Vol. 173, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1990 Pages 186-192

ANTIPROLIFERATIVE EFFECT OF INTERLEUKIN 1 (IL-1) ON TUMOR CELLS: G_0-G_1 ARREST OF A HUMAN MELANOMA CELL LINE BY IL-1

Yoshihiro Morinaga[1], Hidetoshi Hayashi[2], Akihiko Takeuchi[1] and Kikuo Onozaki[2*]

[1]Department of Microbiology, Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki 305, Japan

[2]Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho, Nagoya 467, Japan

Received October 7, 1990

Summary: Interleukin 1(IL-1) has been shown to have antiproliferative or cytocidal effects on several tumor cell lines and this effect is closely related to the induction of terminal differentiation of the target tumor cells. In this study we analyzed the antiproliferative effect of recombinant human IL-1α on a human melanoma cell line A375 in relation to cell cycle. Nutrient-starved cells, most of which were in $G_0 + G_1$, were stimulated by culturing in fresh medium, causing them to enter S. IL-1 treatment induced a slight decrease in the first cell cycle progression from $G_0 + G_1$ to S. In addition IL-1 retarded progression of the cells through G_2M and inhibited progression of the second cell cycle from $G_0 + G_1$ to S. Therefore we concluded that IL-1 exerts its antiproliferative effect by arresting the cells in $G_0 + G_1$.

Interleukin 1 (IL-1) is a 17 KDa protein that is produced by several cell types, but predominantly by macrophages/monocytes (1). IL-1 has multiple biological effects both in vitro and in vivo and is thought to be an important mediator in immunologic, inflammatory, hematopoietic and homeostatic reactions. One of the major functions of IL-1 is its regulatory effect on cell proliferation. IL-1 was first described as a co-mitogenic factor for lymphocytes (2). Currently IL-1 is known to stimulate the proliferation of many cell types including thymocytes (2), B cells (3), fibroblasts (4) and mesangial cells (5). Conversely, IL-1 has an antiproliferative or cytocidal effect on various tumor cell lines including a

* To whom all correspondence should be addressed.

Abbreviations used: IFN, interferon; IL, interleukin; Mn SOD, manganese superoxide dismutase,ODC, ornithine decarboxylase; TNF, tumor necrosis factor.

0006-291X/90 $1.50

human melanoma cell line (A375), several malignant human mammary cell lines, a human myeloid leukemic cell line (K562) and a mouse T lymphoma cell line (Eb)(6-8). Even normal cells, such as rat pancreatic Langerhans cells and mouse two cell embryos, appeared to be sensitive to the IL-1 antiproliferative or cytocidal effects (9,10). The rationale and mechanism of the antiproliferative effect has only been partially clarified.

The effect of IL-1 for A375 cells does not result from immediate cytolysis (6). Therefore, we speculated that the IL-1 effect was related to cell differentiation. This possibility appeared correct as it was demonstrated that a mouse myeloid leukemic cell line (M1) could be induced to differentiate into macrophage-like cells with a concomitant cessation in growth, when they were treated with IL-1 in conjunction with tumor necrosis factor (TNF), interferon (IFN) or IL-6 (11,12). In this paper we investigated the mode of action of IL-1 in relation to cell cycle by using a clone of A375 cells which is very sensitive to the IL-1 antiproliferative effect.

MATERIALS AND METHODS

Reagents: RPMI 1640 was purchased from Nissui Pharmaceutical Co. Ltd., Tokyo, Japan. Fetal calf serum (FCS) was obtained from Hyclone Laboratories (Logan, UT). Recombinant human IL-1α (2×10^7 U/mg based on thymocyte co-mitogenic activity) was provided by Dr. M. Yamada of Dainippon Pharmaceutical Co. Ltd., Osaka, Japan. Colcemid was from Nacalai Tesque Co. Ltd., Kyoto, Japan. Propidium iodide and RNase were from Sigma Chemical Co. (St. Louis, MO). [^{3}H]TdR (2 Ci/mmole) was from New England Nuclear (Boston, MA).

