Interleukin-1 α regulates G₁ cell cycle progression and arrest in thyroid carcinoma cell lines NIM1 and NPA

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Abstract

This study provides the first report that the same cytokine (interleukin-1 (IL-1)) can induce opposite effects on cyclin-dependent kinases (Cdks) and Cdk inhibitors (Cdkis) in the G₁ phase even in the same type of cancer cells (papillary thyroid carcinoma cells). Cell cycle analysis revealed an increase in NIM1 cells and a decrease in NPA cells in the S and G₂+M phases after treatment with IL-1 α . The addition of IL-1 α to NIM1 cells reduced the expression of p16 and p21 protein and induced the expression of Cdk2 and Cdk4 protein, which leads to the

phosphorylation of retinoblastoma protein. The addition of IL-1 α to NPA cells induced the expression of p27 protein and reduced the expression of Cdk2 protein, which leads to induction of p107 protein expression. It is of interest that p21 protein expression was not observed in NPA cells. These results suggest that several Cdks and Cdkis play a regulatory role in the G₁ cell cycle progression and arrest induced by IL-1 α in thyroid carcinoma cell lines.

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Introduction

Thyroid carcinomas vary in aggressiveness, ranging from differentiated carcinomas to anaplastic carcinomas. A correlation has been found between the molecular alteration in an oncogene, a tumor suppressor gene and the histopathology of thyroid carcinomas. Recent investigations of the molecular mechanisms governing the G₁ to S phase cell cycle progression in mammalian cells demonstrated a central role for several cyclin-dependent kinases (Cdks), Cdk inhibitors that bind and inactivate the Cdks (Cdkis; p16, p21 and p27 were examined in the present study) and G₁ cyclins, which are involved in forming holoenzyme complexes with Cdks. The central role appears to be mediated by neutralization of the growth-restraining ability of retinoblastoma (Rb) protein via its phosphorylation or by decreasing the level of the Rb-related protein p107 in the advanced G₁ phase.

With regard to thyroid carcinomas, there are only a few reports on the deletion or mutation of Cdkis (Calabro *et al.* 1996) leading to proliferation of thyroid carcinoma cells. It has also been reported that thyrotropin pretreatment induces the expression of G₁ cyclin, and that insulin-like growth factor-I causes the accumulation of enough G₁ cyclins to drive the cell cycle from the G₁ phase to the S phase in a short time in rat thyroid (FRTL-5) cells (Yamamoto *et al.* 1996). The progression of these cells from quiescence into the cell cycle is associated with a burst of mevalonate synthesis caused by major transcrip-

tional activation of the 3-hydroxy-3-methylglutaryl-CoA reductase gene by thyrotropin (Grieco *et al.* 1990), and, in the presence of the mevalonate metabolite geranylgeranyl pyrophosphate, geranylgeranylated Rho small GTPases promote the degradation of p27 during G₁/S transition in FRTL-5 cells (Hirai *et al.* 1996). In our search of the literature, we were unable to find any reports on the relationship between multiple Cdks, Cdkis and cytokine-induced cell cycle progression or arrest in thyroid carcinomas.

Among the many cytokines, it has been reported that human thyroid cells not only possess interleukin-1 (IL-1) receptors but also secrete IL-1 (Kawabe *et al.* 1989, Yamashita *et al.* 1989, Rasmussen *et al.* 1993, Kayser *et al.* 1995, Watson *et al.* 1995). IL-1 has many effects on the thyroid gland, and these effects are counteracted by the IL-1 receptor antagonist (Rasmussen *et al.* 1993, 1994, 1997, Reimers *et al.* 1996, Nilsson *et al.* 1998). In addition, IL-1 stimulates the growth of normal human thyroid cells, FRTL-5 cells (Zeki *et al.* 1991), and human papillary thyroid carcinoma (NIM1) cells (Zeki *et al.* 1993, Inokuchi *et al.* 1995), whereas it inhibits the growth of other human papillary thyroid carcinoma cell lines, such as NPA and TPC-1 (Kimura *et al.* 1992, Yip *et al.* 1995). The purposes of the present investigation were, first, to examine the relationship between IL-1-induced cell cycle progression or arrest and the changes in several Cdks and Cdkis in thyroid carcinomas, and, secondly, to elucidate the mechanism of thyroid carcinoma cell growth. We

found that the proliferative effect of IL-1 α on NIM1 cells involves inhibition of the expression of p16 and p21 protein and enhancement of the expression of Cdk2 and Cdk4 protein. We also found that the antiproliferative effect of IL-1 α on NPA cells involves enhancement of the expression of p27 protein and inhibition of the expression of Cdk2 protein. No expression of p21 protein was observed in the NPA cells.

