Vol. 178, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1991 Pages 378-384

REDUCTION BY INTERFERON-α OF LEVELS OF c-myc PROTEIN AND DNA SYNTHESIS IN A HUMAN HEPATOMA CELL LINE MEDIATED BY INHIBITION OF PUTRESCINE SYNTHESIS

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Received May 29, 1991

Interferon-α inhibited increases in ornithine decarboxylase, intracellular putrescine, and DNA synthesis as human hepatoma cells were stimulated to grow. Interferon-α inhibited the increase in the c-myc protein level, but not its mRNA level. Added putrescine abrogated the effects on c-myc protein and DNA synthesis. Interferon-α seemed to inhibit the increase in the c-myc protein level post-transcriptionally by reducing the putrescine level, inhibiting DNA synthesis.

Interferons (IFNs) have many biological activities (1). IFNs inhibit cell growth through an unknown mechanism. IFN($\alpha + \beta$) inhibits the induction of ornithine decarboxylase (ODC) activity and DNA synthesis in mouse liver regenerating after partial hepatectomy, and the inhibition of DNA synthesis is prevented by the administration of putrescine (2). These results suggest that IFN suppresses liver cell growth by inhibiting putrescine synthesis. The increases in ODC activity and in the expression of the c-myc proto-oncogene, which codes for a nuclear protein essential for DNA synthesis (3,4), occur in parallel as cells are stimulated to grow (5). To evaluate potential interactions of IFN, polyamines, and c-myc protein, we assessed the effects of IFN-α on DNA synthesis and on the expression of ODC and c-myc protein.

Materials and Methods

Cells: Human hepatoma cells (HLF cell line) were obtained from The Japanese Cancer Research Resources Bank and maintained in Eagle's minimum essential medium containing 10% fetal calf serum. Cells (5 x $10^3/cm^2$) were

Abbreviations: IFN, Interferon; ODC, Ornithine decarboxylase; 2-5AS, 2',5'-oligoadenylate synthetase.

0006-291X/91 $1.50

seeded in plastic dishes (Falcon 3002) and cultured at 37℃ in a CO_2-incubator with air containing 5% CO_2 for 48 h. Then, the culture medium was removed and cells were incubated again with fresh medium containing IFN-α (10^3 IU/ml) or not.

Assay of intracellular levels of polyamines and ODC activity: Polyamines were extracted from cells (1×10^5) with 100 µl of 5% trichloroacetic acid. After centrifugation of the extract at 30,000 x g for 20 min, polyamines in the supernatant were separated by HPLC and measured as described previously (2). ODC activity was measured as the amount of [^{14}C]putrescine formed from L-[5-^{14}C]ornithine as described previously (6).

Assay of mRNA: RNA was extracted from cells by the guanidinium isothiocyanate/cesium chloride method (7). For Northern blotting, total RNA (20 µg) was treated by formamide agarose electrophoresis and transferred to Hybond N filters (Amersham). [^{32}P]-labelled DNA probes were prepared by a multiprime labeling system (Amersham). Northern blotting was performed as described before (6).

Assays of c-myc protein: Cells were fixed in 70% ethanol, incubated with sheep anti-myc antibody at room temperature for 1 h, and then incubated with rabbit anti-sheep immunoglobulin G (IgG) labelled with fluorescein isothiocyanate (FITC) at 4℃ for 30 min. After DNA of the cells was labelled with propidium iodide, the c-myc protein in the cells was measured by two-color flow cytometry (FACS 440, Becton Dickinson; 8), and Western blotting.

Assay of DNA synthesis: During the last 4 h of culture for 24 h, cells were labelled with [^{3}H]thymidine (1 µCi/ml) and DNA was extracted by the method of Schmidt and Thannhauser as modified by Schneider (9). The radioactivity incorporated into DNA was measured with a Beckman liquid scintillation counter. The amount of DNA was measured by the method of Burton (10).