Cells and cell culture: An IL-1-sensitive human melanoma cell line clone (A375-C6) has been obtained by a limiting dilution method. A375-C6 cells were maintained in RPMI 1640 supplemented with 100 U/ml of Penicillin G, 100 µg/ml of Streptomycin, 15 mM HEPES, and 5% heat inactivated FCS. Cells were detached from culture plates with 0.05% trypsin - 0.02% EDTA. The cells were washed with culture medium and adjusted to 2×10^4 cells/ml. A total of 100 µl of the cell suspensions were added to each flat-bottomed well of a 96-well Microtiter plate (Falcon, Lincoln, NJ). Plates containing the cells were precultured for 2 days to allow complete recovery of cells from trypsinization. One hundred µl of medium containing IL-1 were then added to these cells.

Assay for cell proliferation: Cells were cultured for 3 days, and then pulsed with [^{3}H]TdR (0.5 µCi/well) for 4 hr. Radioactivity incorporated into cells was determined as described previously (3). Percent of growth was calculated as follows:

$$\% \text{ control} = \frac{\text{cpm in cells in test sample}}{\text{cpm in cells in medium alone}} \times 100.$$

Synchronized culture: Cells ($0.5 - 1 \times 10^6$) suspended in RPMI 1640 supplemented with 2.5% FCS in a tissue culture dish (100 mm diameter) were cultured at 37C for 5 days. More than 70% of the cells thus cultured appeared to be in $G_0 + G_1$ by flow cytometric analysis. After trypsinization, the cells (5×10^5 cells) were seeded in a tissue culture dish (60 mm diameter) and cultured in fresh medium supplemented with 5% FCS for an indicated period at 37C.

DNA staining and flow cytometric analysis: At each time point, cells were harvested by pipetting in ice cold phosphate buffered saline (PBS, pH 7.4) supplemented with 0.1 mM EDTA and 0.1% NaN_3. The cells were collected by centrifugation and washed with cold PBS. The cell pellet was resuspended in cold 70% ethanol/PBS for more than 30 min. After washed with cold PBS, the cells were treated with 200 µl RNase (1 mg/ml PBS) at 37C for 30 min. After centrifugation, the cells were treated with 2 ml propidium iodide (PI, 50 µg/ml PBS) at 4C in the dark for 1 hr. After washed with PBS, the stained cells were resuspended in PBS. Fluorescence of individual cells stained with PI was measured with a flow cytometer (FACScan, Becton Dickinson Immunocytometry System, Mountain View, CA) and the data was stored in the computer. The cell cycle analysis was conducted by the use of DNA CELL-CYCLE ANALYSIS SOFTWARE (Becton Dickinson Immunocytometry Systems). Under this method it was not possible to distinguish between G_0 and G_1 or G_2 and M, therefore we expressed $G_0 + G_1$ and G_2M, respectively.

RESULTS AND DISCUSSION

As reported previously, when exponentially growing A375-C6 cells were cultured for 3 days, proliferation of the cells was markedly inhibited by recombinant human IL-1α in a dose-dependent manner (Fig. 1). After 1 day culture, IL-1 exhibited little effect on cell proliferation. IL-1 inhibition of [^{3}H]TdR incorporation was detectable after 2 days and became more evident with time. To determine the antiproliferative effect of IL-1 on the cell cycle, the DNA histogram of cells cultured with or without IL-1 for

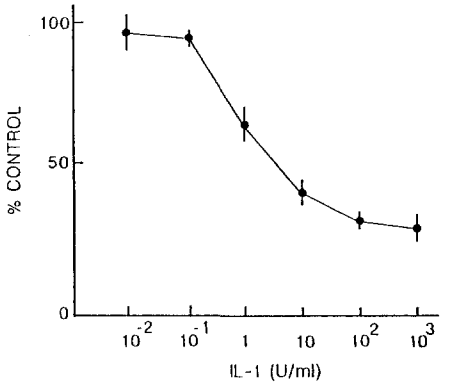


Figure 1. Effect of IL-1α on DNA synthesis in A375-C6 cells. Cells were cultured for 3 days with or without varying doses of IL-1α. Cells were pulsed with [^{3}H]TdR for 4 hr before harvest. Mean + S.E.M. of triplicate cultures is shown.

