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The role of c-Myc on granulocyte colony-stimulating factor-dependent neutrophilic proliferation and differentiation of HL-60 cells

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Abstract

We have previously suggested that phosphatidylinositol 3-kinase (PI3K)/p70 S6 kinase (p70 S6K) plays an important role in the regulation of neutrophilic differentiation of HL-60 cells on the basis of analysis of transferrin receptor (Trf-R)-positive (Trf-R^+) and -negative (Trf-R^-) cells that appear after treatment with dimethyl sulfoxide (DMSO). In the present study, we analyzed the downstream events of p70 S6K in differentiation and proliferation of both cell types, with a particular focus on c-Myc. Similar to p70 S6K, we found that the expression of c-Myc in Trf-R^+ cells is also higher than that in Trf-R^- cells. Wortmannin, a specific inhibitor of PI3K, partially inhibited G-CSF-induced p70 S6K activity, c-Myc expression, and G-CSF-dependent proliferation, whereas rapamycin, an inhibitor of p70 S6K, completely inhibited p70 S6K activity, c-Myc expression, and G-CSF-dependent proliferation, indicating that the extent of c-Myc inhibition by these inhibitors correlates with a reduction in proliferation, and that c-Myc is downstream from PI3K/p70 S6K. We also determined phosphorylation of the 4E-binding protein 1 (4E-BP1), which is regulated downstream of the mammalian target of rapamycin. The addition of G-CSF failed to enhance the phosphorylation state of 4E-BP1 of HL-60 cells 2 days after DMSO differentiation. An antisense oligonucleotide for c-*myc* inhibited both G-CSF-dependent enhancement of c-Myc expression and proliferation in Trf-R^+ cells, but did not enhance the differentiation in terms of O_2^--generating ability or fMLP-R expression. In contrast, antisense oligonucleotide for c-*myc* promoted fMLP-R on non-treated HL-60 cells. We therefore conclude that the PI3K/p70 S6K/c-Myc cascade plays an important role in neutrophilic proliferation in HL-60 cells. Unlike that of rapamycin, however, the antisense oligonucleotide for c-*myc* could not promote differentiation of Trf-R^+ cells cultured with G-CSF, indicating that another target downstream of p70 S6K may control the differentiation of HL-60 cells in terms of the signal transduction of G-CSF.

Keywords: c-Myc; p70 S6 kinase; Phosphatidylinositol 3-kinase; Granulocyte colony-stimulating factor; HL-60 cells; Neutrophilic differentiation

1. Introduction

G-CSF plays a pivotal role in neutrophilic development. Mice deficient in either G-CSF or its receptor genes have a reduced number of neutrophils [1,2]. We have reported that G-CSF enhances the phosphorylation of p70 S6K in DMSO-treated HL-60 cells undergoing neutrophilic differentiation [3]. Furthermore, we found that during the neutrophilic differentiation of DMSO-treated HL-60 cells, Trf-R-positive (Trf-R^+) and -negative (Trf-R^-) cells appeared 2 days after the addition of DMSO, and these Trf-R^+ and Trf-R^- cells were characterized as proliferative- and differentiation-type cells, respectively [4]. We also showed that while G-CSF enhances proliferation of Trf-R^+ cells via stimulation of p70 S6K phosphorylation [4], S6K activity, and PI3K activity [5], and enhances differentiation of Trf-R^- cells via stimulation of tyrosine phosphorylation of STAT3. Rapamycin was found not only to enhance the differentiation of Trf-R^+ and Trf-R^- cells,

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Abbreviations: DMSO, dimethyl sulfoxide; G-CSF, granulocyte colony-stimulating factor; Trf-R, transferrin receptor; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; p70 S6K, protein 70 S6 kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; mTOR, mammalian target of rapamycin; 4E-BP1, 4E-binding protein 1; fMLP-R, formyl-Met-Leu-Phe receptor.

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but to eliminate the difference in differentiation ability between Trf-R^+ and Trf-R^- cells in the presence of G-CSF. From these results, we concluded that downstream signaling components of a mTOR, which includes p70 S6K, play a negative role in the neutrophilic differentiation of HL-60 cells [4].

c-Myc is a transcription factor of the basic helix–loop–helix leucine zipper (bHLH-LZ) family that heterodimerizes with Max, another bHLH-LZ protein. The binding of Myc–Max heterodimers to E boxes (canonical consensus sequence CACGTG) activates the transcription of multiple genes implicated in the regulation of cell metabolism, protein synthesis, cell division, and apoptosis [6]. c-Myc can also repress the expression of a number of genes and decrease cell differentiation by unclear mechanisms. Constitutive c-Myc expression is observed in many human cancers, including lung carcinomas and Burkitt's lymphomas [6]. In differentiated cells, including fibroblasts, smooth muscle cells, and hepatocytes, c-Myc overexpression stimulates cell growth and proliferation, decreases cell differentiation, and sensitizes cells to apoptosis [7–10]. Treating HL-60 cells with c-*myc* antisense oligonucleotide results in the inhibition of cellular proliferation [11–13] and induces differentiation of HL-60 cells [13–15].

