Interleukin 4 Regulates G1 Cell Cycle Progression in Gastric Carcinoma Cells

Takashi Morisaki, Akihiko Uchiyama, Dale Yuzuki, Richard Essner, Donald L. Morton, and Dave S. B. Hoon

ABSTRACT

We have previously reported that interleukin 4 (IL-4) inhibits the growth of human gastric carcinoma cells. To investigate the mechanism for this inhibition we analyzed the effect of IL-4 on cell cycle progression of the IL-4-sensitive gastric carcinoma cell line, HTB-135. IL-4 significantly inhibited cell cycle G1-S-phase progression. To assess the postreceptor molecular events that transduce the negative-growth signals by IL-4, we analyzed the expression of cell cycle nuclear-regulating factors such as retinoblastoma gene product (Rbp), c-myc, c-myc protein (c-mycp), and cyclin D1 expression which are known to be regulators of G1-S-phase transition. IL-4 was found to induce an unphosphorylated form of Rbp within 24 h and significantly reduce the phosphorylated form at 48 h. The transition of Rbp to a hypophosphorylated form occurs with the decrease in c-myc gene expression and c-mycp. In addition, we demonstrated that IL-4 down-regulated p34cdc2, a kinase associated with Rbp phosphorylation and cyclin D1. Cyclin D1, considered as a critical nuclear regulatory factor of G0-G1 to S-phase transition was down-regulated 24 and 48 h post-IL-4 treatment as well. These studies suggest that IL-4 inhibits gastric cell proliferation by blocking cell cycle progression by down-regulating several key G0-G1 cell cycle nuclear-regulating factors.

INTRODUCTION

Interleukin 4, originally described as a B-cell growth factor (1), has now been shown to possess many pleiotropic effects on various normal and neoplastic cells of different lineages. We and other investigators have recently reported that IL-4 can inhibit the growth of human tumor cells of nonhemopoietic cell origin such as melanoma (2), renal cell carcinomas (3, 4), gastric carcinomas (5), breast carcinomas, and colon carcinomas (6). IL-4 has been shown to inhibit the growth of hemopoietic origin malignant cells as well (7-10). Human tumor cell lines have been shown to express IL-4 receptor (4, 5). In a recent study, we demonstrated that IL-4 inhibitory effect is correlated to IL-4 receptor expression on tumor cells (4). However, the mechanism by which IL-4 exerts its growth-inhibitory effect on tumor cells remains unknown.

In this study we sought to determine the mechanism by which IL-4 inhibits the growth of gastric carcinoma cells by examining modifications in specific cell cycle-regulatory events. We present data demonstrating that IL-4 inhibits cell cycle progression from G1 to S phase, and that it may be through the down-regulation of the expression of c-myc, p34cdc2, cyclin D1, and the induction of unphosphorylated Rbp. Our data provide evidence that IL-4 is an important cell cycle-negative regulator of gastric carcinoma cells.

MATERIALS AND METHODS

Cell Culture and Reagents. The human gastric adenocarcinoma cell line, HTB-135, was obtained from American Type Cell Culture Collection (Rockville, MD). Cells were routinely cultured in RPMI 1640 supplemented with 2 mm glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% FBS (Gemini Bioproducts, Calabasas, CA) as described previously (5).

Human recombinant IL-4 was the kind gift of Sterling Drug, Inc. (Malvern, PA). Human recombinant insulin was obtained from Nordisk Nordmark (Princeton, NJ). Mouse anti-Rb protein mAB (3C8) was obtained from Dr. S. F. Wen (Canji, Inc., San Diego, CA). Anti-human p34cdc2 and anti-c-myc (C-33) mAB was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-human cyclin D1 protein polyclonal antibody (31) was obtained from Pharmingen (San Diego, CA). Goat anti-mouse IgG mAB was obtained from Jackson Immune Response, Inc. (West Grove, PA) and goat anti-rabbit IgG antibody was from TAGO, Inc. (Burlingame, CA).

Cell Proliferation Assay. IL-4 growth inhibition of HTB-135 cells was determined by using a standard 72-h [3H]thymidine incorporation assay as previously described (5). Cells were cultured in 96-well round-bottomed microplates; each test was performed in triplicate. Data were expressed in cpm [3H]thymidine uptake. To assess growth of cells in the presence of IL-4 plus insulin, a viability count assay was set up in 96-well round-bottomed microplates (Costar, Cambridge, MA) (2). Briefly, cells were seeded in wells at 10000 cells/well in 200 μl of culture medium containing various concentrations of IL-4 and/or insulin in 0.5% FBS HI plus RPMI 1640 and antibiotics. Analysis of agents at different dilutions were carried out in triplicate after 48 h.

