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INTRODUCTION OF MACROMOLECULES INTO HEMOPOIETIC STEM CELLS WITH AN ERYTHROCYTE-GHOST-MEDIATED SYSTEM

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We have developed a method of introduction of macromolecules into normal human hemopoietic stem cells. The erythrocyte ghosts were loaded with diphtheria toxin fragment A (molecular weight=22,000 daltons), which exerts cytotoxicity only in the intracellular space. Granulocyte-macrophage colonies of human bone marrow cells incubated with the above ghosts in the presence of Sendai virus decreased in number to about 10% of the control. This means that the cell fusion and the subsequent introduction of the fragment A into granulocyte-macrophage progenitors occured at a high incidence (about 90%). This method will be useful to study intracellular events during the proliferation and differentiation of hemopoietic stem cells. © 1985 Academic Press, Inc.

Many factors have been reported which act on hemopoietic cells in an extracellular fashion and affect their proliferation and/or differentiation. However, intracellular growth regulatory mechanisms of hemopoietic cells have not been fully investigated. Microinjection of macromolecules, especially genes or purified gene products, seems to be one of the most reliable tools to study the molecular mechanisms of hemopoietic cell biology. In the present study, we have tried to introduce macromolecules into normal human hemopoietic stem cells efficiently with an erythrocyte-ghost-mediated system using Sendai virus (hemagglutinating virus of Japan, HVJ) as a fusogenic factor (1). We have injected diphtheria toxin fragment A to assess the efficiency of microinjection. Because the fragment A can exert its

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cytotoxicity only in the intracellular space (2), the hemopoietic stem cells fused with the ghosts containing the fragment A can not form colonies in the semi-solid cultures, and we can evaluate the efficiency of cell fusion and subsequent microinjection with the decrease of colony numbers.

MATERIALS AND METHODS

Erythrocyte ghosts containing diphtheria toxin fragment A: Diphtheria toxin fragment A (molecular weight=22,000 daltons, E.Y. laboratories, Inc., San Mateo, CA) was packed into erythrocyte ghosts according to the method of Yamaizumi et al. (2). The concentrations of the fragment A in the ghosts which were shown to be almost the same as that in the dialysis bag (2) were 5 or 50 μg/ml. The ghosts were resuspended at 20%(v/v) in balanced salt solution with calcium (BSS-Ca; 137mM NaCl, 5.4mM KCl, 0.44mM KH_2PO_4, 0.34mM Na_2HPO_4, 13mM Tris, 2mM $CaCl_2$). Control ghosts were prepared in the same way without fragment A.

Sendai virus (hemagglutinating virus of Japan, HVJ): A chicken chorioallantoic fluid (CAF) in which HVJ had been propagated was a source of HVJ. The virus particles were purified from CAF with ultracentrifugation. The hemagglutinating units (HAU) of this purified preparation were determined with chicken red blood cells according to Toister et al. (3). The usual titer of purified HVJ preparation was from 10,000 to 25,000 HAU/ml. The viruses were inactivated by ultraviolet irradiation (100 erg/sec/mm^2) for 3 minutes just before the fusion experiments

Human bone marrow mononuclear cells: Bone marrow specimens obtained from healthy adult volunteers with informed consent were spun on Ficoll-Metrizoate solution (Lymphoprep, Neygaard, Oslo, Norway, density=1.077 g/ml) at 400 x g for 20 minutes. The light density cells at the interface were collected and washed twice with phosphate-buffered solution and twice with BSS-Ca. The concentration of the mononuclear cells was adjusted to 1-8 x 10^6 cells/ml in BSS-Ca.

Cell fusion mediated by HVJ: Bone marrow mononuclear cells were fused with erythrocyte ghosts using HVJ as a fusogenic factor. A mixture of 1 part of bone marrow mononuclear cells, 1 part of erythrocyte ghosts and 2 parts of HVJ, all of which were suspended in BSS-Ca, were incubated on ice for 15 minutes and then at 37°C for 30 minutes with gentle shaking. Free ghosts were separated from the fusion products by centrifugation of the mixture at 200 x g for 4 minutes, and then the cell pellet was layered over 1 ml of fetal calf serum (FCS, Flow Laboratories, Inc., McLean, VA) and centrifuged under the same condition. After repeating this procedure 2-3 times, the bone marrow mononuclear cells were used for the colony assay.

Colony assay for granulocyte-macrophage progenitors (CFU-C): The colony assay for CFU-C of each bone marrow mononuclear cell preparation was done in single-layer soft agar according to the method of Robinson et al. (4) with minor modifications. The bone marrow mononuclear cells obtained after the above cell-fusion procedure were seeded at a concentration of 2 x 10^4 cells/ml in alpha medium (Flow) containing 0.3% bacto agar (Difco Laboratories, Detroit, MI), 20%(v/v) FCS and 10%(v/v) colony-stimulating factor (CSF) solution in 35-mm petri dish (Falcon 1008, Becton Dikinson & Co., Conkeyville, MD). We used GCT-CM (Grand Island Biological Co., Grand Island,

NY) as a source of CSF. The dishes were incubated for 7 days at 37°C in a humidified atmosphere with 5% CO_2 in air and then colonies consisting of 40 or more cells were counted using an inverted microscope.