PI3K is reported to play an important role in the activation of p70 S6K [16–18]. From data obtained using Trf-R^+ and Trf-R^- cells, we have recently reported that, being upstream from p70 S6K, PI3K plays an important role in differentiation and proliferation [5]. Law *et al.* have reported that c-Myc is down-regulated by the inhibition of p70 S6K [19]. However, the role of c-Myc in neutrophilic differentiation and the identity of its upstream regulator, which is induced by G-CSF, remains unelucidated. In the present study, we analyzed the regulation of c-Myc expression and its role in the G-CSF-dependent signaling pathway in differentiation of neutrophilic HL-60 cells in the relationship with p70 S6K.

2. Materials and methods

2.1. Reagents

Recombinant human G-CSF was a kind gift from Chugai Pharmaceutical Co. The magnetic cell-sorting kit, MACS, was from Miltenyi Biotec. DMSO was from Pierce. The mouse anti-human Trf-R (CD 71) monoclonal antibody and the mouse anti-human c-Myc protein monoclonal antibody were purchased from Pharmingen. The rabbit anti-human 4E-BP1 polyclonal antibody was from Cell Signaling Technology Inc. The horseradish peroxidase-conjugated sheep anti-mouse IgG antibody was from Amersham Life Science Corp. Rapamycin was purchased from Calbiochem-Novabiochem Intl. Wortmannin was from Wako Pure Chemical Industries, Ltd. [γ-^{33}P]ATP was from Amersham Pharmacia Biotech.

2.2. Cell culture and differentiation in relation to neutrophilic granulocyte lineage

HL-60 cells were kindly supplied by the Japanese Cancer Research Resources Bank. Cells were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS and 30 mg/L kanamycin sulfate at 37° under moisturized air containing 5% CO_2. HL-60 cells were differentiated into neutrophilic cells by the addition of DMSO. Two days after addition of the differentiating agent, Trf-R^+ and Trf-R^- cells were sorted by a magnetic cell-sorter.

2.3. Magnetic cell-sorting

Magnetic cell-sorting was performed as previously reported [4]. Trf-R^+ and Trf-R^- cells were subsequently cultured with or without 50 ng/mL G-CSF in the conditioned medium. Proliferation and O_2^--generating activity were measured 5 days after magnetic cell-sorting.

To clarify the effects of rapamycin (20 ng/mL) and wortmannin (100 nM) on differentiation of HL-60 cells, both substances were preincubated with the cells for 30 min before the addition of G-CSF.

2.4. Detection of p70 S6K enzymatic activity

The activity of p70 S6K (including p85 isoforms of S6K) was determined by ^{33}P incorporation into the S6 peptide (Upstate Biotechnology). Trf-R^+ and Trf-R^- cells were stimulated by G-CSF (50 ng/mL) for 1 hr in the presence or absence of 20 ng/mL rapamycin or 100 nM wortmannin. The reaction was terminated by the addition of an ice-cold cocktail of protease and phosphatase inhibitors [4]. After centrifugation at 1800 *g* for 3 min at 4°, the cells were suspended in 1 mL of lysis buffer [4] and then sonicated at 40 W for 20 s with a Branson Sonifier. Following further centrifugation, the collected supernatant was incubated with a rabbit anti-human p70 S6K (511–525) peptide polyclonal antibody IgG (5 mg per sample, Upstate Biotechnology) at 4° for 1 hr. The reaction mixture was next incubated with 25 mL of Protein A Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) at 4° for 1 hr. The immune complexes were washed twice with lysis buffer. Protein 70 S6K enzymatic activities in each sample were determined using the p70 S6K assay kit (Upstate Biotechnology).

2.5. O_2^--generating activity and formyl-Met-Leu-Phe receptor (fMLP-R) expression

The O_2^--generating activity of the differentiated cells was measured in terms of the ferricytochrome *c* reduction assay, as previously described [4]. For the fMLP-R expression assay, the differentiated cells were collected and incubated with FITC-fMLP, then were subjected to flow cytometric analysis (FACSCalibur, Becton and Dickinson).

2.6. Preparation of cell lysates and immunoblotting

For immunoblotting with anti-cMyc antibody, Trf-R$^+$ and Trf-R$^-$ cells resuspended in conditioned medium were stimulated with G-CSF (50 ng/mL). After the incubation with G-CSF for 7 hr, the cells were mixed with an equal volume of ice-cold buffer containing a cocktail of protease and phosphatase inhibitors. For immunoblotting with anti-p70/p85 S6K and 4E-BP1 antibodies, HL-60 cells treated with DMSO for 2 days were stimulated by G-CSF for 30 min. The reactions were terminated by addition of the ice-cold buffer. Western blotting analysis was then performed, as described previously [4]. The bands that appeared on X-ray films were scanned and the density of each band was calculated using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet) within the linear range for quantitation.

2.7. Treatment of c-myc antisense or sense oligonucleotides

The sequences of the antisense- and sense-c-*myc* S-oligonucleotides were 5′-AACGTTGAGGGGCAT-3′ and 5′-TTGCAACTCCCCGTA-3′, respectively. These sequences were originally reported by Heikkila *et al.* [20]. The antisense- and sense-c-*myc* S-oligonucleotides were dissolved in water at 1 mM. After cell-sorting, both cells were incubated with 10 mM antisense or 10 mM sense c-*myc* oligonucleotides for 30 min. After the addition of 50 ng/mL G-CSF for 3 hr, 10 mM of each oligonucleotide was again added. Cells were subsequently cultured for 5 days, and then, both proliferation and O_2^- production were determined. Expression of fMLP-R was analyzed 3 days after the addition of antisense oligonucleotides for c-*myc*. Seven hours after the addition of G-CSF, the cells were collected and subjected to the Western blotting analysis for c-Myc expression.

