Effect of Expression of Jagged1 Protein in Stromal Sublayer on the Maintenance of Hemopoietic Precursors in *In Vitro* Coculturing System

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In order to verify the relationship between the expression of Notch ligand in NIH 3T3 fibroblast clones and maintenance of hemopoietic activity *in vitro*, monolayers from three clones were co-cultured with mouse bone marrow mononuclears for 1-4 weeks and the total production of hemopoietic cells and myeloid colony-forming activity in methylcellulose were evaluated. One of the clones exhibited significant hemopoiesis maintaining activity and after 2 weeks of culturing promoted a more than 100-fold increase in the number of colony-forming precursors in comparison with control cells. Hemopoiesis maintenance activity of the second clone was much lower, and that of the third clone virtually did not differ from that of control cells. The results indicate that expression of Notch ligand can radically increase cell strain capacity to maintenance of *in vitro* hemopoiesis in the co-culturing system.

Key Words: *hemopoiesis; stem hemopoietic cells; Notch; colony-forming activity*

Proliferation of blood stem cells (BSC) *ex vivo* is an interesting theoretical and practical problem. *In vitro* culturing of BSC with a cocktail of growth factors usually leads to their rapid loss manifesting in a significant decrease in the number of cells capable of stable restitution of hemopoiesis [9]. On the other hand, BSC are important for practical medicine, because they play the key role in bone marrow transplantation and can be used (on condition of adequate development of the technology) for correction of congenital and acquired diseases of the hemopoietic and immune systems by introduction of therapeutic genes into these cells [11]. For effective treatment by this method one must remember that the status and presence of BSC largely depend on interactions with components of their stromal environment forming the stem cell niche. The niche for BSC is formed by osteoblast and, presumably, endothelial cells [1,4,18]. Many protein factors and signal pathways involved in the maintenance of BSC in an undifferentiated state are now identified [14].

Direct evidence of participation of Notch receptor-mediated signaling in the regulation of BSC functioning in their niches was obtained. For instance, enhanced expression of Jagged1 in the bone marrow stroma was found in transgenic mice whose osteoblasts express activated parathyroid hormone receptor, thus promoting the increase in BSC count. This is paralleled by increased formation of the active form of Notch in BSC ($Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells) [1]. The capacity of the transgenic mice BSC to maintain hemopoietic precursors is reduced to the basal level in the presence of γ-secretase inhibitor, impairing the Notch-mediated signaling. Experiments on transgenic animals carrying promotor elements activated in response to Notch signaling sho-

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wed that this signal pathway was most active for hemopoietic precursors at presumable sites of BSC niche location, *i.e.* in the periosteal area or at sites of contacts with large stromal cells [2].

Sibnce attempts at BSC culturing *in vitro* in the presence of various cytokines failed, it seemed interesting to try model systems of BSC culturing approximating *in vivo* conditions. We started a series of experiments aimed at creation of an artificial hemopoietic niche. In the present work we used NIH 3T3 fibroblasts not maintaining hemopoiesis in culture for obtaining cell clones expressing Jagged1 protein. The capacity of three of these clones to maintain hemopoiesis and mouse bone marrow colony-forming myeloid cells under conditions of co-culturing was studied.

MATERIALS AND METHODS

Preparation of expression plasmids, packaging cells, and stable cell strains. Plasmid encoding full-length Jagged1 cDNA (hJagged1/pcDNA3.1/Hygro+) [13] was used. In order to obtain Jagged1 cDNA coding protein fused with V5 epitope at the C-terminal, the Jagged1 open reading frame was amplified in 8 PCR cycles with thermostable Pfu polymerase (Stratagene) using T7 (5' TAATACGA CTCACTATAGGG) and hJagV5A (5' ACTCTCGA GTACGATGTACTCCATTC) primers. The PCR product was cleaved with BamHl and Xhol restrictases and cloned into pcDNA3.1/V5 HisA vector. In order to obtain a protein fused with V5 epitope, but carrying no hexahistidine sequence, the plasmid was cleaved at the Age 1 restriction site and an oligonucleotide adapter encoding translation termination sequence was inserted between V5 and His6. The insert was then subcloned into pBabe neo retrovirus vector by the corresponding restriction sites.

The Phoenix Ampho packaging strain was transfected with Jagged1-V5/pBabe neo vector and control pBabe neo vector using Lipofectamine 2000 reagent (Invitrogen). Virus supernatants were collected 1-3 days after transfection and used for infection of NIH 3T3 cells. The cells were selected for 5 days in a medium containing 500 μg/ml neomycin (Invitrogen), after which individual clones were isolated using cloning cylinders. The expression of Jagged1-V5 protein in cell lysates was verified by immunoblotting analysis with antibodies to V5 epitope (Invitrogen). Several clones expressing Jagged1-V5 protein were detected among neomycin-resistant clones.