Materials and Methods

Cell culture

The papillary thyroid carcinoma cell lines NIM1 and NPA were maintained in RPMI 1640 medium (Gibco Oriental Co., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS; Gibco Oriental). Cell lysate and culture supernatant were prepared from NPA cells cultured for 48 h as previously reported (Zeki *et al.* 1993). For IL-1 α treatment in the cell cycle analyses, each cell line was treated with recombinant human IL-1 α (10 ng/ml; Dainippon Pharmaceutical Co., Osaka, Japan) for 24–120 h at 24-hourly intervals without FCS for NIM1 cells and with 10% FCS for NPA cells. For IL-1 α treatment in the Western blot analyses, each cell line was treated with recombinant human IL-1 α (10 ng/ml) for 48 h without FCS for NIM1 cells and with 10% FCS for NPA cells. The methods of preparing thyrocytes from patients with Graves' disease, which we used as a negative control in Western blot analyses, are described in detail elsewhere (Matsunaga *et al.* 1986).

Assay of IL-1 α content

The IL-1 α content of the cell lysate and culture supernatant of NPA cells was measured by using an IL-1 α -specific ELISA kit (Otsuka Assay Laboratories, Diagnostic Division, Otsuka Pharmaceutical Co., Tokushima, Japan). Assays were performed according to the manufacturer's instructions.

[3 H]Thymidine ([3 H]TdR) incorporation into DNA

NIM1 and NPA cells (1×10^4 /well) were plated in 96-well culture plates (Falcon 3072; Becton–Dickinson, Oxnard, CA, USA) and grown in RPMI 1640 medium without FCS for NIM1 cells and with 10% FCS for NPA cells. Then 0.1, 1 or 10 ng/ml IL-1 α were added to each cell line and the mixture incubated at 37 °C. After 2 days, [3 H]TdR (0.5 μ Ci/well; Amersham Japan, Tokyo, Japan) was added, and incubation was continued for a further 24 h at 37 °C. After trypsinization, the cells were harvested on glass fiber filters with an automatic cell harvester. The radioactivity in each sample was determined by liquid scintillation counting (Aloka LSC-3500E;

Aloka Co., Tokyo, Japan). The results are expressed as means $\pm$ s.d. of [3 H]TdR c.p.m. in triplicate cultures. Differences in the mean values were examined for statistical significance by Student's *t*-test. A *P* value less than 0.05 denoted a statistically significant difference.

Cell cycle analyses

After the cells had been treated with IL-1 α , they were harvested and fixed in 70% ethanol. After treatment with RNase A (1 mg/ml in 0.1 M phosphate buffer, pH 7.0; Sigma, St Louis, MO, USA), the cells were stained with propidium iodide solution (50 μ g/ml in 0.1% sodium citrate, 0.1% Nonidet P40; Sigma) and then analyzed using an EPICS XL flow cytometer (Coulter Co., Hialeah, FL, USA) as described previously (Mashima *et al.* 1995).

Western blot analyses

Western blot analyses were carried out as described previously (Resnitzky *et al.* 1992). Extracts were prepared, and 20 μ g of the samples were subjected to SDS–PAGE (7.5% gel) and electroblotted. For detection of Rb, p107, Cdk4, p16, p21, p27 or Cdk2 protein, blots were incubated for 1 h at room temperature with 1 μ g/ml anti-Rb (IF8), anti-p107, anti-Cdk4, anti-p16, anti-p21, anti-p27 protein (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or anti-Cdk2 protein (Pharmingen, San Diego, CA, USA) antibodies respectively. Thereafter, a 1 h incubation was performed at room temperature with a 1:2500 dilution of horse radish peroxidase-conjugated F(ab')₂ fragment of sheep anti-mouse immunoglobulin (Amersham Life Science, Tokyo, Japan) for Rb and p107 protein, or horse radish peroxidase-conjugated F(ab')₂ fragment of donkey anti-rabbit immunoglobulin (Amersham Life Science) for Cdk2, Cdk4, p16, p21 and p27 protein. Lastly, each protein was detected by an enhanced chemiluminescence system (Amersham Life Science). We repeated the Western blot analyses twice and obtained similar results. Representative blots of each Western blot analysis are shown in the Results section.