2.8. Statistical analysis

Each experiment was repeated three or more times, and representative data are indicated. Statistical analysis was performed using the unpaired *t*-test. Values of $P < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Effects of wortmannin and rapamycin on proliferation of Trf-R$^+$ and Trf-R$^-$ cells

To clarify the cascade of signal transduction in the proliferation of neutrophilic proliferation, we examined the effects of rapamycin, a specific inhibitor of p70 S6K, and wortmannin, a specific inhibitor of PI3K on the growth

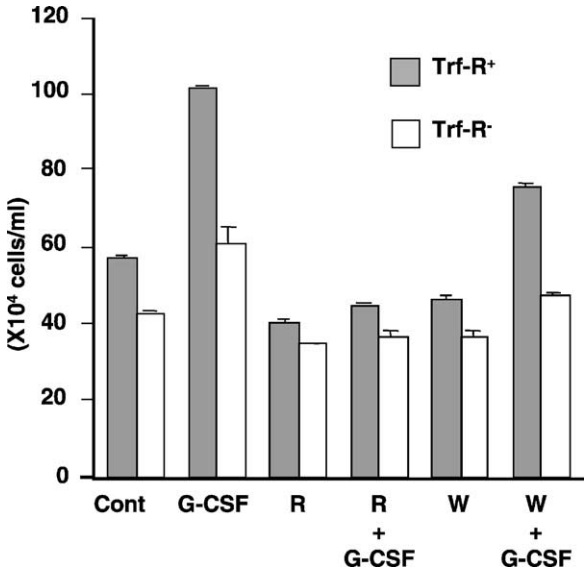

Fig. 1. Effects of rapamycin and wortmannin on G-CSF-induced proliferation in differentiating HL-60 cells. Effects of rapamycin or wortmannin on the proliferation of differentiated HL-60 cells were determined. Trf-R$^+$ cells and Trf-R$^-$ cells sorted from differentiating HL-60 cells were preincubated with or without 20 ng/mL rapamycin (R) or 100 nM wortmannin (W), and then subsequently cultured with G-CSF. After a 5-day culture with G-CSF, the cell numbers in each sample were counted. Open columns denote Trf-R$^-$ cells and gray columns Trf-R$^+$ cells. Columns and bars represent the mean ± SD of triplicate wells.

of Trf-R$^+$ and Trf-R$^-$ cells. We have previously reported that Trf-R$^+$ cells and Trf-R$^-$ cells are proliferative-type and differentiation-type cells, respectively, and that each character is enhanced by G-CSF. As shown in Fig. 1, the growth rate of Trf-R$^+$ cells was greater than that of Trf-R$^-$ cells, and G-CSF markedly enhanced the proliferation of Trf-R$^+$ cells. Rapamycin completely inhibited G-CSF-induced proliferation of Trf-R$^+$ cells, as reported previously [4]. In contrast, 100 nM wortmannin completely inhibited PI3K, but only partially inhibited p70 S6K [5]; in addition, the same concentration of wortmannin only partially inhibited G-CSF-dependent proliferation of Trf-R$^+$ cells (Fig. 1), suggesting that PI3K partially contributes to the G-CSF-dependent proliferation of HL-60 cells.

3.2. Activity of p70 S6K in Trf-R$^+$ and Trf-R$^-$ cells and the effects of wortmannin or rapamycin on p70 S6K activity and 4E-BP1 phosphorylation

To clarify the role of p70 S6K (including p85 isoforms of S6K) in the proliferation of differentiating HL-60 cells, we examined the activities of p70 S6K in Trf-R$^+$ and Trf-R$^-$ cells. As shown in Fig. 2A, the p70 S6K activity of Trf-R$^+$ cells was higher than that in Trf-R$^-$ cells. In the presence of G-CSF, the p70 S6K activity of Trf-R$^+$ cells was 2.7 times higher than that of Trf-R$^-$ cells. In contrast, G-CSF activated ERK in neither Trf-R$^+$ nor Trf-R$^-$ cells [5]. These data confirm the hypothesis that p70 S6K plays an important role in the G-CSF-dependent and -independent proliferation of neutrophils [4,5].