3 days were analyzed. In control cultures 60% of the cells were found to be in $G_0 + G_1$ and 40% were in $S + G_2M$. These values were not affected when the cells were treated with IL-1 (Fig. 2). The effect of a mitotic blocking agent on the cell cycle was also determined. When the cells were treated with colcemid for the last 18 hr of incubation, most of the cells were in $S + G_2M$. However, if the cells were treated with IL-1, the proportion of cells in $G_0 + G_1$ significantly increased. These results suggest that either IL-1 slows the progression of the cell cycle or IL-1 arrests the cells in a particular phase. To investigate the mode of action of IL-1 in more detail the cells were synchronized, and the IL-1 effect on the cells as a function of time was determined. Experiments were carried out three times, and representative data are shown in Figure 3. More than 70% of the synchronized cells were in $G_0 + G_1$. In control cultures, cells began to move into S after 12 hr in culture and the percentage in this phase peaked at 18 hr. The cells then moved into G_2M peaking at 24 hr. After 27-30 hr, the cells again moved into S. If the cells were treated with IL-1, the ratio of cells which moved from $G_0 + G_1$ into S decreased slightly without lengthening the time in S. The

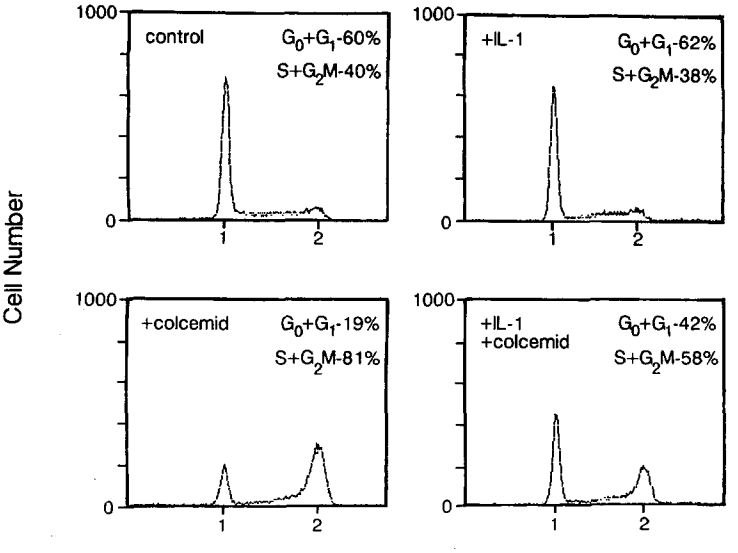
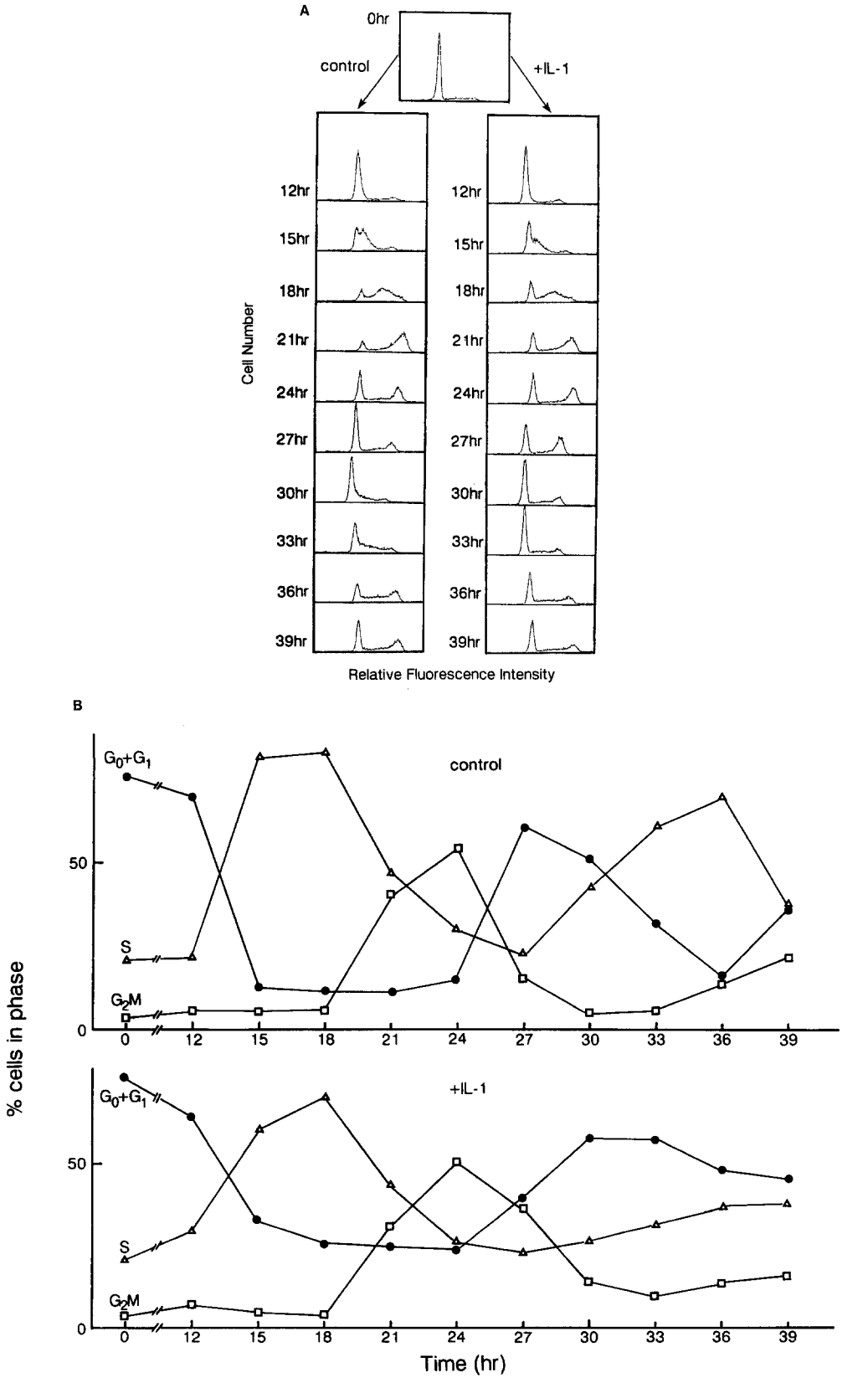


