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MODULATION OF PROTEIN KINASE C ACTIVITY DURING INHIBITION OF TUMOR CELL GROWTH BY IFN-β AND -γ

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SUMMARY: We investigated the effects of human interferon(IFN)-β and -γ on protein kinase C activity in human HEp-2 and KHm-14 tumor cells during IFN-induced inhibition of cell growth. Cytosolic protein kinase C activity in both cell lines was strikingly decreased following treatment with either IFN-β or -γ. In the particulate fraction, IFN-γ decreased protein kinase C activity within 1 hr but it reappeared after 24 hr, whereas IFN-β decreased the activity during the inhibition of cell growth. Furthermore, phorbol-12,13-dibutyrate(PDBu)-binding activity was altered in parallel with the changes in protein kinase C activity induced by the IFNs. In summary, we showed that IFN-β and -γ cause long-term modulation of protein kinase C activity in these cultured tumor cells.

Interferons(IFNs) are currently being tested as antitumor agents in humans(1). Human IFNs are divided into three classes:α, β and γ. The α and β types are both synthesized in response to virus infections, whereas IFN-γ is produced by T-lymphocytes following mitogenic or antigenic stimulation(2). Although all three IFNs have inhibitory effects on tumor cell growth, the mechanism(s) of action is not clear. Meanwhile, it is known that protein kinase C is essential in the intracellular transduction of extracellular signals for cell proliferation and function (3,4,5). Yap et al.(6,7) have recently reported that a rapid and transient increase in levels of diacylglycerol, the physiological activator of protein kinase C, occurs in human diploid fibroblasts and

Abbreviations : IFNs, interferons; ATP, adenosine-5'-triphosphate; PDBu, phorbol-12,13-dibutyrate ; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylen glycol bis-(β -aminoethyl ether) NNN'N'-tetraacetic acid.

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Daudi cells within 30 seconds of their exposure to IFN-β and -γ. However, Mehmet et al.(8) found that in Swiss 3T3 cells IFN-β does not activate protein kinase C even after 10 min although cell growth was inhibited. In those studies, short-term alterations in diacylglycerol and phosphorylation were examined, but the long-term effects of IFNs were not. We therefore investigated protein kinase C activity in human HEp-2 and KHm-14 tumor cells during cell growth inhibition induced by IFN-β and -γ.

MATERIALS AND METHODS

Materials and Chemicals

[γ-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]PDBu (15.8 Ci/mmol) were obtained from New England Nuclear. Phosphatidylserine was obtained from Avanti Polar-Lipid Inc.. Sigma Chemicals Co. supplied diolein and H1 histone (Type III-S). Leupeptin was purchased from Peptide Institute Inc., Japan. DEAE-cellulose (DE-52) was from Whatman. Phosphatidylserine-Affi-Gel was prepared by the method of Wise et al.(9). Human native IFN-β and recombinant IFN-γ were obtained from Toray Co.Ltd., Tokyo. Monoclonal antibodies against IFN-β (Seikagaku Kogyo Co. Ltd., Japan) and against IFN-γ (Y. Tsukui, unpublished) neutralized approximately 3×10^{4} U of IFN-β and 6.5×10^{5} U of IFN-γ, respectively.

Cell cultures

The human epipharyngioma cell line, Hep-2, and the human melanoma cell line, KHm-14 cells(10),were cultured in a 5% CO_2 incubator at 37°C in Eagle's minimum essential medium (Nissui Co., Japan) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum (General Scientific Laboratories). Cell growth inhibition tests were performed by seeding cells in the wells of 6-well tissue culture dishes at 2×10^{4} cells/well in 3 ml of medium. At given intervals the cells were removed by trypsinization and the number of viable cells was determined by trypan blue exclusion assay.

Preparation of cytosolic and particulate cell fractions

Cells ($2\text{-}10\times10^{5}$cells) were seeded in culture flasks (175 cm^2, Falcon) in 50 ml of medium. After they had adhered, they were treated with IFNs and incubated with them for various length of time at 37°C. Subconfluent IFN-treated and untreated cells were then scraped from the flasks using rubber policemen, were sonicated for 30 sec at 4°C in buffer A (20 mM Tris-HCl, pH7.5, 0.25M sucrose, 2mM EDTA, 10mM EGTA, 0.01% leupeptin, 2-mercaptoethanol) and were centrifuged at 100,000 g for 1 hr at 4°C. The supernatant was referred to as the cytosolic fraction. The pellet was rehomogenized in buffer A containing 1% Nonidet P-40, and was centrifuged at 30,000 g for 15 min at 4°C. The supernatant was referred to as the particulate fraction.