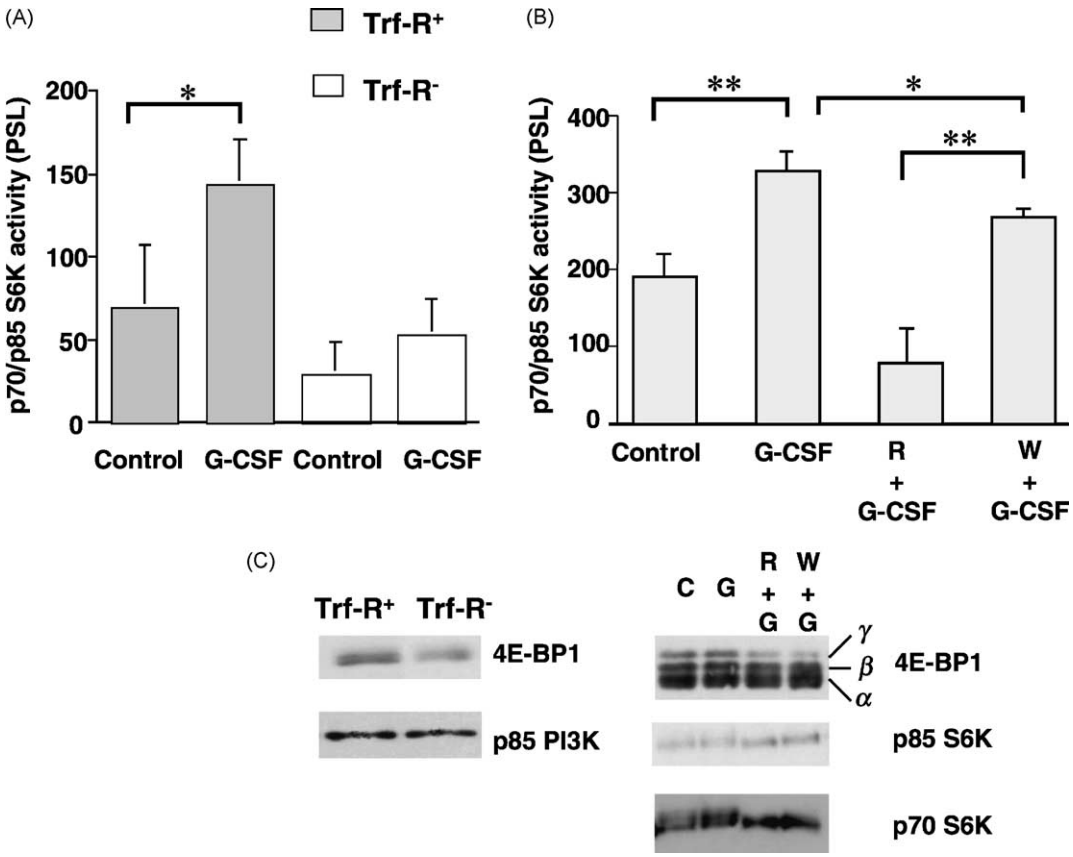

Fig. 2. Effects of rapamycin and wortmannin on p70/p85 S6K activation and 4E-BP1 phosphorylation. (A) Protein 70/p85 S6K activity in Trf-R$^+$ and Trf-R$^-$ cells obtained from DMSO-treated cells. After the addition of G-CSF, both cells were disrupted in lysis buffer, and p70 S6K was immunoprecipitated from the cell lysate with the anti-p70 S6K antibody. A kinase assay was performed using the immunocomplexes. (B) Effects of rapamycin and wortmannin on G-CSF-induced p70/p85 S6K activity of HL-60 cells. Two days after culture with DMSO, HL-60 cells were incubated with rapamycin (R) or wortmannin (W) for 30 min, and then stimulated by G-CSF for 60 min. The kinase assay was performed as described in (A). The *y*-axis of p70/p85 S6K activity represents the radioactivity of the ^{33}P-S6 peptide as the degree of photon-stimulated luminescence (PSL). Quantitation of p70/p85 S6K was performed using data from three separate experiments. Columns and bars represent the means ± SD ($^*P < 0.05$; $^{**}P < 0.01$). (C) Protein level of 4E-BP1 in Trf-R$^+$ and Trf-R$^-$ cells and effect of G-CSF on 4E-BP1 and p70/p85 S6K phosphorylation of HL-60 cells. Left, upper is an illustration of the Trf-R$^+$ and Trf-R$^-$ cells immunoblotted with 4E-BP1. The expression of p85 PI3K protein was analyzed as a loading control (left, lower). 4E-BP1 (right, upper), p85 S6K (right, middle), and p70 S6K (right, lower) phosphorylation was examined by mobility shift on protein immunoblots with each antibody. Two days after differentiation, HL-60 cells were treated with rapamycin (R) and wortmannin (W) before addition of G-CSF (G).

Next, to clarify the role of PI3K in p70 S6K activity, wortmannin, a specific inhibitor of PI3K, was added before the addition of G-CSF. In contrast to rapamycin, wortmannin partially inhibited the activity of p70 S6K in Trf-R$^+$ cells (Fig. 2B). As reported previously [5], the same concentration of wortmannin completely inhibited PI3K, suggesting that PI3K may partially regulate p70 S6K.