Cell Cycle Analysis. The DNA content of the cell suspensions prepared by trypsinization was determined by propidium iodide labeling with the use of a Cell Cycle Analysis Kit (Becton Dickinson, Mountain View, CA) following the manufacturer’s instructions. Briefly, cells were cultured at 105 cells/cm2 in mammalian cells (16-18). c-myc is a protooncogene and is considered as an early response gene in which its product plays a major role in nuclear signal transduction (12). Deregulation of c-myc gene expression (amplification) occurs in a variety of cancers including gastrointestinal carcinomas (19, 20). Down-regulation of c-myc has been associated with differentiation and is believed to be necessary for withdrawal from the cell cycle (12, 21). Antiproliferative effect on cells by inhibitory polypeptides such as interferons has been associated with selective reduction of both c-myc mRNA and Rbp dephosphorylation (17). The Rb gene product is posttranslationally regulated (18); functional activity is modified depending on its level of phosphorylation (22-24). Unphosphorylated Rbp has been shown to inhibit G0-G1 cell cycle progression causing cell growth arrest (18, 24).

The CDKs regulate cell cycle progression (24-26). The prototypic CDK p34cdc2 is suggested to be responsible for phosphorylation of Rbp (24, 25). Cyclins interact and regulate specific kinases such as p34cdc2 (24, 25, 26) and tumor suppressor gene products such as Rbp (28, 29). Recently several types of cyclins (A, B, C, D, and E) in mammalian cells have been identified and shown to individually play key roles in regulating particular stages of the cell cycle (25, 27, 30, 31).

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2 To whom requests for reprints should be addressed, at Division of Molecular and Cellular Immunology, John Wayne Institute For Cancer Treatment and Research, 2200 Santa Monica Blvd., Santa Monica, CA 90404.

3 The abbreviations used are: IL-4, recombinant interleukin 4; mAB, monoclonal antibody; Rb, retinoblastoma gene; Rbp, retinoblastoma gene product; c-myc, c-myc protein; CDK, cyclin-dependent protein kinase; HI, heat-inactivated; FBS, fetal bovine serum; SSC, standard saline-citrate; PBS, phosphate-buffered saline.
IL-4 EFFECT ON CELL CYCLE

RESULTS

IL-4 Effect on Cell Cycle. We previously demonstrated that IL-4 inhibited the growth of gastric carcinoma cells in a dose-dependent manner from 0 to 100 units/ml IL-4 in a 3-day proliferation assay (5). Using the IL-4 sensitive cell line HTB-135, we examined whether IL-4 can inhibit cell cycle progression. Cells were treated with 0, 10, or 100 units/ml IL-4 for 24, 48, or 72 h in RPMI 1640 plus 10% FBS medium and analyzed for DNA distribution analysis by flow cytometry. The DNA distribution histogram of the cells treated with or without IL-4 at 24 h is shown in Fig. 1. HTB-135 cells incubated with 100 units/ml IL-4 for 24 h had a markedly reduced S-phase population and increased G1-phase population compared to control cells incubated in culture medium only (Fig. 1). Table 1 shows DNA cell cycle distribution in response to IL-4 concentration and duration of treatment. The study indicates that the effect of IL-4 increased with dose and time. IL-4 significantly increased the G0/G1 population and reduced S-phase population in a dose-dependent manner. The G0/G1:S-phase ratio population significantly increases with IL-4 concentration and incubation time (Table 1). At 72 h, 100 units/ml IL-4 treated cells, the G0/G1:S ratio was 4.2 times greater compared to 24 h, 0 unit/ml IL-4 treated cells. Respective growth inhibition of HTB-135 cells in the presence of IL-4 was demonstrated in a 72-h [3H]-thymidine incorporation assay (Fig. 2).

Insulin is a cell cycle progression factor which induces cell cycle progression from G1 to S phase (11, 34, 35). To further assess IL-4 effect on cell cycle progression we examined its effect in the presence of insulin. The effect of insulin was assessed in low-concentration serum medium; HTB-135 cells were precultured in 0.5% FBS plus RPMI 1640 culture medium for 48 h. Cultures were then treated with IL-4 (100 units/ml) or insulin (100 ng/ml) or combined and incubated for 48 h in the same type of culture medium. Insulin enhanced growth and IL-4 inhibited growth, whereas IL-4 combined with insulin inhibited the cell growth which was induced by insulin (Fig. 3). The
Table 1. Effect of IL-4 on DNA cell cycle distribution

Subconfluent HTB-135 cells were harvested by trypsinization and resuspended in culture medium containing 10% FBS HI in the presence of 0, 10, or 100 IL-4 (units/ml). Cells were further cultured for 24, 48, or 72 h, and then harvested for assay. Cells were prepared for DNA cell cycle analysis as described in "Materials and Methods." Data express the percentage of cells in each cell cycle phase.