Materials: Natural human IFN-α was supplied by Sumitomo Pharmaceutical Co. Antibodies for c-myc protein and FITC-labelled rabbit anti-sheep IgG were obtained from Cambridge Research Biochemicals Co. and from EY Laboratories, respectively. Plasmids carrying ODC cDNA and 2',5'-oligoadenylate synthetase (2-5AS) cDNA were the kind gift of Dr. C. Kahana (Weissman Institute of Science, Rehovot, Israel) and from Dr. M. Sokawa (Kyoto University of Industrial Art and Textile, Kyoto), respectively.

Results

Incubation with fresh medium caused increases in ODC activity; the enzyme activity increased biphasically, peaking at 8 and 24 h. When IFN-was added, the increase in ODC activity was reduced by 38% at 8 h and by 46% at 24 h compared with cells not so treated (Fig. 1).

Figure 2 shows the effect of IFN-α on gene expression in HLF cells. ODC mRNA increased in cells incubated with fresh medium. The increase was diminished by IFN-α . Conversely, 2-5AS mRNA was increased by IFN-α . c-myc mRNA increased when cells were incubated with fresh medium and was not decreased by IFN-α . The effect of IFN-α on the level of c-myc

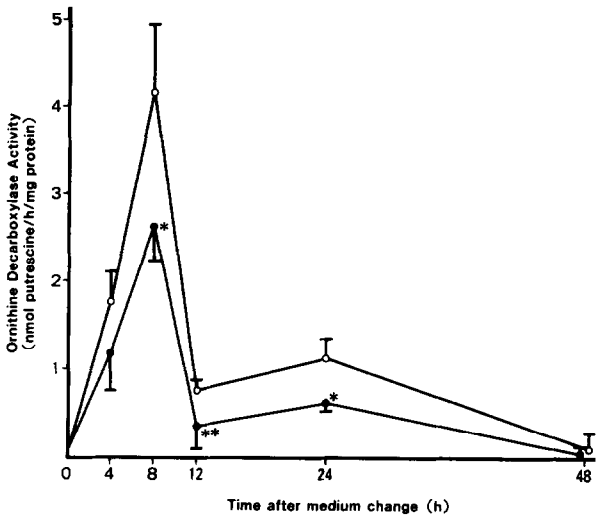

Fig. 1. Effect of IFN-α on ODC activity. Cells were incubated in the presence of fresh medium with (●) or without IFN-α (○). The ODC activity of the cells harvested at the times indicated was measured. The points and the vertical bars show the mean and SD of six experiments, respectively. *$p < 0.01$ and **$p < 0.05$.

protein seen by flow cytometry is shown in Fig. 3. The c-myc protein increased after the addition of fresh medium, peaking at around 24 h. IFN suppressed the increase in this protein. To test whether the reduced level of c-myc protein arose from a decrease in the putrescine level, we examined the effect of putrescine on the reduction of c-myc protein level caused by

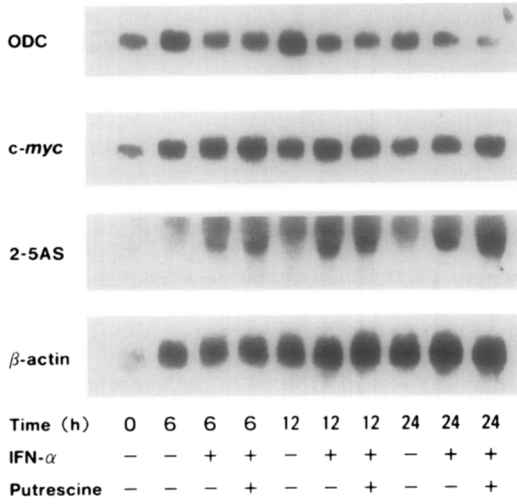

Fig. 2. Effects of IFN-α on gene expression. Total RNA was isolated from the cells at the times indicated. Then 20 μg of the RNA was electrophoresed, and mRNAs for ODC, c-myc, 2',5'-oligoadenylate synthetase (2-5AS), and β-actin were analyzed by Northern blotting.