In order to investigate the role of mTOR, the effects of rapamycin and wortmannin on phosphorylation of 4E-BP1 in DMSO-treated HL-60 cells were determined. 4E-BP1 phosphorylation is regulated by mTOR. Rapamycin binds to FKBP 12 and inhibits p70 S6K binding to a molecule termed mTOR (FRAP or RAFT). Addition of rapamycin inhibits protein synthesis and, more specifically, translation of mRNAs encoding ribosomal proteins, suggesting that p70 S6K and S6 are important in these events. However, rapamycin also inhibits phosphorylation of 4E-BP1, which is another regulatory molecule for mRNA translation. Rapamycin induces accumulation of the dephosphorylated species of 4E-BP1 that binds to the translation initiation factor eIF-4E and suppresses translation initiation of cap-dependent mRNAs via inhibition of mTOR. The amount of 4E-BP1 proteins in Trf-R$^+$ cells was higher than that in Trf-R$^-$ cells (Fig. 2C, left, upper), as was the case with p70 S6K. There was no difference in the expression of p85 PI3K, a loading control, in both types of cells, as we previously reported [5] (Fig. 2C, left, lower). On the other hand, there was no difference in the phosphorylation of 4E-BP1 of HL-60 cells 2 days after DMSO-differentiation between the control and the G-CSF-treated cells (Fig. 2C, right); rapamycin and wortmannin inhibited the phosphorylation of 4E-BP1. In contrast, phosphorylation of both p70 S6K (Fig. 2C, right bottom) and p85 isoforms of S6K (Fig. 2C, right middle) were enhanced by G-CSF, inhibited completely by rapamycin, and inhibited partially by wortmannin in accordance with their respective activities (Fig. 2B). These data indicated that G-CSF-induced

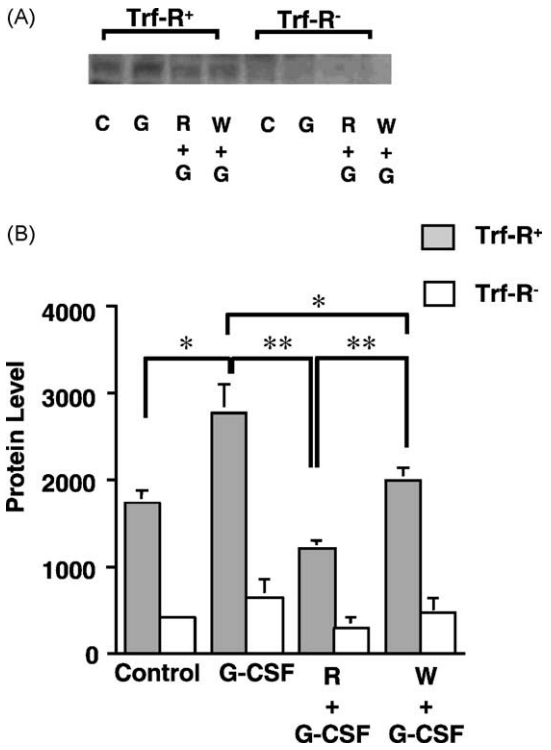

Fig. 3. Effects of rapamycin and wortmannin on G-CSF-induced c-Myc protein expression in Trf-R$^+$ and Trf-R$^-$ cells. (A) Effects of rapamycin (R) and wortmannin (W) on the expression of c-Myc protein induced by G-CSF (G) for 7 hr was analyzed by immunoblotting with an anti-c-Myc antibody. (B) Quantitation of G-CSF-induced protein was performed using data from three separate experiments. Open columns denote Trf-R$^-$ cells and gray columns Trf-R$^+$ cells. Columns and bars represent the mean ± SD ($^*P < 0.05$; $^{**}P < 0.01$).

proliferation mainly depends not on mTOR but on p70 S6K (also the p85 isoforms).

3.3. The role of c-Myc in events DownStream from p70 S6K

It has been reported that c-Myc expression is regulated at a position downstream from p70 S6K [19]. Antisense oligonucleotide to c-*myc* mRNA induced the differentiation of HL-60 cells [13–15]. To clarify the role of c-Myc in the G-CSF-induced enhancement of differentiation and proliferation in DMSO-treated HL-60 cells, the expression of c-Myc was determined by Western blotting. Figure 3A shows a typical pattern of c-Myc expression at 7 hr after the addition of G-CSF, and the density of each band was quantitated by the NIH Image program (Fig. 3B). Under all conditions, the expression of c-Myc in Trf-R$^+$ cells was much greater than that in Trf-R$^-$ cells. G-CSF significantly induced an enhancement of c-Myc expression in Trf-R$^+$ cells ($*$; $P < 0.05$). Rapamycin completely inhibited G-CSF-induced expression of c-Myc ($**$; $P < 0.01$), while wortmannin only partially inhibited the G-CSF-induced increase in c-Myc expression ($*$; $P < 0.05$). These data suggest that the expression of c-Myc is regulated downstream from p70 S6K, but is partially regulated by PI3K.

