N-*myc* suppression and cell cycle arrest at G_1 phase by prostaglandins

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Effects of cyclopentenone prostaglandins, Δ^{12}-prostaglandin (PG) J_2 and PGA_2 on the expression of N-*myc* in relation to the effects on cell cycle progression were investigated using human neuroblastoma cell line GOTO. Both PGs suppressed N-*myc* expression within several hours prior to inducing G_1 arrest. The N-*myc* suppression with Δ^{12}-PGJ_2 was continued but with PGA_2 it was gradually released, followed by the release of G_1 arrest. These results suggest that Δ^{12}-PGJ_2 and PGA_2 inhibit cell cycle progression in strong association with N-*myc* suppression and Δ^{12}-PGJ_2 is more potent and has a longer effect than PGA_2.

Δ^{12}-Prostaglandin J_2; Prostaglandin A_2; N-*myc*; Cell cycle; Human neuroblastoma cell

1. INTRODUCTION

Cyclopentenone prostaglandins (PGs), Δ^{12}-prostaglandin J_2 (Δ^{12}-PGJ_2) and PGA_2 are active metabolites of PGD_2 and PGE_2, respectively [1,2]. They act directly on nuclei and arrest the cells at the G_1 phase of cell cycle [3,4]. The inhibitory effect on cell cycle progression of Δ^{12}-PGJ_2 is somewhat irreversible, whereas that of PGA_2 is reversible [4]. Though the precise mechanisms of the cell cycle arrest induced by PGs are still not known, using human promyelocytic leukemia cell line HL 60, Ishioka et al. reported that PGA_2 suppresses c-*myc* expression completely prior to the cell cycle arrest and the effects are reversible [5]. Here we report that Δ^{12}-PGJ_2 and PGA_2 suppressed N-*myc* mRNA expression in human neuroblastoma GOTO cells whose N-*myc* is amplified and that the suppression and the restoration of N-*myc* were closely associated with the changes of cell cycle progression.

2. MATERIALS AND METHODS

2.1. *Chemicals*

PGA_2 was purchased from Funakoshi Chemicals Co., Ltd., Tokyo, and Δ^{12}-PGJ_2 was a kind gift from Fuji Pharmaceutical Co., Ltd., Toyama and from Ono Pharmaceutical Co., Ltd., Osaka. PGs were dissolved in absolute ethanol and stocked at a concentration of 10 mg/ml at -20°C and were diluted to appropriate concentrations at the time of use. The final concentration of ethanol used to dissolve PGs was less than 1.0%. Each equivalent ethanol was added to control dishes and it had no effects on GOTO cells. Dulbecco's modified Eagle medium (DMEM) was obtained from Nissui Seiyaku Co., Ltd., Tokyo, and fetal calf serum (FCS) from Flow Laboratories, North Ryde. RNase A was obtained from Sigma, St Louis, MO. Propidium iodide was obtained from Calbiochem Corp., La Jolla, CA. [α-^{32}P]-d CTP was obtained from Amersham Japan. All other chemicals used were of reagent grade.

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2.2. *Cell culture*

Human neuroblastoma cell line GOTO, established by Sekiguchi et al. [6], was used. Cultures were maintained in DMEM containing 10% FCS and were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were seeded at 0.5×10^5 cells/ml in a total volume of 4 ml onto 60 mm ∅ dishes for cell cycle analysis and Northern blot analysis. The medium was renewed 2 days after inoculation and PGs were added at various concentrations.

2.3. *Flow cytometric analysis*

Cells were removed at the indicated time from culture dishes by trypsinization and centrifuged. After washing twice with phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS (−)), they were suspended in PBS (−) containing 0.1% Triton X-100. Then the suspension was filtered through 50 μm nylon mesh and incubated with 0.1% RNase and 50 μg/ml propidium iodide for 30 min. DNA contents in stained cells were analyzed with FACScan, Becton Dickinson. The suspension of 1.0×10^4 cells was analyzed for each DNA histogram. Cell numbers in each phase were measured according to the S fit program in the FACScan [7].

2.4. *Northern blot analysis*

Total cellular RNA was prepared by the acid guanidium thiocyanate-phenol-chloroform extraction as described by Chomczynski et al. [8]. Each total cellular RNA (3 μg) sample was denatured and electrophoresed and then transferred to a Nitro Plus 2000 membrane purchased from Micron Separations Inc., Westboro, MA. After prehybridization, hybridization was performed as described before [9]. Hybridization probe was 1.0 kb *Eco*RI/*Bam*HI fragment (Nb-1) from human neuroblastoma cell line Kelly [10] and actin probe was used as an internal control [11]. Both probes were purchased from Oncor, Gaithersburg, MD. Probes were labelled with [α-^{32}P]d CTP to a specific activity of $>10^8$ cpm/μg by nick translation. The filter was exposed to Fuji RX film. The autoradiograph was scanned by densitometric tracing, CS-9000 from Shimadzu Co. Ltd., Kyoto.