Results

IL-1 α content of the cell lysate and culture supernatant of NPA cells

IL-1 α concentration in the lysate of NPA cells was 21.3 pg/10⁶ cells, and in the culture supernatant of NPA cells it was less than 0.5 pg/10⁶ cells. The limit of detection for the specific ELISA kit is 0.5 pg/ml.

Effect of IL-1 α on DNA synthesis

In agreement with previous reports (Zeki *et al.* 1993, Inokuchi *et al.* 1995, Kimura *et al.* 1992), we confirmed

Table 1 Effects of IL-1 α on the growth of the human papillary thyroid carcinoma cell lines NIM1 and NPA. Values are means $\pm$ S.D. of triplicate cultures

IL-1 α (ng/ml)	[³ H]TdR incorporation (c.p.m./well)	
	NIM1	NPA
-	12 153 $\pm$ 1178	11 053 $\pm$ 1088
0.1	15 273 $\pm$ 2627	11 664 $\pm$ 1729
1	16 770 $\pm$ 2761*	8817 $\pm$ 638*
10	24 313 $\pm$ 4843**	6055 $\pm$ 607**

* $P < 0.05$, ** $P < 0.01$ vs without IL-1 α .

that IL-1 α stimulates the growth of NIM1 cells and inhibits the growth of NPA cells in a dose-dependent manner (Table 1).

Cell cycle analyses

Cell cycle analyses revealed that the treatment with IL-1 α for 48 h resulted in an increase in NIM1 cells and a decrease in NPA cells in the S and G₂+M phases (Fig. 1). Pre-G₀/G₁ peaks of NPA cells were observed independently of the IL-1 α treatment; these were thought to be apoptotic fractions. Treatment times of 24, 48, 72, 96 and 120 h were tested, and 48 h was chosen for the experiments in Fig. 1 because the changes observed were most evident after this length of treatment (Table 2).

Western blot analyses

Western blot analysis of the NIM1 cells revealed inhibition of p21 protein expression and enhancement of Cdk2 protein expression (Fig. 2), and inhibition of p16 protein expression and enhancement of Cdk4 protein expression (Fig. 3) following the treatment with IL-1 α . IL-1 α also increased phosphorylated Rb protein, and the unphosphorylated Rb protein almost disappeared after the treatment with IL-1 α in NIM1 cells (Fig. 4); the total amount of Rb protein in the IL-1 α -treated cells was decreased (Fig. 4). IL-1 α treatment did not change the level of p107 protein (Fig. 5) in the NIM1 cells. The thyrocytes from Graves' disease patients which we used as a negative control always showed results similar to those for NIM1 cells without IL-1 α treatment.

In the NPA cells, IL-1 α increased the expression of p27 protein and reduced the expression of Cdk2 protein. It is of interest that no p21 protein expression was observed in NPA cells (Fig. 2). Expression of p16 and Cdk4 protein was not changed by the treatment with IL-1 α in NPA cells (Fig. 3), although it did increase the level of p107 protein in these cells (Fig. 5). IL-1 α had no effect on the phosphorylation of the Rb protein (Fig. 4).

Discussion

IL-1 is a representative cytokine that possesses a wide range of biological activities, not only in immunological reactions but also in the growth and differentiation of different cell types. There have been several reports that IL-1 has stimulatory or inhibitory effects on the growth of human thyroid carcinoma cell lines (Kimura *et al.* 1992, Zeki *et al.* 1993, Inokuchi *et al.* 1995, Yip *et al.* 1995), although it is believed to have an inhibitory effect on human thyroid-specific gene expression (Yamashita *et al.* 1989, Ashizawa *et al.* 1989). In the present study, we confirmed that IL-1 α has a stimulatory effect on the growth of NIM1 cells and an inhibitory effect on the growth of NPA cells. Therefore the cells used in this study maintained the character of the original clones.