We next examined the effects of the c-Myc on the proliferation and differentiation of Trf-R$^+$ and Trf-R$^-$ cells using an antisense nucleotide for c-*myc*. The sequences of antisense and sense oligodeoxynucleotides have been reported previously by Heikkila *et al.* [20]. Figure 4A

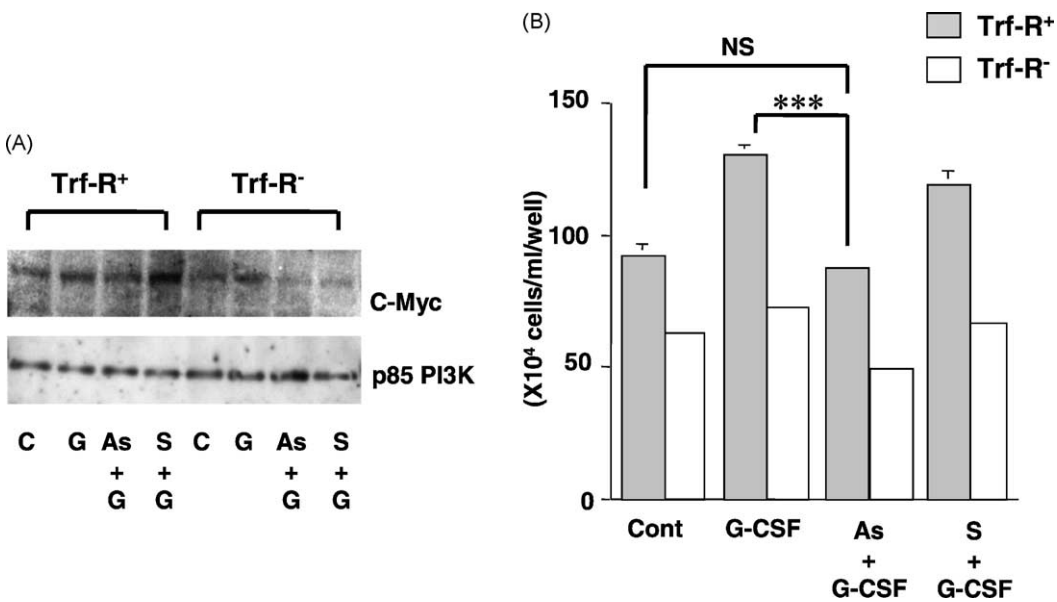

Fig. 4. Effects of c-*myc* antisense oligonucleotides on G-CSF-induced c-Myc protein expression and proliferation in Trf-R$^+$ and Trf-R$^-$ cells. (A) Cells were treated with 10 mM c-*myc* sense (S) or antisense (As) oligonucleotides. Seven hours after the addition of G-CSF (G), the expression of c-Myc protein was determined by Western blotting analysis (upper). The expression of p85 PI3K protein was used as a loading control (lower). (B) The proliferation assay was determined. Trf-R$^+$ and Trf-R$^-$ cells were treated with 10 mM antisense oligonucleotides (As) or sense oligonucleotides (S), and then subsequently cultured with G-CSF. The cell number after 5 days of culture was examined. Open columns denote Trf-R$^-$ cells and gray columns Trf-R$^+$ cells. Columns and bars represent the mean ± SD of triplicate wells ($^{***}P < 0.001$; NS, not significant).

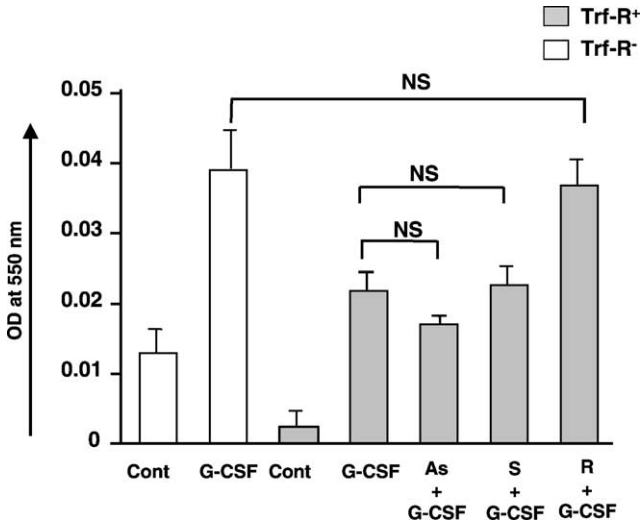

Fig. 5. Comparison of rapamycin or c-*myc* antisense oligonucleotide effects on G-CSF-induced O_2^- production in Trf-R^+ and Trf-R^- cells. Cells were treated with rapamycin (R), sense (S) or antisense (As) oligonucleotides, and were subsequently cultured with G-CSF. O_2^--generating activity stimulated by opsonized zymosan was examined after 5 days of culture using the ferricytochrome *c* reduction assay. Open columns denote Trf-R^- cells and gray columns Trf-R^+ cells. Columns and bars represent the mean ± SD of triplicate wells (NS, not significant).

showed the change in c-Myc protein level (upper) in Trf-R^+ and Trf-R^- cells using antisense or sense oligonucleotides for c-*myc*, compared with p85 PI3K protein level (lower), which is expressed at the same level in both types of cells [5], as a loading control. While the G-CSF induced enhancement of c-Myc expression in Trf-R^+ cells was partially inhibited by the addition of the antisense oligonucleotide for c-*myc* (Fig. 4A), the culture with the antisense oligonucleotide completely inhibited the G-CSF-induced proliferation of these cells to the control level (Fig. 4B; ***; $P < 0.001$). In contrast, neither the antisense nor sense oligonucleotide had any affect on the O_2^--producing ability of Trf-R^+ cells cultured with G-CSF (Fig. 5). The antisense and sense oligonucleotide also had no effects on the O_2^--producing ability of Trf-R^- cells cultured with G-CSF (data not shown). In contrast, rapamycin enhanced the O_2^--producing ability of Trf-R^+ cells cultured with G-CSF to the same level of Trf-R^- cells cultured with G-CSF, as reported previously [5]. These data indicate that while the inhibition of p70 S6K promotes neutrophilic differentiation, this enhancement does not depend on the role of c-Myc.