<table>
<thead>
<tr>
<th>Treatment (h)</th>
<th>IL-4 (units/ml)</th>
<th>G0/G1</th>
<th>S</th>
<th>G0/G1: S ratio</th>
<th>G2 + M</th>
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<tr>
<td>24</td>
<td>0</td>
<td>41.8</td>
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<td>1.07</td>
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<td></td>
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<td>24.5</td>
<td>2.5</td>
<td>14.0</td>
</tr>
<tr>
<td>48</td>
<td>0</td>
<td>51.8</td>
<td>34.1</td>
<td>1.52</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>60.1</td>
<td>22.1</td>
<td>2.72</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>64.5</td>
<td>19.1</td>
<td>3.38</td>
<td>16.4</td>
</tr>
<tr>
<td>72</td>
<td>0</td>
<td>54.3</td>
<td>36.9</td>
<td>1.47</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>67.0</td>
<td>19.3</td>
<td>3.47</td>
<td>13.7</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>69.5</td>
<td>15.1</td>
<td>4.60</td>
<td>15.4</td>
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</table>

To further confirm the down-regulation of c-myc expression by IL-4 in HTB-135 cells, we examined the effect of IL-4 on c-myc mRNA expression. c-myc mRNA expression was significantly reduced after 48-h IL-4 treatment (Fig. 5). Densitometric analysis of Northern blots of c-myc mRNA expression demonstrated significant reduction after 48-h IL-4 treatment compared to the control. Control cultures not treated with IL-4 showed reduction of c-myc mRNA but not as significantly as IL-4-treated cultures.

Rbp. We examined Rbp, a nuclear factor that also is a key G0/G1 cell cycle inhibitor depending on its phosphorylation state. Previous work in various cell systems has shown that hyperphosphorylated Rbp forms (Mr 112,000-116,000) migrate more slowly in gels than the unphosphorylated Rbp form (Mr, 110,000) (29). We examined IL-4 effect on the expression of Rbp-phosphorylated protein in HTB-135 cells. Cells were treated with IL-4 and cell protein lysates were prepared, and then Rbp phosphorylation was determined by Western blotting analysis with the use of a specific mAb. Rbp expression after 0-, 4-, 24-, and 48-h IL-4 treatment of cells was assessed (Fig. 6, top). At 24 and 48 h the Mr, 110,000 band is very distinct, whereas the Mr, 112,000-116,000 band almost disappears completely at 48 h. In Fig. 6, bottom, a comparison of Rbp expression of the phosphorylated forms and unphosphorylated form of control (nontreated) and IL-4-treated cells at 4 and 24 h are shown. The unphosphorylated form (Mr, 110,000) is absent in both controls and appears in the IL-4 24-h-treated cells. There is a very faint Mr, 110,000 band in the 4-h IL-4-treated cells. In the IL-4-treated cells the phosphorylated Rb band is large, suggesting multiple forms (different levels of phosphorylation).

p34<sup>cdc2</sup> Kinase and Cyclin D1. Recently p34<sup>cdc2</sup> kinase has been shown to be a phosphorylation kinase which can convert unphosphorylated Rbp to the phosphorylated form. We examined whether IL-4 can down-regulate this kinase in HTB-135 cells. Total protein from IL-4-treated cells was extracted and analyzed for the expression of p34<sup>cdc2</sup> by Western blot analysis. IL-4 treatment markedly decreased this kinase expression in a time-dependent manner (Fig. 7), corresponding to Rbp hypophosphorylated form expression.

Cyclin D1 has been recently suggested as one of the key G0/G1 cell cycle-positive regulators which interacts with p34<sup>cdc2</sup>. We therefore examined cyclin D1 could be down-regulated by IL-4. Cell lysates were prepared of HTB-135 cells after 0-, 24-, and 48-h IL-4 treatment, and Western blot analysis was performed by using anti-human cyclin

experiments demonstrated that IL-4 can interfere with the growth-stimulating effects of insulin. IL-4 treatment of cell cultures did not modulate expression of 125I-labeled insulin binding to cells in a radioimmunoassay (data not shown). The effect of IL-4 (100 units/ml) plus insulin (100 ng/ml) on cell cycle was examined at 48 h (Fig. 4). Insulin significantly enhanced the S-phase population and decreased the G0/G1 population. IL-4 inhibited cell cycle progression from G0/G1 to S phase induced by insulin. The G0/G1:S-phase ratios for insulin, IL-4, and IL-4 plus insulin, were 0.69, 2.1, and 1.89, respectively. These results suggest that IL-4 can antagonize insulin-induced growth of HTB-135 cells through blocking G0/G1 to S-phase cell cycle progression.