Although the growth of NIM1 cells is reported to be stimulated by IL-1 α via an autocrine mechanism (Zeki *et al.* 1993), the growth of NPA cells is not believed to be regulated by IL-1 α in an autocrine fashion, because IL-1 α levels in the lysate and culture supernatant of NPA cells are very low, as demonstrated in this study, suggesting that they do not synthesize IL-1 α .

With regard to cell cycle progression or arrest in thyroid carcinoma cells, it was reported that c-Myc has growth-stimulating effects, especially the G₀ to G₁ stage progression, and its mRNA expression is inhibited by IL-1 in NPA cells, indicating an inhibitory role for IL-1 on c-Myc-mediated DNA synthesis in these cells (Kimura *et al.* 1992). The present cell cycle analyses revealed an increase in the percentage of NIM1 cells and a decrease in the percentage of NPA cells in the S and G₂+M phases after treatment with IL-1 α . Of the treatment times tested, the changes observed were most evident after 48 h. Therefore we examined, by Western blot analysis, the effects of IL-1 α on the expression of Rb and the Rb-related proteins, Cdk and Cdk i , using the same treatment time of 48 h because the changes observed in the G₁ checkpoint control proteins should be most evident several hours before 48 h.

The mechanisms of cell cycle progression and arrest have been extensively studied, and the involvement of Rb and Rb-related proteins such as p107 and p130 (Knudsen *et al.* 1998), and also of several Cdks and Cdk i s, in the G₁ phase has been reported. With regard to the relationship between these G₁ checkpoint control proteins and IL-1-induced cell cycle progression or arrest, there has only been one report that IL-1 induces growth arrest of human melanoma cells by hypophosphorylation of the Rb protein (Muthukumar *et al.* 1996). We therefore examined the changes in expression of Rb, p107, Cdk2, Cdk4, p16, p21 and p27 proteins in order to study the molecular events involved in thyroid cancer cell cycle progression and arrest induced by IL-1 α treatment. The addition of IL-1 α to NIM1 cells reduced the expression of p16 and p21 protein, and the unphosphorylated form of Rb protein

Figure 1 DNA distribution in NIM1 cells and NPA cells. NIM1 cells before (A) and after (B) treatment with 10 ng/ml IL-1 α for 48 h were fixed and stained with propidium iodide, and the DNA content was analyzed by flow cytometry, as described in Materials and Methods. The same procedure was performed in NPA cells before (C) and after (D) treatment with IL-1 α . The percentage of NIM1 cells in the S and G₂+M phases was increased from 16.2 (A) to 21.5 (B). The percentage of NIM1 cells in the G₀/G₁ phase was decreased from 83.2 (A) to 77.6 (B), whereas that of NPA cells was increased from 64.8 (C) to 75.2 (D). Pre-G₀/G₁ peaks were observed in the NPA cells independently of the IL-1 α treatment; 2.5% (C) and 2.3% (D). These pre-G₀/G₁ peaks were less than 1% in the NIM1 cells independently of the IL-1 α treatment.

disappeared. We could not rule out the possibility that the unphosphorylated form of Rb protein was not detected because the total amount of Rb protein in the IL-1 α -treated cells was decreased. It also induced a large increase in the expression of Cdk2 and Cdk4 protein and the phosphorylated form of Rb protein. This result suggests that the proliferative effect of IL-1 α on NIM1 cells involves enhancement of Cdk2 and Cdk4 expression, which leads to phosphorylation of Rb protein and the

release of E2F transcription factor, resulting in increased expression of a number of genes required for the S phase, including *c-myc*.