The effects of antisense oligonucleotides for c-*myc* on the fMLP-R expression in DMSO-treated HL-60 cells were examined. After 2 days cultivation with DMSO, HL-60 cells were subsequently cultured for 3 days in the presence of G-CSF and antisense oligonucleotides for c-*myc* or rapamycin. Whereas rapamycin promoted the fMLP-R expression of HL-60 cells treated with DMSO (Fig. 6A, upper left), antisense oligonucleotides for c-*myc* did not promote the fMLP-R expression (Fig. 6A, upper right). Culturing with G-CSF enhanced the fMLP-R expression

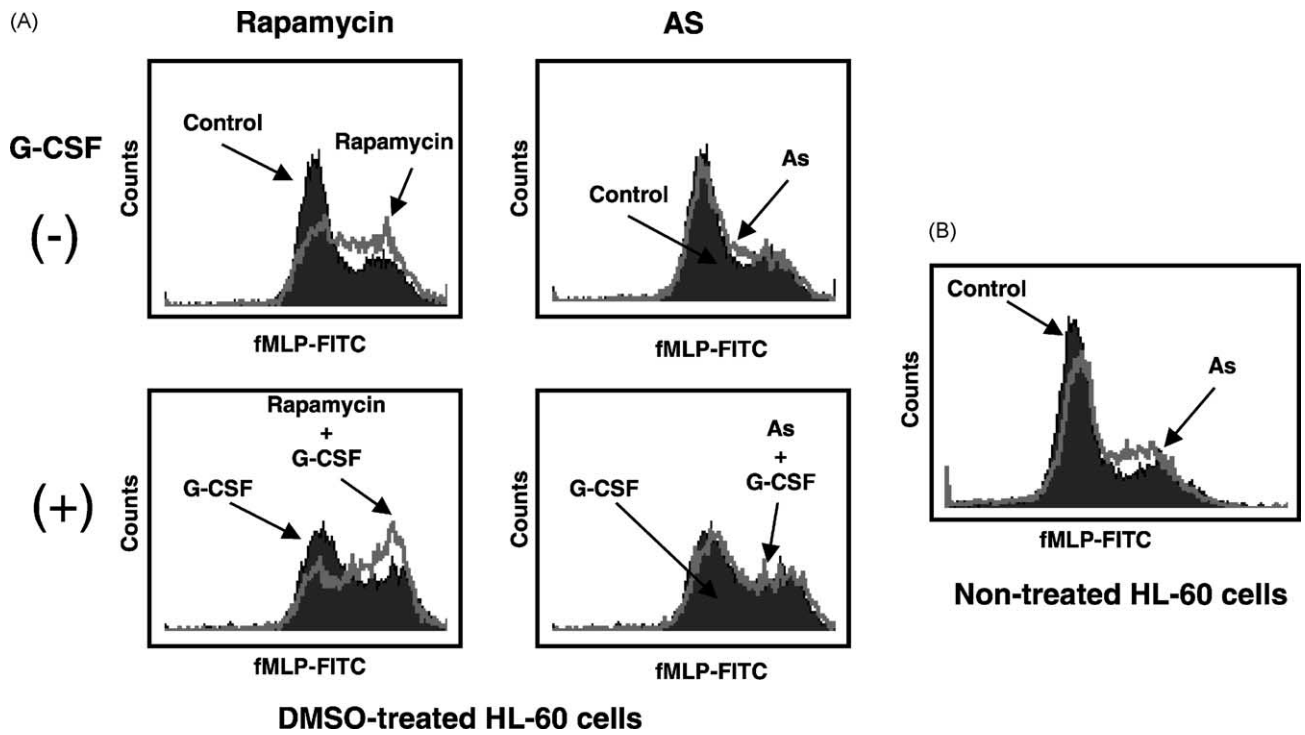

Fig. 6. Effect of antisense oligonucleotides for c-*myc* and rapamycin on fMLP-R expression in DMSO-treated and non-treated HL-60 cells. (A) fMLP-R expression in DMSO-treated HL-60 cells. Two days after addition of DMSO, cells were treated with antisense oligonucleotides for c-*myc* (As) either rapamycin (R), and then cultured for 3 days with or without G-CSF. (B) fMLP-R expression in non-treated HL-60 cells. Cells were treated with antisense oligonucleotides for c-*myc* (As) for 5 days. For the assay of fMLP-R expression, cells were incubated with FITC-conjugated fMLP, and then analyzed by flow cytometry.

on DMSO-treated HL-60 cells, and rapamycin further promoted it (Fig. 6A, lower left). In the presence of G-CSF, antisense oligonucleotides for c-*myc* had no effects (Fig. 6A, lower right). These data corresponded with O_2^- production ability (Fig. 5), indicating that the G-CSF-induced c-Myc plays an important role for proliferation but may not be a negative regulator of differentiation.