Figure 2. Cell cycle distribution after IL-1α and colcemid treatment of growing A375-C6 cells. Cells were plated at 1 x 10^5 cells per 60 mm dish and cultured for 3 days with or without IL-1α(100 U/ml). Colcemid (0.2 μg/ml) was added for the last 18 hr.


A
0hr
control
+IL-1
12hr
15hr
18hr
21hr
24hr
27hr
30hr
33hr
36hr
39hr
Cell Number
Relative Fluorescence Intensity
B
control
+IL-1
G_0+G_1
S
G_2M
% cells in phase
Time (hr)

peak of G_2M continued to appear at 24 hr. However, a prolongation of the period needed to move from G_2M to $G_0 + G_1$ accompanied by the delay of the peak of $G_0 + G_1$, and a marked inhibition of progression into the second S was noted. Similar results were obtained in all experiments. These results indicate that a major effect of IL-1 is to arrest cells in $G_0 + G_1$, thereby preventing cells from entering into second S. In contrast, the cell cycle was not altered by IL-1 in another colne A375-C5 which is resistant to IL-1 antiproliferative effect (13)(data not shown). Therefore the changes of the cell cycle appeared to be related to the antiproliferative effect of IL-1.

There are a number of cytokines which inhibit the proliferation of cells, these include IL-1, IL-6, TNF and IFN. The IFN effect in relation to cell cycle is heterogeneous, prolonging specific stages or several phases of the cell cycle (14). TNF is reported to lyse a mouse fibroblast cell line (LM) in M concomitant with a delay in cell progression through G_2 to M (15). The antiproliferative effect of IL-1 and IL-6 in relation to cell cycle has not yet been elucidated. In this study we demonstrated that a major effect of IL-1 is in preventing the cells from entering into S. It was of note that IL-1 does not affect cell movement into the first S. This effect is unique to IL-1 because such kind of inhibitory action is not reported in IFN and TNF (14,15). This also concurs with our previous finding that IL-1 effect is only noted after a minimum of one day culture (6). Early events which occur in A375-C6 cells in response to IL-1 are complex. IL-1 treatment of these cells for 6 to 8 hr induces mRNA for mitochondrial manganese superoxide dismutase (Mn SOD) and IL-6 (13,16). Since IL-1 also induces Mn SOD mRNA in fibroblasts which proliferate in response to IL-1(16), Mn SOD induction appeared to be unrelated to the antiproliferative effect of IL-1. Recently we have reported that induced IL-6 contributes to the antiproliferative effect of IL-1 by acting as an autocrine factor (13). At present it is not known how

Figure 3. Cell cycle transition of A375-C6 cells in the presence or absence of IL-1α. Nutrient-starved cells were stimulated at time 0 with fresh medium (5% FCS) in the presence or absence of IL-1α (100 U/ml). Samples were harvested at 3 hr intervals and analyzed by FCM. A. DNA histograms are shown. B. The proportions of cells in $G_0 + G_1$, S and G_2M are shown.