Co-culturing of hemopoietic cells and NIH 3T3 fibroblasts. Bone marrow cells were obtained from 4-8-week-old C57Bl/6 mice. For co-culturing of mouse bone marrow nuclear cells with a monolayer of fibroblasts expressing Jagged1-V5, the fibroblasts were inoculated in 25-cm^2 flasks with ventilated caps and cultured until confluence, after which mouse bone marrow cells (final concentration of 10^6 cell/ml) were layered onto the monolayer (erythrocytes were lysed by hypotonic shock). The cultures were grown in DMEM with 10% fetal calf serum (FCS) and 2 mM glutamine at 37°C and 5% CO_2 for 4 weeks. The medium was replaced simultaneously with cell counting 1-2 times a week.

Analysis of colony forming capacity of hemopoietic cells. In order to evaluate colony-forming capacity of cells during co-culturing, aliquots of nonadherent cells were collected from flasks and inoculated into semiliquid medium containing 1.5% methylcellulose (Sigma), 25% FCS, 8.5% conditioned medium from WEHI3B and L929 cells in 2:1 ratio, 50 mg/ml bovine serum albumin, 1.4×10^{-4} M β-mercaptoethanol, 2 mM glutamine, 100 μg/ml penicillin G (Sigma), and 100 μg/ml streptomycin sulfate (Sigma). Culturing was carried out for 11 days at 37°C and 5% CO_2 in 24-well plates, after which the colonies were counted under an inverted microscope.

RESULTS

No appreciable increase in hemopoiesis-maintaining activity of total NIH 3T3 cell culture was detected after retroviral Jagged1 transduction and neomycin selection. This was presumably due to low level of Jagged1 expression in the total culture. In subsequent co-culturing experiments we used individual cell clones with high Jagged1 expression. A plasmid expressing Jagged1 protein fused with V5 epitope in the C-terminal (Jagged1-V5) was obtained for this purpose. After viral transduction and selection, a series of cell clones was obtained, in which the levels of Jagged1 expression were evaluated by immunoblotting analysis with subsequent visualization of the protein with anti-V5 antibodies. Subsequent experiments were carried out on three clones (Jagged3, Jagged13, and Jagged21), expressing Jagged1-V5 in amounts sufficient for the analysis (Fig. 1).

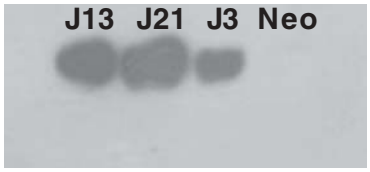

Fig. 1. Immunoblotting analysis of the expression of Jagged-V5 protein in cell lysates of clones Jagged13 (J13), Jagged21 (J21), Jagged3 (J3), and in control clone (neo).

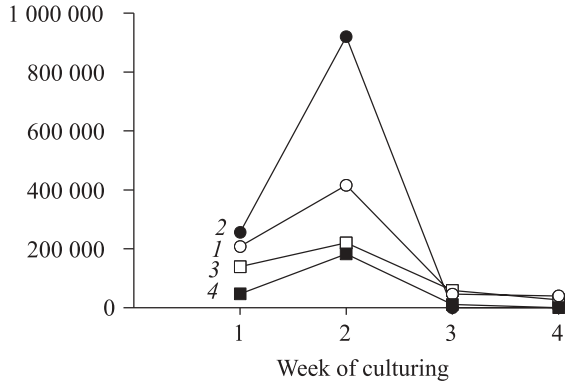

Fig. 2. Relationship between Jagged1 overexpression in NIH 3T3 cells and their capacity to maintain *in vitro* hemopoiesis. Ordinate: total count of live nucleated cells in nonadherent fraction. *1*) Jagged13; *2*) Jagged21; *3*) Jagged3; *4*) neo (control clone).

Evaluation of proliferation of bone marrow cells cultured with FCS for 4 weeks on a sublayer of Jagged1-expressing NIH 3T3 fibroblasts showed significant differences in the capacity of clones to maintain the production of hemopoietic cells in the total bone marrow cell fraction (Fig. 2). Clone Jagged21 exhibited the highest activity; the count of nucleated cells was maximum after 3 weeks of culturing on this clone. In the control (culturing on NIH 3T3 cells transduced with empty vector), the formation of hemopoietic cells completely stopped by this term. Clone Jagged13 exhibited intermediate activity, while clone Jagged3 virtually did not differ from control cells carrying empty vector.

Morphologically these differences between the clones manifested by the appearance of numerous large adherent accumulations of cells, presumably individual clones descendant from individual hemopoietic precursors (Fig. 3). Clone Jagged3 and control cells contained virtually no adherent accumulations of this kind.