RESULTS

As shown in Figure 1, the number of colonies derived from human bone marrow CFU-C decreased by the incubation of bone marrow cells with erythrocyte ghosts containing diphtheria toxin fragment A in the presence of HVJ. Although the colony formation from bone marrow cells incubated with control ghosts in the presence of HVJ was also reduced to about 60% of control cultures without HVJ, the extent of this decrease did not change at three different concentrations of HVJ (Figure 2). On the other hand, the decrease of colony formation from the bone marrow cells incubated with erythrocyte ghosts containing fragment A was more significant at higher concentrations

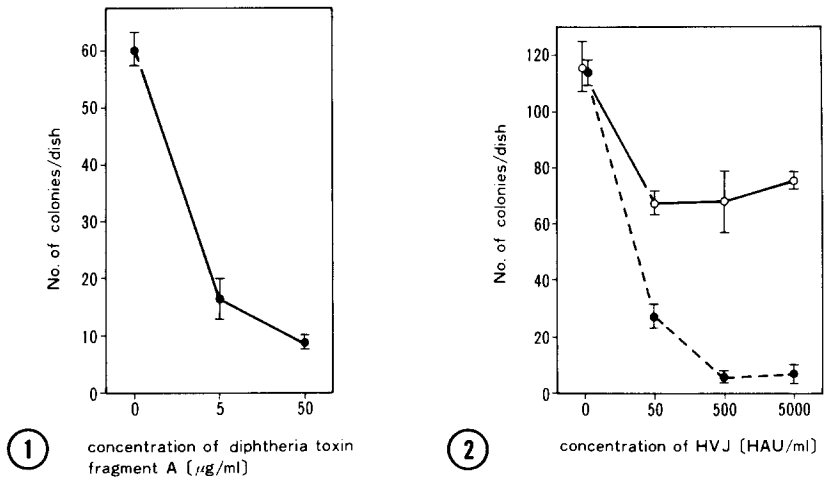
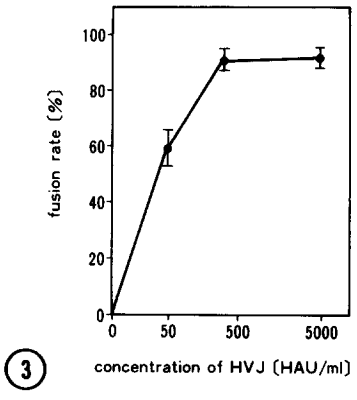


Figure 1. Effects of microinjection of diphtheria toxin fragment A into CFU-C on their growth. Bone marrow mononuclear cells were fused with erythrocyte ghosts containing different concentrations of fragment A using HVJ (500 HAU/ml) and then cultured for CFU-C assay. Mean ± S.D. of triplicate cultures.

Figure 2. Microinjection of diphtheria toxin fragment A into CFU-C at different concentrations of HVJ. Bone marrow mononuclear cells were fused with erythrocyte ghosts containing (●---●) or not containing (o—o) diphtheria toxin fragment A (50 μg/ml) using various concentrations of HVJ and then cultured for CFU-C assay. Mean ± S.D. of triplicate cultures.



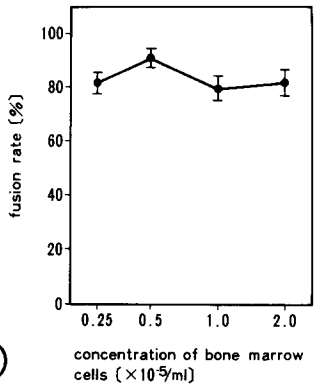


Figure 3. Effects of various concentrations of HVJ on the rate of fusion between CFU-C and erythrocyte ghosts. Fusion rate was calculated as written in "RESULTS" on the basis of the data in Figure 2. Mean ± S.D. of triplicate cultures.

Figure 4. Relationship between concentration of bone marrow mononuclear cells and fusion rate. Various concentrations of bone marrow mononuclear cells were incubated with erythrocyte ghosts containing or not containing diphtheria toxin fragment A (50 μg/ml) in the presence of HVJ (500 HAU/ml), and they were cultured for CFU-C assay. The fusion rate was calculated as written in "RESULTS". Mean ± S.D. of triplicate cultures.

of HVJ. These results suggest that cell fusion and subsequent microinjection of fragment A into bone marrow CFU-C occurred very efficiently.

We evaluated the efficiency of microinjection into CFU-C by calculating the "fusion rate" as follows; fusion rate = $(N_C - N_O)/N_C$ x 100 (%), where N_C equals the number of colonies from bone marrow cells incubated with control ghosts and N_O equals the number of observed colonies from bone marrow cells treated with ghosts containing diphtheria toxin fragment A. As Figure 3 demonstrates, we obtained about a 90% fusion rate with several hundred HAU/ml or more of HVJ. The fusion rate was not affected by the concentration of bone marrow cells during fusion experiments (Figure 4).

DISCUSSION

Many strategies of microinjection of macromolecules into intact cells have been reported. Because normal hemopoietic stem cells can not be usually isolated from blood or bone marrow cells, the

methods, especially those using membraneous carriers, which enable us to treat large numbers of cells simultaneously, would be more suitable than the other methods in which one must treat individual cells (e.g. mechanical injection). We have shown that erythrocyte ghosts can be used as a carrier of macromolecules into normal hemopoietic stem cells. Also the other membraneous carriers (liposomes, bacterial protoplasts and reconstituted envelopes from fusogenic animal virus) (5, 6, 7) may be useful for the same purpose.

Recently, some functions of the oncogene were analysed by injecting the gene product or monoclonal antibodies directed against it into the established cell line cells, and an important correlation between oncogene and transformation and/or proliferation of cells was demonstrated (8,9). It would be possible to study the role of proto-oncogene in normal cellular function, especially in the normal hemopoietic cell proliferation and differentiation, if we use the ghost-mediated system to microinject proto-oncogene products and/or the monoclonal antibodies against them. In addition, we can employ the system presented here to examine the pathway of intracellular signals following the external stimulation on hempoietic stem cells by injecting purified antibodies against the probable second messenger (10). Ghost-mediated microinjection of macromolecules would be valuable to study the molecular biology of hemopoietic stem cells.

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