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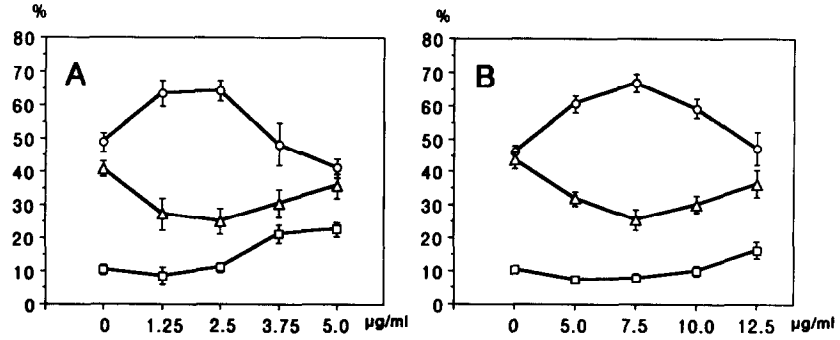

Fig. 1. Dose-dependent effects of Δ^{12}-PGJ_2 (A) and PGA_2 (B) on cell cycle progression. After 24 h from the addition of PGs, the percentages of G_1 (○), S (△), and G_2 + M (□) phases were obtained by flow cytometric analysis. 0 μg/ml represents control culture without PGs. DATA are shown as means ± SD (n = 5 for Δ^{12}-PGJ_2, n = 4 for PGA_2).

3. RESULTS

3.1. *Effects of PGs on cell cycle progression*

To investigate the effects of PGs on the cell cycle progression of GOTO cells, we treated cells with PGs after 2 days of inoculation. At 24 h from the addition of PGs, DNA contents of the cells were measured by flow cytometric analysis. As shown in Fig. 1, the peaks of G_1 phase cell accumulation were obtained with 2.5 μg/ml (7.5 μM) of Δ^{12}-PGJ_2 and 7.5 μg/ml (22.4 μM) of PGA_2, respectively, at which doses the cells remained viable. The growth curves of GOTO cells (data not shown) showed that the IC_{50} value of Δ^{12}-PGJ_2 was 2.5 μg/ml and of PGA_2 between 7.5 μg/ml and 10.0 μg/ml. At higher doses, the percentages of G_1 cells were similar to those of the controls, and the PGs completely inhibited the cell growth of GOTO cells. So we made following experiments with 2.5 μg/ml of Δ^{12}-PGJ_2 and 7.5 μg/ml of PGA_2, which arrested the cell cycle specifically in G_1/S boundary. Fig. 2 shows DNA histograms at 24 h, the percentages of G_1 phase cells increased, whereas those of S phase cells decreased. These suggest that PGs blocked the cell cycle at the G_1/S boundary as mentioned before [4,12]. Next we followed the cell cycle progression under the existence of these PGs. As shown in Fig. 3, the accumulation of G_1 phase cells reached a peak at 24 h, and then it decreased but the effect of Δ^{12}-PGJ_2 continued up to 72 h from the addition. PGA_2 showed the same effect with Δ^{12}-PGJ_2 up to 36 h, but thereafter G_1 phase cells gradually decreased to the control level.

3.2. *Effects of PGs on the expression of N-myc*

To investigate the effects of PGs on the expression of N-*myc*, we used exponentially growing cells in the same condition which served for cell cycle analysis. Total cellular RNA was extracted from each sample and the expression of N-*myc* mRNA was analysed using Northern blot analysis described in Materials and Methods. As shown in Fig. 4, the expression of N-*myc* was suppressed dose-dependently at 24 h after the addition of PGs. At the concentration of 2.5 μg/ml, Δ^{12}-PGJ_2 suppressed N-*myc* expression to about 40% of the control level and at 5.0 μg/ml, Δ^{12}-PGJ_2 almost completely suppressed it. In the case of PGA_2, the addition of 7.5 μg/ml caused about 50% suppression, and at the concentration of 12.5 μg/ml, PGA_2 suppressed the expression of N-*myc* almost completely. The expression of actin gene as an internal control was not suppressed under the same conditions. These findings suggest that PGs selectively suppressed N-*myc* expression. Fig. 5 shows the time course of the expression of N-*myc* after 1–72 h from the addition of PGs using the doses of 2.5 μg/ml

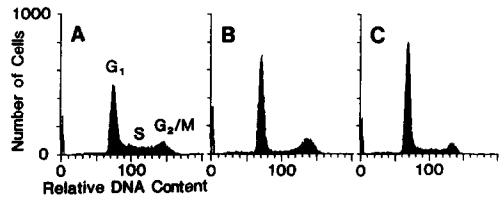

Fig. 2. DNA histograms of GOTO cells without PGs (A) or with either 2.5 μg/ml of Δ^{12}-PGJ_2 (B) or with 7.5 μg/ml of PGA_2 (C). Data shown are representative DNA histograms of two duplicate experiments.