For evaluation of the capacity of the resultant clones to maintain hemopoietic precursors, we determined colony-forming activity of cells. Equal aliquots of cells from long-term cultures were placed into a semisolid medium with methylcellulose and myeloid growth factors, after which cell proliferation and formation of individual myeloid colonies were analyzed under a microscope. Our data indicate that Jagged21 clone much more actively stimulated *in vitro* colony formation compared to

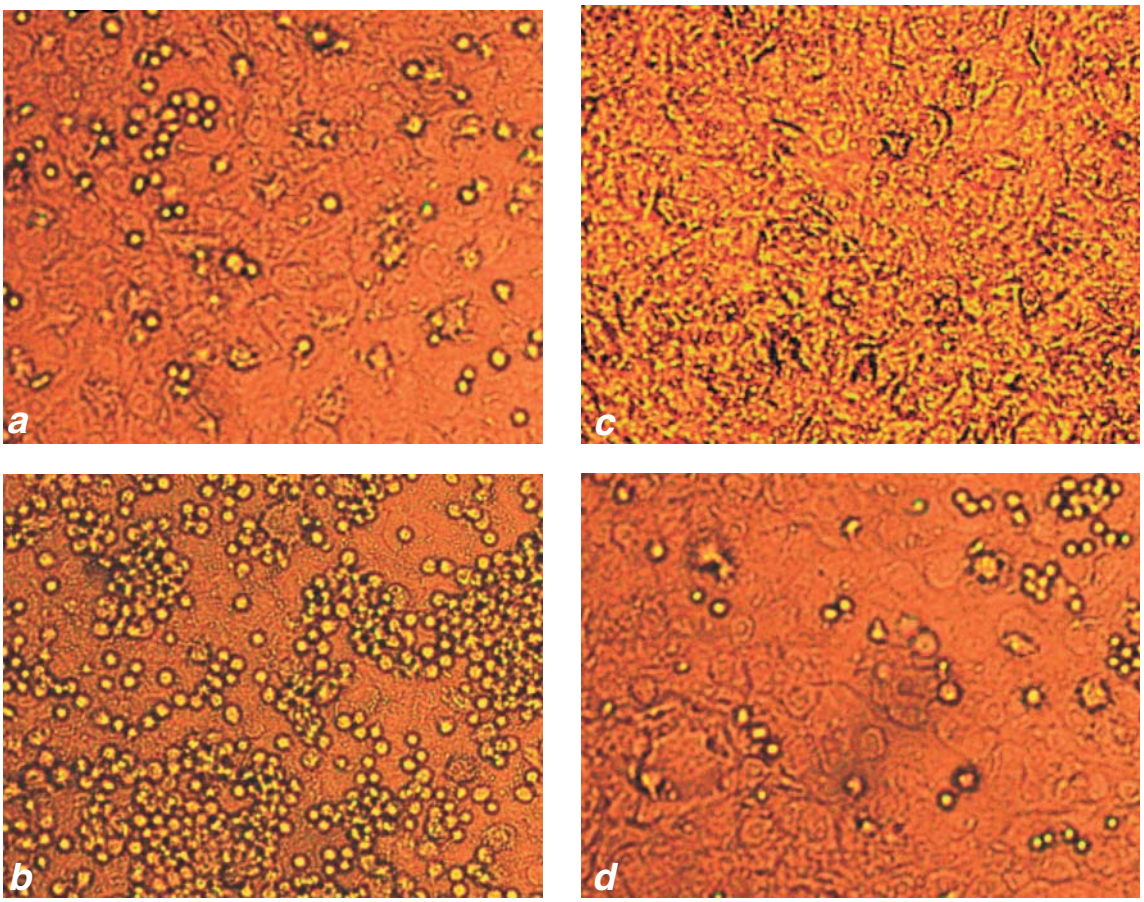

Fig. 3. Morphology of bone marrow cells after their 7-day culturing with Jagged1-expressing NIH 3T3 clones. *a*) Jagged13; *b*) Jagged21; *c*) Jagged3; *d*) neo (control clone).

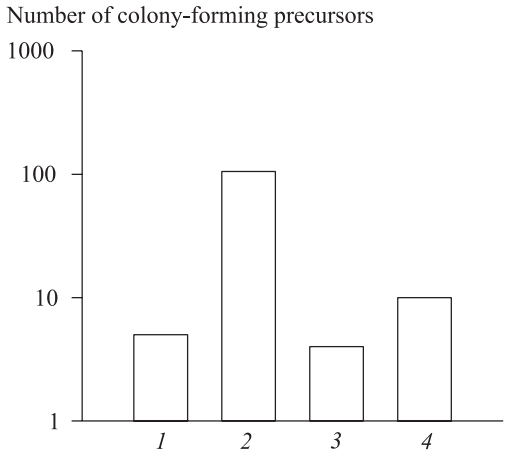

Fig. 4. Comparative analysis of NIH 3T3 clones expressing Jagged1 to maintain colony-forming myeloid precursors after 16-day co-culturing with bone marrow cells. *1*) Jagged13; *2*) Jagged21; *3*) Jagged3; *4*) neo (control clone).

other clones. For example, after 16 days of culturing on the sublayer the maintenance of colony formation for clone Jagged21 was 100-fold higher than in control (Fig. 4). The morphology of cultures is presented in Figure 5.