DEAE-cellulose chromatography

Cytosolic and particulate fractions were applied to a DE-52 column (0.9x1.8 cm) equilibrated with buffer A without 0.25M sucrose (buffer B). Protein kinase C was eluted with a linear (0-0.5M) NaCl gradient, its activity in HEp-2 and KHm-14 cell extracts eluting in the fractions containing 0.04-0.12 M NaCl and 0.06-0.15 M NaCl, respectively. In this study, enzyme preparations were eluted batchwise with 3 ml of 0.2M NaCl

in buffer B, according to the method described by Kraft and Anderson(11). This batch elution yielded more than 90% of the total protein kinase C detectable by salt gradient elution. Protein was measured using the method of Bradford(12).

Protein kinase C assay

Protein kinase C was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into H1 histone in the presence of Ca^{2+}, phospholipids and diolein, according to the method of Kikkawa et al.(13).

[^{3}H]PDBu-binding assay

[^{3}H]PDBu-binding activity was assayed by the method described by Hoshijima et al.(14), using phosphatidylserine-Affi-Gel.

RESULTS

The effects of IFN-β and -γ on the growth of HEp-2 and KHm-14 cells are shown in Fig.1. Both IFN-β and -γ inhibited the cell growth in a dose-dependent manner, inhibition reaching approximately 50 % when the cells were exposed to IFNs at a concentration of 1000 U/ml for 3 days.

Figure 2 shows the protein kinase C activity detected in the cytosolic and particulate fractions of HEp-2 and KHm-14 cells after 24-hr treatments with IFN-β and -γ. IFN-β strikingly decreased protein kinase C activity in both the cytosolic and particulate fractions in a dose-dependent manner. Monoclonal antibody against IFN-β greatly diminished this inhibition, but had no effect on enzyme activity in control cells. Meanwhile, when the tumor cells were exposed to IFN-γ for 24 hr, althouth protein kinase C activity was found in the particulate fraction, its cytosolic activity was drastically decreased. Monoclonal antibody against

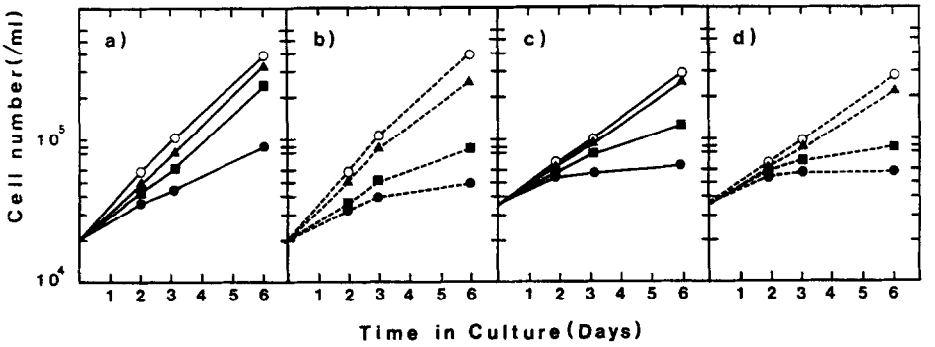

Figure 1. Growth curve of HEp-2 and KHm-14 cells treated with IFN-β and -γ. HEp-2(a,b) and KHm-14(c,d) cells were treated with IFN-β (—) and -γ (---) at the concentrations of 0(○), 10(▲), 100(■) and 1000(●) U/ml. The number of viable cells was determined by trypan blue exclusion assay. The figure represents the mean of three exeriments.