c-myc and c-myc Expression. Since HTB-135 cells were blocked at G0/G1 phase by IL-4 we then examined several G0/G1 cell cycle nuclear-regulating factors such as c-myc known to regulate cell cycle progression from G1 to S phase. HTB-135 cells before and after IL-4 treatment were assessed for c-myc expression by flow cytometry by using a specific mAb. Cells were cultured in 10% FBS HI plus RPMI with 10 units/ml or 100 units/ml IL-4 for 24 and 48 h. c-myc expression decreased in a dose-dependent manner with higher concentration of IL-4 at 24 and 48 h as expected (Table 2). c-myc expression in nontreated cells decreased after 48 h. However, after 48 h of IL-4 treatment c-myc expression decreased more than non-treated cells compared to 24-h IL-4 treatment. These experiments showed that IL-4 can down-regulate c-myc expression in HTB-135 cells.
Fig. 4. DNA distribution histograms of HTB-135 cells incubated in culture medium alone (A); insulin, 100 ng/ml (B); IL-4, 100 units/ml (C); insulin, 100 ng/ml plus IL-4, 100 units/ml (D) for 48 h.

Table 2 c-myc expression post-IL-4 treatment

<table>
<thead>
<tr>
<th>Treatment time (h)</th>
<th>0 unit/ml</th>
<th>10 units/ml</th>
<th>100 units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>24.3</td>
<td>16.1</td>
<td>10.6</td>
</tr>
<tr>
<td>48</td>
<td>8.1</td>
<td>3.1</td>
<td>2.2</td>
</tr>
</tbody>
</table>

D1 antibody. IL-4 down-regulated cyclin D1 to some extent after 24- and 48-h treatment (Fig. 8).

**DISCUSSION**

Identifying peptide factors which exert negative control on cell proliferation of carcinomas have recently drawn much attention. Analysis of these factors and their mechanism of controlling cell cycle, particularly at the G0-G1 phase, may provide important insights in controlling cancer growth. In the present study, we demonstrated that IL-4 inhibited cell cycle progression of HTB-135 cells at the G0-G1 to S-phase transition that may be correlated to our previous observation that IL-4 treatment induced differentiation of HTB-135 cells (5).

IL-4-inhibitory effect on G0-G1-phase progression was further analyzed in the presence of insulin, a well-known G1 cell cycle progression factor. Insulin and related growth factors are autocrine and amplified in many types of carcinomas (34, 35). IL-4 blocking of cell proliferation and G1 cell cycle progression induced by insulin in low-concentration serum-containing medium suggests that the IL-4 effect may be at a checkpoint in the G0-G1 to S-transition phase. Our findings of IL-4 plus insulin growth inhibition on gastric carcinoma cells are paradoxical to the recent findings that IL-4 plus insulin stimulates DNA synthesis of murine myeloid cell lines (36). The latter studies suggest that IL-4 and insulin are synergistic and use a signal transduction pathway that may have a common factor(s).

Several factors involved in cell cycle progression were investigated to determine the mechanism by which IL-4 inhibits progression from G0-G1 to S phase. We demonstrated that IL-4 reduced c-myc mRNA and c-myc expression in HTB-135 cells. In general, c-myc mRNA and c-myc expression is high in proliferating cells such as neoplastic cells, and the expression tends to decrease with differentiation and with loss of cell-proliferative activity (12, 13, 37). c-myc is found to be deregulated in many cancers and thought to behave as a transcriptional factor (12). Transcriptional down-regulation of c-myc is observed in many hematopoietic and nonhematopoietic cell lines following treatment with inducers of differentiation such as interferon (17). The effect of IL-4 depends on cell type and perhaps stage of...
was used for analysis of Rbp-phosphorylated state. HTB-135 cells (1 x 10⁶ cells) were treated with IL-4 (100 units/ml) and cellular extracts were prepared at specific times as described in "Materials and Methods." Anti-Rbp mAb at 0, 4, 24, and 48 h compared to untreated controls at 0 h. IL-4-treated cells at 4 h showed 112,000-116,000, unphosphorylated Rbp, Mr 110,000.

