Vol. 130, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1985 Pages 257-263

# MICROINJECTION OF MACROMOLECULES INTO LEUKEMIC CELLS BY CELL FUSION TECHNIQUE : SEARCH FOR INTRACELLULAR GROWTH-SUPPRESSIVE FACTORS

Keiya Ozawa*, Takayuki Hosoi, Chao-Jung Tsao, Akio Urabe, Tsuyoshi Uchida** and Fumimaro Takaku

The Third Department of Internal Medicine, University of Tokvo, Tokyo 113, Japan

**Institute for Molecular and Cellular Biology, Osaka University, Osaka 565, Japan

Received May 31, 1985

To investigate the intracellular molecular events during leukemic cell proliferation, we have examined the method of ghost-mediated microinjection of macromolecules into leukemic cell line cells (HL-60). Samples were packed into red cell ghosts. Micro-injection was performed by the fusion of ghosts and HL-60 cells using the hemagglutinating virus of Japan (HVJ). Fusion rate was about 80-90%, when determined by the injection of FITC-labeled globulins (IgG) or diphtheria toxin fragment A into HL-60 cells. When the nuclear protein extract from normal granulocytes was injected into HL-60 cells, their growth was significantly suppressed. The injection of the nuclear protein extract from HL-60 itself into HL-60 cells did not inhibit their growth. This finding suggests that leukemic cells may be deficient in intracellular regulatory factors which have suppressive activity on cell growth. 

In contrast to the limited proliferative capacity of normal granulocytes, leukemic cells proliferate in an unregulated fashion without normal differentiation. This may be due to some abnormality in intracellular control mechanisms of gene expression. Micro-injection of gene products or purified antibodies against them into viable cells would be a valuable tool for the study of intracellular molecular events during leukemic cell proliferation and differentia-

* Present address : Cell Biology Section, Clinical Hematology Branch, NHLBI, National Institute of Health, Bethesda, Maryland 20205, USA.

Abbreviations : HL-60, a human promyelocytic leukemia cell line; HVJ, hemagglutinating virus of Japan; PBS, phosphate-buffered saline; rPBS, reverse PBS; BSS-Ca, balanced salt solution supplemented with $CaCl_2$; FITC, fluorescein isothiocyanate; RSB, reticulocyte saline buffer; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; HAU, hemagglutinin unit.

0006-291X/85 $1.50

tion (1-4). In the present study, we have examined the basic conditions of ghost-mediated microinjection of macromolecules into leukemic cell line cells (HL-60). Fusion of ghosts and HL-60 cells was performed with high efficiency by using the hemagglutinating virus of Japan (HVJ). This method was applied to explore the intracellular factors regulating the proliferation of myeloid cells.

## MATERIALS AND METHODS

Packing of macromolecules into red cell ghosts: The procedure was based on a method described by Yamaizumi et al. (5) with minor modifications. Human red blood cells (RBC) were washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4$, 1 mM $KH_2PO_4$) and twice with reverse PBS (rPBS) (3mM NaCl, 137 mM KCl, 8 mM $Na_2HPO_4$, 1 mM $KH_2PO_4$, 4 mM $MgCl_2$). Mixtures of 1 vol of packed RBC (50-200 µl) and 4 vol of rPBS containing samples (200-800 µl) were dialysed against 6-fold diluted rPBS for 30 min at 4°C. RBC were resealed by dialyzing against isotonic PBS for 30 min at 37°C. RBC ghosts containing samples were then washed twice with PBS and twice with balanced salt solution supplemented with $CaCl_2$ (BSS-Ca; 137 mM NaCl, 5.4 mM KCl, 0.34 mM $Na_2HPO_4$, 0.44 mM $KH_2PO_4$, 13 mM Tris-HCl, 2 mM $CaCl_2$).