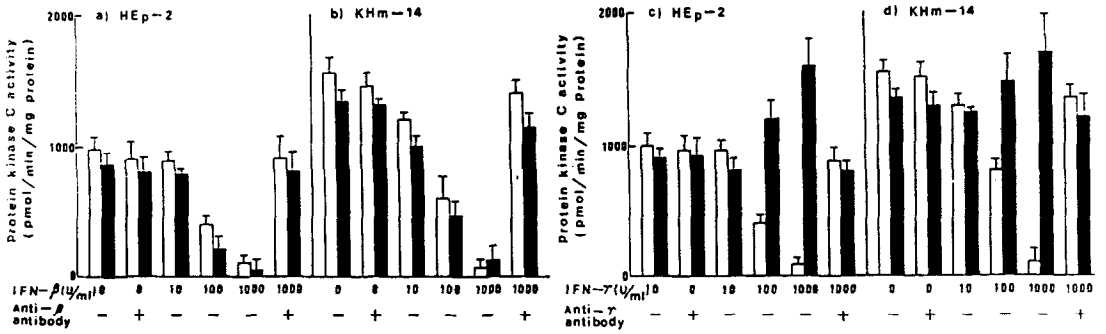

Figure 2. Dose response of the effects of IFN-β and -γ on protein kinase C activity in HEp-2 and KHm-14 cells after 24-hr treatments. HEp-2(a,c) and KHm-14(b,d) cells were treated with various concentrations of IFN-β (a,b) and -γ (c,d) for 24 hr at 37 °C. IFNs were preincubated with or without the monoclonal antibody for 1 hr before use. The cytosolic(□) and particulate(■) fractions in each cell were assayed for protein kinase C activity. The column represent the mean ± S.E.

IFN-γ eliminated the effects of IFN-γ on protein kinase C activity.

We next examined the time courses for the effects of IFN-β and-γ , both at a concentration of 1000 U/ml, on the tumor cells' protein kinase C activities. As shown in Fig.3, IFN-β diminished the activity in both

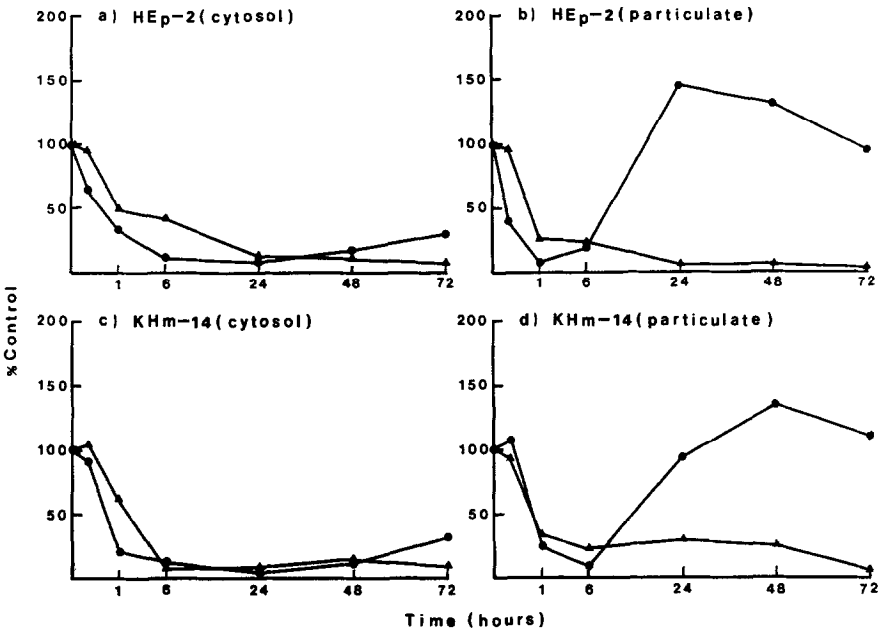

Figure 3. Time courses for the effects of IFN-β and -γ on protein kinase C activity in HEp-2 and KHm-14 cells. HEp-2(a,b) and KHm-14(c,d) cells were treated with 1000 U/ml of IFN-β (▲) and -γ (●) for various length of time at 37 °C. The cytosolic(a,c) and particulate(b,d) fractions in each cell were assayed for protein kinase C activity. The figure represents the mean of at least three experiments and all of the values of S.E. were less than 5 %.

fractions in a time-dependent manner to 24 hr, and the diminution continued to 72 hr. With IFN-γ , on the other hand,though it considerably depressed the activity in the particulate fraction within 1 hr,the enzyme reappeared after 24 hr.