Fig. 6. Western blot analysis of the IL-4-induced response on Rbp in HTB-135 cells. HTB-135 cells (1 x 10⁶) were treated with IL-4 (100 units/ml) and cellular extracts were prepared at specific times as described in "Materials and Methods." Anti-Rbp mAb was used for analysis of Rbp-phosphorylated state. Top, serial analysis of Rbp expression at 0, 4, 24, and 48 h (Lanes 1 to 4, respectively). Bottom, comparison of control cells (untreated) to IL-4-treated cells: control cells at 4 h (Lane 1), IL-4-treated cells at 4 h (Lane 2) and 24 h (Lane 3). Rbp-phosphorylated forms, Mr 112,000-116,000, unphosphorylated Rbp, Mr 110,000.

Fig. 7. Western blot analysis of p34cdc2 kinase expression in HTB-135 cells. Cells were treated with IL-4 (100 units/ml) for 0, 24, and 48 h (Lanes 1, 2, 3, 4, respectively). Western blotting analysis was as described in "Materials and Methods" with anti-p34cdc2 mAb. Densitometric analysis of bands at 0, 4, 24, 48, and 72 h were 51665, 29925, 28230, 10702, 2652, and 3629, respectively.

Recent studies suggest that Rbp which negatively regulates cell growth can bind to the positive growth regulator c-myc (12, 37). Rbp gene encodes a nuclear phosphoprotein found in the hyperphosphorylated form in late G₂, S, and M phases of the cell cycle and in a hypophosphorylated form in the G₁ phase (22-24). In this study we demonstrated that IL-4 induced a hypophosphorylated form of Rbp 24 h after treatment. Rbp in its hypophosphorylated form has been shown to be a critical inhibitor of Go-G₁-phase transition in normal cells and tumor cells (22-24). Studies on B-lymphoblastoid cells have shown that interleukin 6 and α-interferon treatment induced a hypophosphorylated Rbp form and reduced c-myc mRNA that correlated with Go-G₁ arrest (17). However, studies with transforming growth factor β inhibition of cell growth also has been correlated with reduced phosphorylation of Rbp but increase in c-myc mRNA (39). The relation between Rbp phosphorylation state and c-myc activity is unclear. It has been suggested that c-myc specifically binds to Rbp and that they functionally antagonize each other (12, 40). Studies suggest that mechanisms in which Go-G₁ inhibitors suppress c-myc transcription are independent of the presence of hypophosphorylated Rbp (17). The Rbp and c-myc may both be intermediary factors regulating Go-G₁ cell cycle progression via overlapping or independent regulatory networks.

p34cdc2 kinase has been suggested to convert hypophosphorylated Rbp to its phosphorylated form (41, 42). Several consensus sequence recognition sites for this kinase are present in Rbp (22). The increase in the level of unphosphorylated Rbp form by IL-4 may be through down-regulation of p34cdc2 kinase. Recently, it has been shown that colon and gastric carcinomas possess high levels of p34cdc2 kinase (43). This kinase may potentially be one of the critical targets of IL-4 postreceptor action. CDK-cyclin complexes are required for the transverse of controls and progression of cell cycle (25, 27). In our studies we observed that IL-4 induced down-regulation of cyclin D1. In general it has been observed that D-type cyclins are amplified in continuously proliferating cells and that their expression is growth factor dependent (27). Overexpression of cyclin D1 has been associated with several types of carcinomas (28, 44). Recently, cyclins D1 have been shown to bind to Rbp and regulate its function (30, 31). Cyclin D1 binds to CDKs and has been suggested to regulate their phosphorylation activity and cell cycle progression. Future studies will involve determining whether these events induced by IL-4 are interrelated or of independent pathways, and how these events lead up to Go-G₁ arrest.

Our studies implicate that IL-4 is a regulator of Go-G₁ cell cycle progression and growth of gastric cell carcinoma. This event may be part of a regulatory mechanism in normal gastric epithelial cells that has become altered relative to the state of malignant transformation of the cell. IL-4 response by the cells may be autocrine as well as paracrine (5). However, during transformation of the cells the loss of IL-4 receptor or function may release the cell from this regulatory growth control. IL-4 tumor growth-regulatory activity may be similar to that of the interferons (2, 3). Further studies on the mechanism of IL-4 effect may lead to development of therapeutics toward intracellular growth-regulatory elements regulated by IL-4.

Fig. 8. Western blot analysis of cyclin D1 expression in HTB-135 cells. Cells were treated with IL-4 (100 units/ml) for 0, 24, and 48 h (Lanes 3, 2, and 1, respectively). Protein lysate was analyzed by Western blotting with anti-human cyclin D1 polyclonal antibody. Densitometric analysis of bands at 0, 24, and 48 h were 75208, 34679, and 42396, respectively.

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