In contrast, when non-differentiated HL-60 cells were cultured with antisense oligonucleotides, the fMLP-R expression was markedly promoted (Fig. 6B), which result is in agreement with those in previous reports [13–15].

4. Discussion

c-Myc plays an important role in cell growth and differentiation and c-Myc is known to inhibit granulocyte/neutrophil differentiation. In myeloid cells, constitutive expression of v-Myc blocks phorbol ester-induced differentiation of human U-937 monoblasts [21]. Nakajima and Ihle [22] reported that the induction of C/EBPe, an important transcription factor for promoting differentiation of granulocytes, is completely abrogated in 32Dcl3 cells overexpressing c-Myc. Moreover, an oligomer complementary to c-*myc*-mRNA induces differentiation of HL-60 cells [13–15].

In our previous study [4], we reported the appearance of Trf-R^+ and Trf-R^- cells in DMSO-treated HL-60 cells. The Trf-R^+ and Trf-R^- cells were characterized as proliferative- and differentiation-type cells, respectively. Based on an analysis of the G-CSF effect on both cell types, we suggested that p70 S6K plays a negative role in neutrophilic differentiation of HL-60 cells. In this study, we examined the role of c-Myc involved in neutrophilic differentiation, focusing specifically on the p70 S6K cascade and/or the G-CSF-dependent signal transduction pathway.

The present and previous results [5] suggest that the PI3K/p70 S6K cascade plays an important role in higher proliferative ability in Trf-R^+ cells (Figs. 1 and 2). Furthermore, G-CSF-dependent enhancement of proliferation is also dependent on the PI3K/p70 S6K cascade. In this study, the expression of c-Myc in Trf-R^+ cells was greater than that in Trf-R^- cells, and G-CSF markedly enhanced the expression of c-Myc in Trf-R^+ cells (Figs. 3A and 4A). Law *et al.* have reported that c-Myc expression is regulated at a position downstream from p70 S6K [19]. Because the high proliferation ability of Trf-R^+ cells coincides with the augmentation of PI3K and p70 S6K activity and c-Myc expression, the PI3K/p70 S6K/c-Myc cascade plays an important role in the proliferation ability of Trf-R^+ cells. Since G-CSF did not influence the phosphorylation of 4E-BP1 (Fig. 2C), G-CSF-induced proliferation may not depend on mTOR. However, as we did not measure the activity of mTOR, further investigation is needed.

Many reports suggest a negative correlation between c-Myc expression and cellular differentiation, with the Myc oncoprotein (c-, L- and N-Myc) is down-regulated during differentiation in many cells [23–26]. We also showed that the expression of c-Myc in Trf-R^- cells is lower than that of Trf-R^+ cells (Fig. 3). When Trf-R^+ cells were treated with rapamycin or wortmannin, the G-CSF-induced proliferation in Trf-R^+ cells was completely inhibited by rapamycin and partially by wortmannin (Fig. 1). The inhibition of p70 S6K in Trf-R^+ cells by rapamycin or wortmannin showed the same tendency as the proliferation in these cells. We have reported that during the neutrophilic differentiation of HL-60 cells, G-CSF stimulates the activity of p70 S6K, which is located in downstream from PI3K [5]. These data are consistent with previous reports that p70 S6K acts downstream from PI3K [16,27–29]. Furthermore, the G-CSF-induced protein expression of c-Myc was found to be completely inhibited by rapamycin and partially by wortmannin. It has also been reported that c-Myc expression is regulated downstream from Ras/Raf/MEK/ERK or STAT3 [30,31]. Therefore, the G-CSF-induced c-Myc expression may be partially regulated by PI3K/p70 S6K.

Antisense oligodeoxynucleotides of c-*myc* inhibited not only the expression of c-Myc protein (Fig. 4A), but also the G-CSF-induced proliferation in Trf-R^+ cells (Fig. 4B). Heikkila *et al.* [20] and Holt *et al.* [15] have also successfully inhibited cell growth using antisense oligonucleotides of c-*myc*. On the other hand, as reported previously [4], rapamycin enhanced the differentiation of Trf-R^+ cells in the absence or presence of G-CSF, and there was no difference in neutrophilic differentiation between Trf-R^+ and Trf-R^- cells. Wortmannin enhanced the differentiation of Trf-R^+ cells only in the presence of G-CSF [5]. Antisense oligonucleotides for c-*myc*, however, could not stimulate the differentiation in terms of O_2^--production ability (Fig. 5) or fMLP-R expression in DMSO-treated HL-60 cells (Fig. 6A). Several investigators have reported that antisense oligonucleotides for c-*myc* inhibit the proliferation of HL-60 cells and induce differentiation into neutrophilic cells [13–15]. In those reports, antisense oligonucleotides for c-*myc* were used as a differentiation drug such as DMSO or retinoic acid. In this study, we confirmed that antisense oligonucleotides for c-*myc*, when added to non-differentiated HL-60 cells, also induced the fMLP-R expression (Fig. 6B). These data suggest that the G-CSF-dependent proliferation of Trf-R^+ cells may occur through the PI3K-p70 S6K-c-Myc pathway, but another factor(s) located downstream from PI3K/p70 S6K may contribute to the G-CSF-induced enhancement of differentiation in HL-60 cells.

Acknowledgments

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