Microinjection by cell fusion: Samples were injected into HL-60 cells by the fusion of ghosts containing samples and HL-60 cells using HVJ (5). HL-60 cells cultured in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum (Flow Laboratories. Inc., McLean, VA) were washed once with PBS and twice with BSS-Ca. HVJ was partially purified from embryonated eggs and titrated using chick red blood cells (6). It was inactivated by ultraviolet (UV) irradiation (100 erg/sec/$mm^2$) for 3 min just before use and was diluted with BSS-Ca. Mixtures of 0.2 ml of 15% (V/V) ghost suspension, 0.2 ml of HL-60 suspension and 0.4 ml of UV-inactivated HVJ were incubated at 4°C for 15 min and then at 37°C for 30 min with shaking. These fusion experiments were performed at various concentrations of HL-60 cells and HVJ. Free ghosts were removed from the fusion products by density centrifugation on fetal calf serum cushion. The efficiency of microinjection was determined by the introduction of fluorescein isothiocyanate (FITC)-labeled globlins (IgG 5 mg/ml; Cappel Worthington Biochemicals, Malvern, PA) or fragment A of diphtheria toxin (10 µg/ml) (E.Y. Laboratories, Inc., San Mateo, CA), which can exert its cytotoxicity only in the intracellular space (7). Proportion of HL-60 cells containing FITC-IgG in the cytoplasm was examined using a fluorescence microscope. Cytotoxic effect of diphtheria toxin fragment A was examined by counting the viable cells using a trypan blue dye exclusion method after the culture of 24 hrs.

Preparation of nuclear protein extracts: Peripheral blood from normal human volunteers was separated by Ficoll-Metrizoate (Lymphoprep; Nyegaard, Oslo, Norway) density centrifugation at 400 x g. Granulocytes were obtained from high-density fraction after the lysis of contaminated red cells using ammonium chloride solution (0.13 M $NH_4Cl$, 0.017 M Tris base, 0.01 M $KHCO_3$). Normal granulocytes and HL-60 cells were washed twice with PBS and were

suspended in RSB buffer (10 mM NaCl, 15 mM $MgCl_2$, 10 mM Tris-HCl) containing 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, MO), $5 \times 10^{-3}$ M 2-mercaptoethanol (Sigma) and Triton X-100 (0.5% for granulocytes and 0.1% for HL-60 cells) (Wako Chemicals, Tokyo, Japan). The cells were then homogenized with a Dounce homogenizer (pestle A, 50 strokes). Homogenates were centrifuged at 400 x g for 10 min, and nuclear pellets were rinsed twice with 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM PMSF, Triton X-100 (0.5% for granulocytes and 0.1% for HL-60 cells) and twice with 0.25 M sucrose, 5 mM $MgCl_2$, 1 mM PMSF. Purity of nuclei was examined with a phase contrast microscopy. Washed nuclear pellets were suspended in nuclear buffer (70 mM NaCl, 25 mM EDTA, pH 7.6) containing 1 mM PMSF at a concentration of 20% (V/V), and were disrupted by sonication. Nuclear protein extracts were separated by centrifugation at 12,000 x g for 15 min and dialyzed against rPBS. These samples were stored at -70°C until use in microinjection experiments. Bovine serum albumin (BSA; Sigma) solution (1 mg/ml in rPBS) was used as a control.

## RESULTS

### Microinjection of FITC-labeled IgG into HL-60 cells

To determine the efficiency of microinjection, FITC-labeled IgG was loaded into red cell ghosts and injected into HL-60 cells by the fusion of ghosts and HL-60 cells at various concentrations of UV-inactivated HVJ (Figure 1). About 80% of HL-60 cells contained FITC-labeled IgG after the fusion at 500 and 1000 hemagglutinin

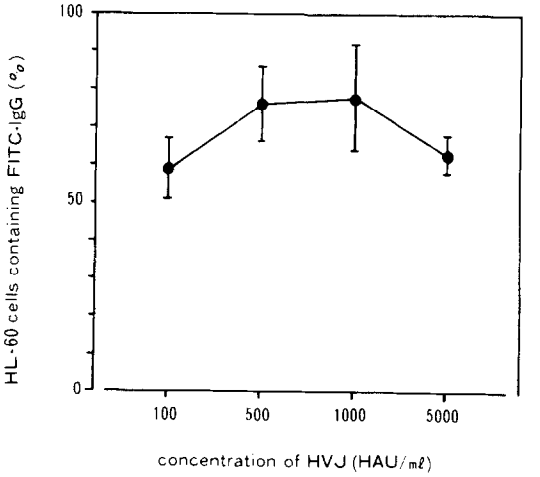


Figure 1. Microinjection of FITC-IgG molecules into HL-60 cells at different concentrations of HVJ. HL-60 cells ($5 \times 10^5$/ml) were fused with ghosts containing FITC-labeled IgG using HVJ as a fusogenic agent. After fusion, HL-60 cells containing FITC-IgG in the cytoplasm were counted using a fluorescence microscope. Mean ± S.D.