IL-6 contributes to the IL-1 effect in relation to the cell cycle. IL-1 treatment of A375-C6 cells for 12 hr inhibits the activity of ornithine decarboxylase (ODC)(17) which is a key enzyme in the synthesis of polyamines. A decrease in the levels of intracellular polyamines by an inhibitor of ODC is reported to result in a specific cell cycle arrest at G_1 or S, depending on the cell types (18). Thus, our finding may be related to a decrease in polyamine synthesis. IL-1 has antiproliferative or cytocidal effects on both tumor cells and normal cells. Our study is important in understanding the molecular basis of IL-1 effects in pathological and homeostatic reactions accompanying tissue or cell injury and differentiation.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. N. Tamura of University of Tsukuba for helping us to perform this study, and Dr. M. Snyder of Columbia University for review and help for preparing this manuscript. This work was supported in part by grants from the Ministry of Culture, Education and Science 01571233 and from Japan Rheumatoid Foundation.

REFERENCES

1. Oppenheim,J.J., Kovacs,E.J., Matsushima,K. and Durum,S.K. (1986) Immunol. Today 7:45-57.
2. Gery,I., Gershon,R. and Waksman,B.H. (1972) J. Exp. Med. 136:128-142.
3. Freedman,A.S., Freeman,G., Whitman,J., Segil,J., Dley,J. and Nadler,L.M. (1988) J. Immunol. 141:3398-3404.
4. Schmidt,J.A., Mizel,S.B., Cohen,D. and Green,I. (1982) J.Immunol. 128: 2177-2182.
5. Lovette,D.H., Szamel,M., Ryan,J.L., Sterzel,R.B., Gemsa,D. an Resch,K. (1986) J. Immunol. 136:3700-3705.
6. Onozaki,K., Matsushima,K., Aggarwal,B.B. and Oppenheim,J.J. (1985) J. Immunol. 135:3962-3968.
7. Gaffney,E.V. and Tsai,S.C. (1986) Cancer Res. 46:3834-3837.
8. Lovette,D., Kozan,B., Hadman,M., Resch,K. and Gemsa,D. (1986) J.Immunol. 136:340-347.
9. Bendtzen,K., Mandrup-Poulsen,T., Nerup,J., Nielsen,J.H., Dinarello,C.A. and Svenson,M. (1986) Science 232:1545-1547.
10. Fakih,H., Baggett,B., Holtz,G., Tsang,K., Lee,J.C. and Williamson,H.O. (1987) Fertil. Steril. 47:213-217.
11. Onozaki,K., Urawa,H., Tamatani,T., Iwamura,Y., Hashimoto,T., Baba,T., Suzuki,H., Yamada,M., Yamamoto,S., Oppenheim,J.J. and Matsushima,K. (1988) J. Immunol. 140:112-119.
12. Onozaki,K., Akiyama,Y., Okano,A., Hirano,T., Kishimoto,T., Hashimoto,T., Yoshizawa,K. and Taniyama,T. (1989) Cancer Res. 49:3602-3607.
13. Morinaga,Y., Suzuki,H., Takatsuki,F., Akiyama,Y., Taniyama,T., Matsushima,K., and Onozaki,K. (1989) J.Immunol. 143:3538-3542.
14. Lundblad,D. and Lumdgren,E. (1981) Int. J. Cancer 27:749-754.
15. Darzynkiewicz,Z., Williamson,B., Carswell,E.A. and Old,L.J. (1984) Cancer Res. 44:83-90.
16. Masuda,A., Longo,D.L., Kobayashi,Y., Appella,E., Oppenheim,J.J. and Matsushima,K. (1988) FASEB J. 2:3087-3091.
17. Endo,Y., Matsushima,K., Onozaki,K. and Oppenheim,J.J. (1988) J. Immunol. 141:2342-2348.
18. Sunkara,P.S., Fowler,S.K. and Nishioka. (1981) Cell Biol. Int. Rep. 5: 991-997.