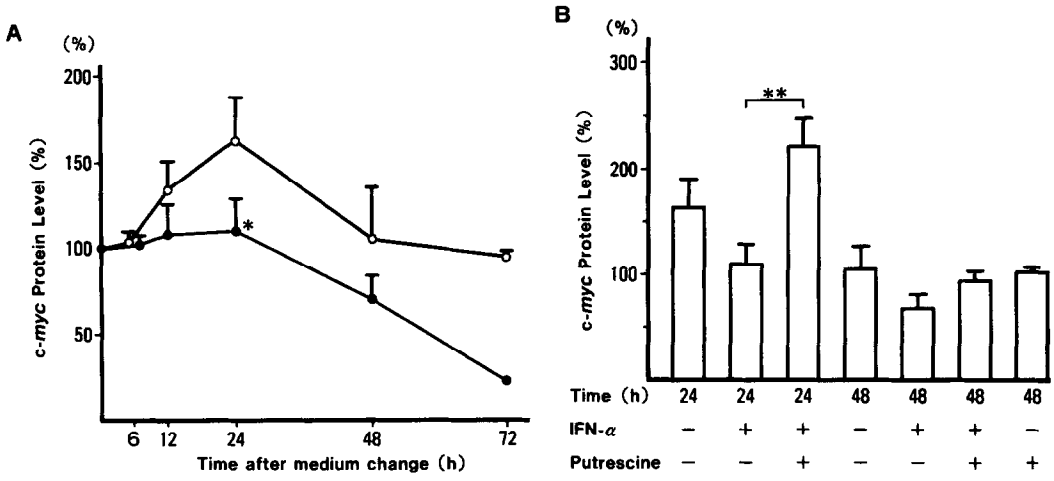

Fig. 3. Effects of IFN-α and putrescine on the c-myc protein level. Cells were harvested at the indicated times after incubation with fresh medium. The level of c-myc protein was measured by two-color flow cytometry and was taken to be 100% at time zero. Changes in c-myc protein of cells treated (●)and not treated (○) with IFN are shown in panel A and the effect of putrescine is shown in B. Putrescine (100 nM) was added at the same time as IFN-α . The points and the vertical bars show the mean and SD of triplicate experiments, respectively. *$p < 0.01$ and **$p < 0.05$ as compared with cells not treated with IFN.

IFN. When putrescine was added to the medium, the c-myc protein level did not decrease. By Western blotting also (data not shown), the increase in c-myc protein caused by the fresh medium was reduced by IFN-α , and the addition of putrescine abrogated this reduction.

To find whether inhibition of putrescine synthesis by IFN is related to its antiproliferative action, the effects of putrescine on DNA synthesis in cells treated with IFN-α were studied. In preliminary studies, incorporation of [^{3}H]thymidine into DNA was maximum when measured 24 h after replacement of the medium (data not shown). Such incorporation was inhibited by IFN-α , and the inhibition was prevented by putrescine (Fig. 4).

ODC activity was suppressed by IFN-α , and the IFN-induced reductions in c-myc expression and DNA synthesis were prevented by putrescine, so the reductions might be due to suppressed putrescine synthesis. To check this, we examined the effect of IFN-α on intracellular levels of putrescine, spermidine, and spermine. Levels of all three polyamines increased soon after cells were incubated with fresh medium. IFN-α significantly

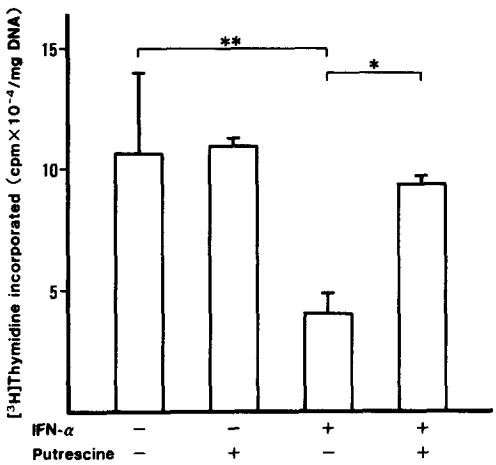

Fig. 4. Effects of IFN-α and putrescine on DNA synthesis. The incorporation of [^{3}H]thymidine into DNA was measured 24 h after replacement of the medium. Putrescine (100 nM) was added at the same time as IFN. The values and the vertical bars show the mean and SD of triplicate experiments. $^*p < 0.01$ and $^{**}p < 0.05$.

suppressed the increase in the putrescine level, but not the increases in spermidine or spermine. The addition of putrescine abrogated the suppression of the putrescine level (Table I).