Table 1. Fusion reaction at different concentrations of HL-60 cells

| Concentrations of HL-60 (No. of cells/ml) | HL-60 cells containing FITC-IgG (%)* | HL-60 cells containing 2 or more nuclei (%)** |
|---|---|---|
| $0.25 \times 10^6$ | 82 ± 8 | 3.5 ± 1.5 |
| $0.5 \times 10^6$ | 83 ± 11 | 3 ± 1 |
| $1.0 \times 10^6$ | 79 ± 13 | 8.5 ± 1.5 |
| $2.5 \times 10^6$ | 81 ± 6 | 13.5 ± 2.5 |

HL-60 cells were fused with ghosts containing FITC-labeled IgG using HVJ (1000 HAU/ml).
*HL-60 cells containing FITC-IgG in the cytoplasm were counted using a fluorescence microscope. Mean ± S.D.
**HL-60 cells containing 2 or more nuclei were counted using a light microscope after Wright-Giemsa staining of cytocentrifuge preparations. Mean ± S.D.

units (HAU)/ml of HVJ. At the lower (100 HAU/ml) and higher (5000 HAU/ml) concentrations of HVJ, fusion rates slightly decreased. Almost equal efficiency of microinjection was obtained at various concentrations of HL-60 cells in fusion reaction mixtures. However, at higher concentrations of HL-60 cells, the proportion of HL-60 cells containing two or more nuclei increased, which means that HL-60 x HL-60 fusion occurred (Table 1). At $5.0 \times 10^5$ cells/ml or lower, the proportion of multinucleated HL-60 cells was same as that of untreated HL-60 cells. Therefore, the following fusion experiments were performed at 1000 HAU/ml of HVJ and at 2.5 - 5.0 $\times 10^5$ cells/ml of HL-60 cells. Almost all fused cells ( > 95%) were viable as determined by dye exclusion tests.

Microinjection of nuclear protein extracts from normal granulocytes into HL-60 cells

Nuclear protein extracts from normal granulocytes and from HL-60 cells were loaded into red cell ghosts and then injected into HL-60 cells. Their effects on HL-60 growth were investigated by short-term cultures of 24 hrs (Figure 2). As a negative control, ghosts containing BSA were fused with HL-60 cells. The growth of HL-60 cells injected with BSA was slightly suppressed compared with that of untreated HL-60 cells. When diphtheria toxin fragment

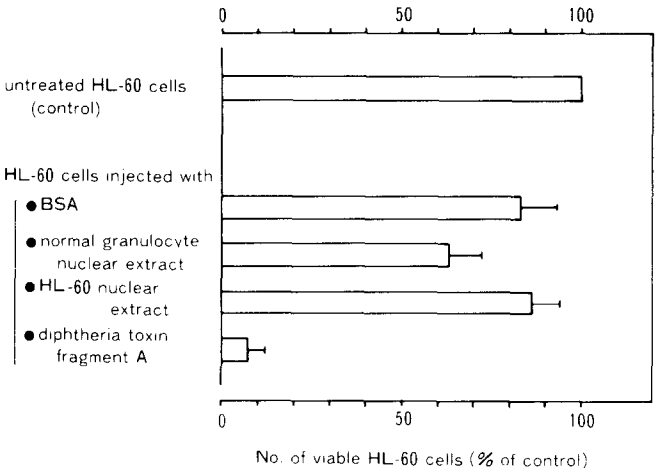


Figure 2. Effects of microinjection of nuclear proteins into HL-60 cells on their growth. HL-60 cells were fused with ghosts containing BSA, normal granulocyte-derived nuclear protein extract, HL-60-derived nuclear protein extract or diphtheria toxin fragment A, using HVJ (1000 HAU/ml). After the culture of 24 hrs, viable HL-60 cells were counted by a dye exclusion method. Results are expressed as means ± S.D. of five experiments. The injection of the granulocyte-nuclear extract suppressed HL-60 growth significantly ($p < 0.01$) compared with that of HL-60 nuclear extract.