Indirect evidence of the involvement of Notch and its ligands into hemopoiesis was reported more than 10 years ago [3-7,12,16]. Activation of Notch in hemopoietic cells leads to inhibition of differentiation-specific genes and the cells become insensitive to induction signals.

Jagged1 protein is expressed at a high level in bone marrow precursor cells and many stromal strains [5,8,10,17], which is in good agreement with its possible involvement into hemopoiesis regulation. The data indicating that addition of soluble Jagged1 to primary culture of mouse bone marrow $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ hemopoietic precursors leads to an appreciable increase in the number of colonies with high proliferative potential during subsequent culturing on methylcellulose [16] is one more proof in favor of the important role of Jagged1 in the maintenance and multiplication of hemopoietic precursors. Similar results were obtained with AGM cells and fetal liver cells [3]. Increased capacity of hemopoietic cells, cultured on a sublayer of Jagged1-expressing fibroblasts, to colony formation was also described previously [15].

Our data indicate that overexpression of Jagged1 cardinally enhances the function of hemopoietic precursor support by stromal cells in the co-culturing system. In general, this fact does not contradict modern concepts on the key role of Notch signaling in hemopoiesis regulation. However, our data make important amendments to these concepts, indicating that Jagged1 expression in the sublayer cells is insufficient for the appearance of hemopoiesis-maintaining activity. Our experiments demonstrated a general correlation between the level of Jagged1 expression and maintenance activity (the highest level of expression in Jagged21 clone and the lowest in Jagged3 clone), but the differences in the level of protein expression in clones differed only 3-5-fold, which was much lower than the differences in the levels of maintenance of colony-forming and total hemopoietic cells. In addition, our data indicate that the appearance of NIH 3T3 clones capable of *in vitro* hemopoiesis maintenance is a rare phenomenon. This explains the absence of appreciable stimulation of hemopoiesis during the use of total NIH 3T3 cultures expressing Jagged1-V5.

Several explanations of the detected phenomenon are possible. Hemopoiesis maintaining activity can have a concentration threshold and its level can be nonlinearly related to Jagged1 protein concentration on cell surface. The efficiency of Jagged1 transportation to the surface can greatly vary in different clones. Jagged1 expressed on cell surface can be inert. In some clones, the expression of additional proteins with hemopoiesis-maintaining activity and cooperating with Jagged1 can be induced. Further experiments are needed for clearing out the true causes of the detected phenomenon.

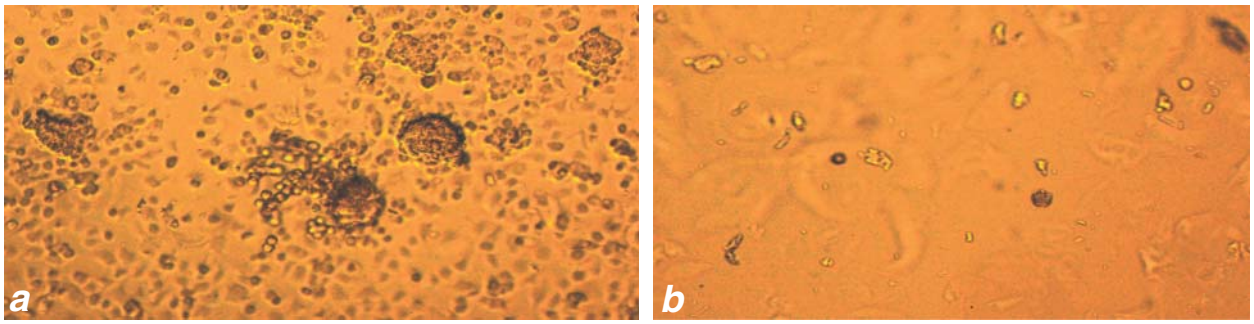

Fig. 5. Morphology of cultures after 16-day culturing with Jagged21 clone and subsequent 11-day culturing in methylcellulose. *a*) control clone (neo); *b*) Jagged21 clone.

The possibilities of "improving" stromal cells for creating an *in vitro* system for BSC maintenance and insertion of expression elements in these cells are rarely used. Our experiments demonstrated the possibility of creating cell strains maintaining hemopoietic precursors *in vitro* from cells initially having no activity of this kind using simple genetic manipulations. This opens new vistas for creation of optimal conditions for *in vitro* culturing and viral transduction of BSC using stromal cultures carrying hemopoiesis-stimulating membrane-bound factors.

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