Meanwhile, IFN-γ depressed the cytosolic activity for at least 72 hr. In addition, M-kinase, produced by hydrolysis by Ca^{2+}-dependent protease, and which is active even in the absence of $Ca^{2+}$and phospholipids, was not detected during the time course experiment if the enzyme preparation was eluted from the column with 0.5 M NaCl in buffer B (data not shown).

As it is known that phorbol esters, such as PDBu, bind to protein kinase C complexed with Ca^{2+} and phospholipids,the effects of the IFNs on [^{3}H] PDBU-binding activity was examined. As shown in Table 1, after 1-hr and 24-hr treatments both IFN-β and -γ diminished the [^{3}H] PDBu-binding activity in both fractions, except,that is,for binding in the particulate fraction after treatment with IFN-γ for 24 hr. Thus it was concluded that these IFNs modulate [^{3}H] PDBu-binding activity in parallel with the changes in protein kinase C activity.

Table 1. Effects of IFN-β and -γ on [^{3}H]PDBu-binding activity in HEp-2 and KHm-14 cells

Treatment (1000 U/ml)		[^{3}H] PDBu-binding activity (pmol/mg protein)			
		HEp-2		KHm-14	
		cytosolic	particulate	cytosolic	particulate
1 hr	Control	11.8 ± 0.3	12.8 ± 0.3	18.6 ± 0.2	16.4 ± 0.4
	IFN-β	8.5 ± 0.2	2.2 ± 0.1	6.6 ± 0.1	5.2 ± 0.2
	IFN-γ	1.3 ± 0.1	0.4 ± 0.1	0.9 ± 0.2	0.7 ± 0.2
24 hr	Control	12.2 ± 0.1	16.3 ± 0.2	18.5 ± 0.3	21.1 ± 0.2
	IFN-β	2.4 ± 0.1	1.1 ± 0.1	1.9 ± 0.2	2.2 ± 0.1
	IFN-γ	1.5 ± 0.2	18.2 ± 0.7	5.2 ± 0.2	17.3 ± 0.6

HEp-2 and KHm-14 cells were treated with 1000 U/ml of IFN-β and -γ for 1 hr or 24 hr at 37°C. The cytosolic and particulate fractions in each cell were assayed for [^{3}H]PDBu-binding activity. The data represent the mean ± S.E.

DISCUSSION

It is well known that IFNs inhibit the growth of a range of tumor cells, but the mechanism(s) of inhibition is not yet clear. The studies by Yap et al.(6,7) demonstrating a rapid rise in diacylglycerol levels following IFN treatment, suggest a correlation between IFN's effects on cell growth and protein kinase C activity. The lack of such a correlation in the studies of Mehmet et al.(8) led them to conclude that protein kinase C activation is not involved in IFN-induced inhibition of cell growth. In our present study, however, IFN-β clearly decreased protein kinase C activities in both cellular cytosolic and particulate fractions over a period of up to 72 hr. Meanwhile, it is known that the translocation of protein kinase C is induced within 1 hr by interleukin 2 and phorbol esters(15,16). In this study, IFN-γ also decreased the cytosolic protein kinase C activity, but the activity reappeared in the particulate fraction after 24 hr. However, it is difficult to conclude that IFN-γ caused the long-term translocation of protein kinase C,since it decreased the activities in both the cytosolic and particulate fractions within 1 hr.

It is also difficult to explain why the effects of IFN-γ on protein kinase C activity were different from those of IFN-β ,though the two are known to have some disparate effects, IFN-γ , for example, more strongly modulating HLA-DR antigen expression and immune regulatory activity than does IFN-β . Such differences may reflect the different effects observed in this study of IFN-β and -γ on protein kinase C activity in the particulate fraction.

Protein kinase C has been suggested to play an integral role in the proliferation of many cell types(3,4,5). The present study demonstrated IFN-β and -γ induce long-term modulation of protein kinase C activity in tumor cells, though whether this modulation of the enzyme is linked to IFN-induced inhibition of cell growth remains to be resolved.

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