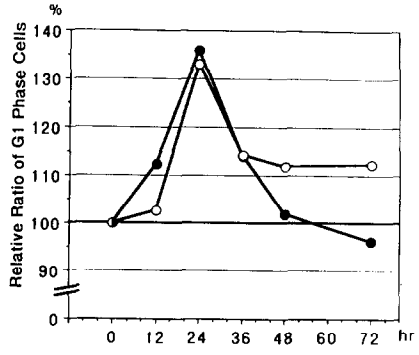

Fig. 3. Relative ratio of G_1 phase cells treated with PGs against control cells during continuous exposure to 2.5 μg/ml of Δ^{12}-PGJ_2 (○) and 7.5 μg/ml of PGA_2 (•). Relatives ratios of G_1 phase were plotted against time. Data represent means of two duplicate experiments.

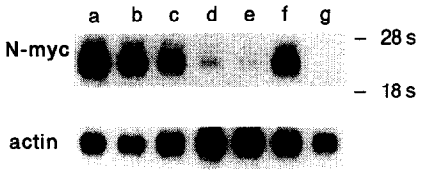

Fig. 4. Effects of PGs on the expression of N-*myc*. GOTO cells were untreated (a) or treated with 1.25 (b), 2.5 (c), 3.75 (d), and 5.0 (e) μg/ml of Δ^{12}-PGJ$_2$ and with 7.5 (f), and 12.5 (g) μg/ml of PGA$_2$ for 24 h, and then Northern blot analysis was performed.

of Δ^{12}-PGJ$_2$ and 7.5 μg/ml of PGA$_2$, The N-*myc* expression was suppressed rapidly to about 20% of the control level up to 6 h with both Δ^{12}-PGJ$_2$ and PGA$_2$. Then it recovered to about 40% or more, but remained at that level with Δ^{12}-PGJ$_2$, though with PGA$_2$ it recovered gradually after 24 h and further recovered up to about 70% at 72 h.

4. DISCUSSION

This is the first report that Δ^{12}-PGJ$_2$, a J series prostaglandin, suppressed the expression of N-*myc* gene which acts in nuclei. Since it has been reported that PGA$_2$ suppresses the expression of c-*myc* gene, which is also *myc* gene family oncogene [5], we compared the effects of J and A series prostaglandins and found that both Δ^{12}-PGJ$_2$ and PGA$_2$ suppressed N-*myc* expression

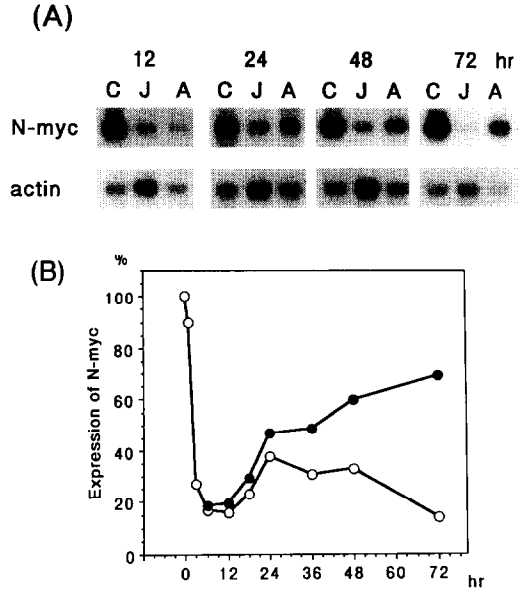

Fig. 5. Effects of PGs on N-*myc* expression during continuous exposure to 2.5 μg/ml of Δ^{12}-PGJ$_2$ and 7.5 μg/ml of PGA$_2$. (A) shows the representative autoradiographs at 12, 24, 48, 72 h from the addition of PGs (C, control; J, Δ^{12}-PGJ$_2$; A, PGA$_2$). The degrees of N-*myc* expression are shown in (B) with Δ^{12}-PGJ$_2$ (○) and PGA$_2$ (●). They were obtained by densitometric analysis of autoradiographs. The percentages of N-*myc* expression are relative ratios of PGs treated cells against control cells at various time points. Data shown are means of two experiments.

and the effect of Δ^{12}-PGJ$_2$ was longer than that of PGA$_2$ with equivalent effective doses. J and A series prostaglandins have an α,β-unsaturated carbonyl group in the cyclopentenone ring which was reported to be the active moiety for cell growth inhibiting activity [13,14]. Though both Δ^{12}-PGJ$_2$ and PGA$_2$ have the same reactive structure, most of PGA$_2$ is present as free molecules in nuclei. On the other hand, most of Δ^{12}-PGJ$_2$ binds firmly to nuclear proteins [3]. These results suggest that the modes of binding to cell nuclei may reflect on the action of PGs.