Discussion

IFNs have antiproliferative effects on eukaryotic cells, but the extent of the effects depend on the cell lines used (11). The effects of IFNs on c-myc expression also differ. IFNs decrease the c-myc mRNA level in Daudi cells (12), but increase this level in chronic lymphocytic leukemia cells (13) and human malignant glioma cells (14); they do not affect this level in small cell lung cancer cells (11) or normal human fibroblasts (15). IFN-α did not affect the c-myc mRNA level in human hepatoma cells, but it decreased the c-myc protein level, probably post-transcriptionally.

Polyamine synthesis and c-myc gene expression are closely related to cell growth, so it is of interest to know whether polyamine synthesis is necessary for c-myc expression or vice versa (or neither). Dean et al.

Table I
Effects of IFN-α on intracellular levels of polyamines

Polyamine	Additions to medium	Polyamine levels (nmol/10^6 cells) Time after medium replacement(h) 0	4	8	12	24
Putrescine	None	0.25 ± 0.09	2.12 ± 0.51	4.94 ± 0.40	4.87 ± 0.40	4.62 ± 0.51
	IFN		1.47 ± 0.29	3.08 ± 0.19*	3.94 ± 0.48**	3.56 ± 0.46**
	IFN + putrescine		3.88 ± 0.51	5.60 ± 0.20	9.90 ± 0.24	9.93 ± 0.48
Spermidine	None	0.75 ± 0.10	1.87 ± 0.17	3.92 ± 0.79	4.19 ± 0.27	5.26 ± 0.25
	IFN		1.57 ± 0.10#	3.22 ± 0.16	3.88 ± 0.30	4.85 ± 1.19
	IFN + putrescine		1.95 ± 0.15	3.22 ± 0.21	3.77 ± 0.19	5.32 ± 0.28
Spermine	None	3.41 ± 0.18	3.97 ± 0.19	6.52 ± 0.61	5.75 ± 0.12	6.32 ± 0.21
	IFN		3.60 ± 0.04#	6.05 ± 0.37	5.91 ± 0.30	7.19 ± 0.17
	IFN + putrescine		4.22 ± 0.23	6.15 ± 0.84	5.78 ± 0.09	6.95 ± 0.24

IFN-α (10^3 IU/ml) and putrescine (100 nM) were added at the same time as fresh medium and cells were incubated for the times indicated. The cells were harvested and the levels of polyamines were measured. Values are the mean ± SD of quadruplicate experiments. *$p < 0.01$, **$p < 0.05$, and #$p < 0.1$ compared with cells not treated with IFN.

(16) reported that ODC gene expression comes to be constitutive when a myeloid cell line is infected by c-myc virus, suggesting that polyamine synthesis is dependent on c-myc expression. However, Celano et al. (17) reported that the decrease in the levels of polyamines caused by a ODC inhibitor, 2-difluoromethylornithine, inhibits c-myc gene transcription in human colon carcinoma cells. Our results also showed that putrescine is needed to increase the c-myc protein level by post-transcriptional regulation, suggesting that putrescine is important for c-myc protein synthesis. The contradictory results may have arisen from the difference in the cells used in the experiments. It is possible that IFN-α causes decrease in the putrescine level that occurs when ODC expression is inhibited, leading to the reduction in the c-myc protein level at the post-transcriptional stage, which in turn inhibits DNA synthesis.

Acknowledgments

We thank Ms. Yoshiko Okamoto, Ms. Yasuko Shimazu, and Ms. Hisako Fujimoto for technical assistance and Ms. Caroline Latta for critical reading of the manuscript.

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