A, a positive control, was injected, the number of viable HL-60 cells were less than 10% of that of untreated HL-60 cells and approximately 10% of that of HL-60 cells injected with BSA. This means that the efficiency of microinjection was about 90%. This figure was confirmed in the experiment of colony formation by HL-60 cells in semi-solid agar cultures (data not shown). The effect of injection of nuclear protein extract from HL-60 itself into HL-60 cells was similar to that of BSA. However, the injection of normal granulocyte-derived nuclear protein extract into HL-60 cells caused slight but significant ($p < 0.01$) suppression of their growth, compared with that of HL-60 nuclear extract.

## DISCUSSION

Microinjection of biologically active macromolecules into viable cells seems to be a powerful tool to study complex biologic phenomena that can not be approached with conventional *in vitro* systems (1-4,

8-10). Among various microinjection methods, the ghost-mediated method is most suitable for the microinjection of protein molecules into suspension cells, although it is difficult to inject DNA or RNA into cells by this method (8-10). In the present study, we demonstrated that protein molecules could be microinjected into leukemic cell line cells (HL-60) through fusion with red cell ghosts containing the samples. Very high efficiency of microinjection was obtained under an optimal experimental condition; i.e. about 80-90% of HL-60 cells were injected with samples. Because of a low cytotoxicity of UV-inactivated HVJ, the viability of fused HL-60 cells was very high. Moreover, microinjected cells retained the capacity to form colonies in semi-solid agar cultures (data not shown).

We have applied this method to investigate the abnormality of leukemic cell growth. It has already been demonstrated that nuclear proteins injected into cytoplasm rapidly transfer and localize to the cell nucleus following microinjection (11,12). Therefore, the ghost-mediated cell fusion technique seems to be efficient for the study of biological role of nuclear proteins. The present study showed that the injection of normal granulocyte-derived nuclear protein extract into HL-60 cells caused significant suppression of their growth. On the other hand, the injection of nuclear protein extract from HL-60 itself into HL-60 cells did not inhibit their growth. Although additional experiments will be required, this study suggests the possibility that leukemic cells are deficient in nuclear regulatory factors which have suppressive activity on cell growth. The identify of these factors is currently under investigation.

Recently the biological role of transformation-related protein p53 and ras oncogene product has been clarified using the glass-capillary microinjection method (1,3,4). However, the glass-

capillary method is difficult to apply for suspension cells. Moreover, it is impossible to treat many cells with this technique. Our method will enable us to investigate the biological role of oncogene products in leukemic cells. Biochemical analysis will also be possible after the microinjection, because a large number of cells can be treated by this ghost-mediated microinjection method.

## ACKNOWLEDGMENT

The authors would like to thank Miss Akiko Toyozumi for her excellent technical assistance and Mrs. Masami Ozawa for typing the manuscript. This study was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare and from the Ministry of Education, Science, and Culture of Japan.

## REFERENCES

1. Mercer, W. E., Nelson, D., DeLeo, A. B., Old, L. J., and Baserga, R. (1982) Proc. Natl. Acad. Sci. USA 79, 6309-6312.
2. Einck, L., and Bustin, M. (1983) Proc. Natl. Acad. Sci. USA 80, 6735-6739.
3. Stacey, D. W., and Kung, H. F. (1984) Nature 310, 508-511.
4. Mulcahy, L. S., Smith, M. R., and Stacey, D. W. (1985) Nature 313,241-243.
5. Yamaizumi, M., Furusawa, M., Uchida, T., Nishimura, T., and Okada, Y. (1978) Cell Struc. Funct. 3, 293-304.
6. Okada, Y., and Murayama, F. (1966) Exp. Cell Res. 44, 527-551.
7. Yamaizumi, M., Mekada, E., Uchida, T., and Okada, Y. (1978) Cell 15, 245-250.
8. Ohara, J., Sugi, M., Fujimoto, M., and Watanabe, T. (1982) J. Immunol. 129, 1227-1232.
9. Kishimoto, T., Miki, Y., Kishi, H., Muraguchi, A., Kishimoto, S., and Yamamoto, Y. (1982) J. Immunol. 129, 1367-1371.
10. Eda, Y., Ohara, J., and Watanabe, T. (1983) J. Immunol. 131, 1294-1299.
11. Yamaizumi, M., Uchida, T., Okada, Y., Furusawa, M., and Mitsui, H. (1978) Nature 273, 782-784.
12. Krippl, B., Ferguson, R., Rosenberg, M., and Westphal, H (1984) Proc. Natl. Acad. Sci. USA 81, 6988-6992.