In the present study, we used the equivalent effective doses of PGs, which caused the strongest accumulation of G_1 cells. At higher doses the percentages of G_1 cells were similar to those of the control. Bhuyan et al. reported a similar observation; i.e. PGs caused G_1 arrest of melanoma cells and at higher doses, which strongly suppressed the cell growth, no obvious G_1 cell accumulations were seen [12]. This phenomenon was also seen with other substances. Crampton et al. showed that Didemnin B induced G_1 arrest and at higher doses it caused 'frozen' cell cycle [15]. These results suggest the possibility that in the present study the PGs arrested the cells non specifically in each phase of the cell cycle at higher doses, resulted in so called 'frozen' cell cycle, and thus G_1 cell accumulation was not observed.

We demonstrated that the inhibition of N-*myc* expression by PGs was associated with G_1 arrest. The patterns of the accumulation of G_1 phase cells by Δ^{12}-PGJ$_2$ and PGA$_2$ were similar up to 36 hours from the addition. Thereafter the effect of Δ^{12}-PGJ$_2$ continued up to 72 h, while that of PGA$_2$ disappeared until 48 h. We also showed that the inhibition and the restoration of N-*myc* expression preceded the changes of the cell cycle progression. These results indicate that cyclopentenone PGs arrest the cell cycle progression of GOTO cells at G_1 phase in close association with modulating N-*myc* gene expression through binding to nuclear proteins, although the possibility that N-*myc* gene suppression is one of the results led by G1 arresting process triggered by PGs is not excluded.

Recently, Δ^{12}-PGJ$_2$ is detected in human urine as a metabolite of PGD$_2$ [16], which raises the possibility that Δ^{12}-PGJ$_2$ acts on cellular proliferation in some physiological conditions.

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REFERENCES

[1] Narumiya, S. and Fukushima, M. (1985) Biochem. Biophys. Res. Commun. 127, 739–745.
[2] Ohno, K., Fujiwara, M., Fukushima, M. and Narumiya, S. (1986) Biochem. Biophys. Res. Commun. 139, 808–815.
[3] Narumiya, S., Ohno, K., Fukushima, M. and Fujiwara, M. (1987) J. Phamacol. Exp. Ther. 242, 306–311.

[4] Ohno, K., Sakai, T., Fukushima, M., Narumiya, S. and Fujiwara, M. (1988) J. Pharmacol. Exp. Ther. 245, 294–298.
[5] Ishioka, C., Kanamaru, R., Sato, T., Dei, T., Konishi, Y., Asamura, M. and Wakui, A. (1988) Cancer Res. 48, 2813–2818.
[6] Sekiguchi, M., Oota, T., Sakakibara, K., Inui, N. and Fujii, G. (1979) Japan J. Exp. Med. 49, 67–83.
[7] Dean, P.N. (1980) Cell Tissue Kinet. 13, 299–308.
[8] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[9] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, pp. 326–328. Cold Spring Harbor Laboratory, New York.
[10] Schwab, M., Alitalo, K., Klempnauer, K-H., Varmus, H.E., Bishop, J.M., Gilbert, F., Brodeur, G., Goldstein, M. and Trent, J. (1983) Nature 305, 245–248.
[11] Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., Rutter, W.J. and Kirschner, M.W. (1980) Cell 20, 95–105.
[12] Bhuyan, B.K., Adams, E.G., Badiner, G.J., Li, L.H. and Barden, K. (1986) Cancer Res. 46, 1688–1693.
[13] Fukushima, M., Kato, T., Ota, K., Arai, Y., Narumiya, S. and Hayaishi, O. (1982) Biochem. Biophys. Res. Commun. 109, 626–633.
[14] Honn, K.V. and Marnett, L.J. (1985) Biochem. Biophys. Res. Commun. 129, 34–40.
[15] Crampton, S.L., Adams, E.G., Kuentzel, S.L., Li, L.H., Badiner, G. and Bhuyan, B.K. (1984) Cancer Res. 44, 1796–1801.
[16] Hirata, Y., Hayashi, H., Ito, S., Kikawa, Y., Ishibashi, M., Sudo, M., Miyazaki, H., Fukushima, M., Narumiya, S. and Hayaishi, O. (1988) J. Biol. Chem. 263, 16619–16625.