamethasone was added to the culture medium for 3-48 h and RNA was extracted from cultured cells at different time points. The extracted RNA was amplified by RT-PCR and PCR product was separated in 1.5% agarose gel. This is a representative of at least three independent experiments.

cription factor in an inactive complex. Therefore, although further evidences were not tested in this study, this result suggests possibility that glucocorticoid-induced growth inhibition of AR42J cell is due to induction of p21$^{waf1/cip1}$, which inactivates cyclinD/Cdk complexes and arrest cell cycle at G1 phase. Similar results have been observed in glucocorticoid-treated hepatoma cells (Cha *et al*., 1998), and fibroblast cells (Ramalingam *et al*., 1997). However, different mechanism for glucocorticoid-induced growth inhibition has also been reported that inhibition of G1 progression is due to the effects of glucocorticoids upon G1 cyclin gene expression in lymphoid cells (Rhee *et al*., 1995; Greenberg *et al*., 2002). The abundance of p21$^{waf1/cip1}$ gene expression reproducibly decreased to near basal expression within 48 h after dexamethasone treatment as shown in Figure 3. Glucocorticoid-mediated down-regulation of the glucocorticoid receptor has been observed in pancreatic AR42J cells (Kaiser *et al*., 1996) and might account for the transient activation of p21$^{waf1/cip1}$ gene expression. The inhibition of MAP kinase pathway by pretreatment of 20 M PD98059 slightly enhanced p21$^{waf1/cip1}$ expression level and shortened time course of dexamethasone-induced p21$^{waf1/cip1}$ gene expression (Figure 3). The maximal elevation of p21$^{waf1/cip1}$ was observed within initial 3 h after stimulation, and then declined gradually toward basal level by 48 h. This result suggests the inhibitory role of MAP kinase pathway on p21$^{waf1/cip1}$ expression as well as amylase mRNA expression during the dexamethasone-induced differentiation, although its exact mechanism has not been elucidated.

Finally, we investigated the changes of *c-fos* and *c-myc* expressions by RT-PCR to identify the possible involvement of nuclear oncogenes in pancreatic AR-42J cell differentiation. RNA expression of the *c-fos* gene was transiently diminished by 6 h and then returned to basal level by 24 h after dexamethasone treatment (Figure 4). This result indicates that dexamethasone inhibits the *c-fos* gene expression transiently in AR42J cells. The protooncogene *c-fos* belongs to the group of immediate early gene of which transcription is induced immediately in response to various growth-promoting signals and expressed c-Fos protein serves as a component of AP-1. It has been suggested that dexamethasone suppressed cell proliferation through selective inhibition of ERK/Elk-1 integrated AP-1 activation (Miyazaki *et al*., 2000). The time course of *c-myc* gene expression by dexamethasone was different from that of *c-fos*. Increased *c-myc* gene expression levels were detected after 3 h, peaked by 12 h, and remained elevated during the rest of 36 h of observation (Figure 4). Although the mechanism remains unknown, it is likely that dexamethasone slightly stimulates *c-myc* gene expression in AR42J cells. The nuclear oncogene *c-myc* executes its multiple activities mostly through the transcriptional regulation of target genes. c-Myc is well known as a multifaceted protein that controls regulation of the cell cycle, cell growth, activities genomic instability, and stimulates angiogenesis, cell transformation, and apoptosis (Kim *et al*., 2001; Oster *et al*., 2002). At this time, it is unclear what roles are played by each of these nuclear oncogenes during the dexamethasone-induced differentiation. Further studies will be required to determine the mechanism of these nuclear oncogene responses to dexamethasone and its biological significance. It is possible that these oncogene products as transcription factors play key roles in mediating the biological effects of glucocorticoids on processes of growth arrest and differentiation in pancreatic AR42J cells.

In summary, this study provided evidences for the involvement of p21$^{waf1/cip1}$ and nuclear oncogenes dur-

ing the pancreatic AR42J cell differentiation induced by dexamethasone. Furthermore, we showed that dexamethasone inhibited the ERKs phosphorylation and inhibition of MAP kinase pathway accelerated the conversion of undifferentiated AR42J cells into amylase-secreting exocrine cells.

Acknowledgement

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