In contrast, the addition of IL-1 α to NPA cells induced the expression of p27 protein, which leads to inhibition of Cdk2 expression and enhancement of p107 expression. It is of interest that p21 protein was not expressed in the NPA cells. However, this is reasonable because NPA cells have been reported to have a p53 mutation (codon 266;

Table 2 Time course of percentage of cells in the S and G₂+M phases in NIM1 and NPA cells before and after treatment with IL-1 α

Time (h)	NIM1		NPA	
	– IL-1 α	+ IL-1 α	– IL-1 α	+ IL-1 α
24	20.8	21.4	38.7	33.3
48	16.2	21.5	32.8	22.3
72	12.2	10.3	29.4	31.2
96	10.5	9.3	29.2	28.1
120	9.5	6.4	28.6	23.6

GGA (Gly) to GTA (Glu); Fagin *et al.* 1993) and p53-dependent increases in p21 mRNA; the subsequent protein levels are associated with increased gene transcription secondary to wild-type p53 binding to its consensus sequence. Taken together, these findings suggest that the mechanism of cell cycle arrest induced by IL-1 α in NPA cells involves inhibition of Cdk2 protein expression and stimulation of expression of p27 and p107 protein. We did not detect any changes in the expression levels of Cdk4 and p16 protein in NPA cells after the treatment with IL-1 α . We suspect that the p27–Cdk2–p107–E2F transcription factor pathway may have a role and that the p16–Cdk4–pRb pathway may not play a significant role in the inhibition of NPA cell proliferation by the treatment with IL-1 α , although this hypothesis has not yet been confirmed. It will be worth while determining the expression of cyclin E–A and cyclin D (G₁ cyclins), as they are involved in forming holoenzyme complexes with Cdk2 and Cdk4 respectively. Such experiments are at present being devised.

Figure 2 Western blot analysis of the expression of Cdk2 protein and p21 and p27 proteins in NIM1 cells and NPA cells. The arrow indicates the specific expression of Cdk2 protein, and the broken arrows indicate the specific expression of p21 and p27 protein. Graves' indicates thyrocytes from patients with Graves' disease, and IL-1 α (–) and (+) indicate without or with IL-1 α treatment respectively. Size markers (kDa) are shown on the left. The data were obtained by the sequential incubation of one filter with the indicated primary antibodies for Cdk2 and p21.

There have been several reports about the relationship between the changes in several Cdks and Cdk i s in the G₁ phase (which result in changes in the phosphorylated state of the Rb protein) and cytokine-induced cell cycle progression or arrest (Tsuji *et al.* 1993, Firpo *et al.* 1994, Nourse *et al.* 1994, Ewen *et al.* 1995, Tiefenbrun *et al.* 1996, Blanchard *et al.* 1997, Liu *et al.* 1997, Urashima *et al.*

Figure 3 Western blot analysis of the expression of Cdk4 protein and p16 protein in NIM1 cells and NPA cells. The arrow indicates the specific expression of Cdk4 protein, and the broken arrow indicates the specific expression of p16 protein. Graves' indicates thyrocytes from patients with Graves' disease, and IL-1 α (–) and (+) indicate without or with IL-1 α treatment respectively. Size markers (kDa) are shown on the left. The data were obtained by the sequential incubation of one filter with the indicated primary antibodies.

Figure 4 Western blot analysis of the expression of the phosphorylated and unphosphorylated forms of Rb protein in NIM1 cells and NPA cells. The arrow indicates the specific expression of the phosphorylated form of Rb protein, and the broken arrow indicates the specific expression of the unphosphorylated form of Rb protein. Graves' indicates thyrocytes from patients with Graves' disease, and IL-1 α (–) and (+) indicate without or with IL-1 α treatment respectively. Size markers (kDa) are shown on the left.

Figure 5 Western blot analysis of the expression of p107 protein in NIM1 cells and NPA cells. The arrow indicates the specific expression of p107 protein. IL-1 α (–) and (+) indicate without or with IL-1 α treatment respectively. Size markers (kDa) are shown on the left.

1997). There have also been several reports on the relationship between the changes in the phosphorylated state of the Rb protein in the G₁ phase and cytokine-induced cell cycle progression or arrest (Resnitzky *et al.* 1992, Morisaki *et al.* 1994). In summary, we have demonstrated that the same cytokine (IL-1) can induce opposite changes in Cdks and Cdk i s in the same type of cancer cells (papillary thyroid carcinoma cells). Further clarification of the mechanism of these effects of IL-1 may lead to a new approach not only to the control of cancer cell growth but also the treatment of some thyroid cancer patients.

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