Role of Transforming Growth Factor-β in Erythroid Cell-Mediated Suppression of B-Cell Blastogenesis

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Soluble products spontaneously secreted by murine nuclear erythroid cells directly suppress the proliferation of activated B-lymphocytes induced by bacterial lipopolysaccharides. Blocking of the transforming growth factor-β synthesis in nuclear erythroid cells by antisense oligonucleotides binding mRNA and blocking of this factor's functional activity by neutralizing antibodies were associated with a marked decrease in suppressor activity of the medium conditioned by nuclear erythroid cells.

Key Words: *erythroblast; natural immunosuppression; transforming growth factor-β*

Nuclei-containing erythroid cells (NEC) suppress humoral immune response induced by both thymus-dependent and thymus-independent antigens [2-4]. This immunoregulatory effect of NEC is mediated, at least partially, by secretion of soluble suppressor factors [10]. We tried to prove that transforming growth factor-β (TGF-β) is one of such factors.

MATERIALS AND METHODS

(CBA×C57Bl/6) F_1 (CBF_1, H-2^k/H-2^b) mice aged 4-8 months from Breeding Center of Siberian Division of the Russian Academy of Medical Sciences were used.

Splenic erythropoiesis was induced by intraperitoneal injection of phenylhydrazine (1.2 mg/ml, Sigma) as described previously [2,3,10]. At least 60% of suspended cells of erythropoietic spleen were NEC [3,10]. NEC were separated from other cells as described elsewhere [1]: blast cells were isolated by centrifugation in a Percoll density gradient (1.075 g/liter), treated with anti-Thy 1.2 monoclonal antibodies (clone 5a-8, IgG2b, ascites dilution 1:500, Cedarlane), macrophages, and T- and B-lymphocytes were removed by adhesion to plastic coated with anti-immunoglobulin antibodies, and the cells that failed to adhere to the plastic were treated with leucyl-methyl ether (Fluka) toxic for large granular lymphocytes and macrophages [14]. Cell viability was assessed by the trypan blue exclusion test. Analysis of cell smears stained by the Nocht—Maksimov method showed that at least 95% of the resultant cell population were basophilic NEC.

For preparing the supernatant, NEC were cultured in serum-free RPMI-1640 medium with 20 mM HEPES, 2 g/liter $NaHCO_3$, 4 mM L-glutamine, and antibiotics (all reagents from Sigma) in a humidified atmosphere with 5% CO_2. After 24 h, the culture medium was collected by centrifugation, sterilized by filtration through 0.22 μ filter, and stored at -20°C.

Lymphocytes were cultured in RPMI-1640 with 2 g/liter $NaHCO_3$, 20 mM HEPES, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, antibiotics, and 7% fetal calf serum in humidified atmosphere with 5% CO_2.

For preparing activated B-lymphocytes, 10 ml of splenocyte suspension (5×10^6 cells/ml) was cultured

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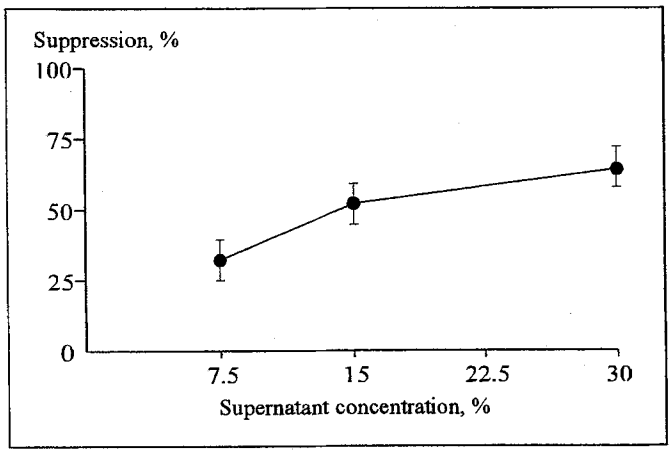

Fig. 1. Suppressive effect of culture supernatant from nuclear erythroid cells on B-cell blastogenesis. Data of three independent experiments are shown.

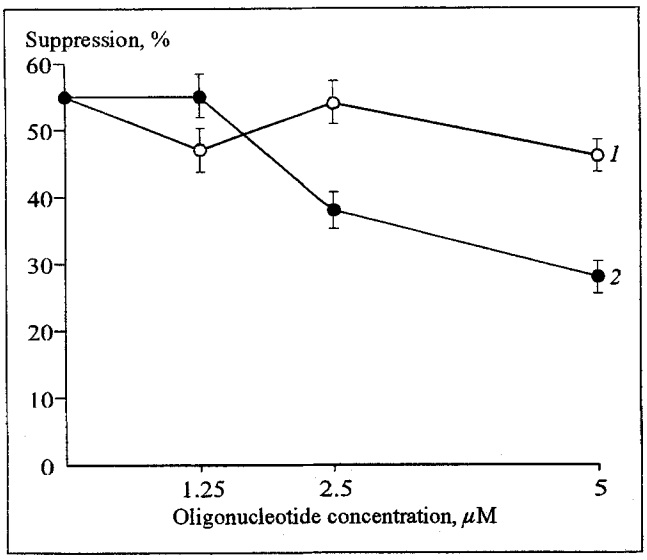

Fig. 2. Effect of antisense oligonucleotides specific to the TGF-β mRNA on the formation of soluble suppressor activity by nuclear erythroid cells (NEC). TGF-β control (*1*) or antisense to mRNA (*2*) oligonucleotides were added to NEC at the beginning of their culturing. Data of two experiments are shown.

in the presence of 20 μg/ml lipopolysaccharide (LPS *E. coli* 055:B5, Sigma) in 25 cm² plastic flask (Linbro) for 24 h. After culturing, washed cells (no more than 40×10^6) were applied onto a Petri dish (90 mm) and incubated in the culture medium for 2 h at 4°C. Then cell population free from adhering cells was applied onto plastic coated with anti-immunoglobulin antibodies, and penning was carried out as described previously [9,12]. After the procedure, nonadherent cells were washed with culture medium, and the remaining B-cells were suspended using a Costar policeman, washed, and then used in tests. Immunofluorescence microscopy with fluorescein isothiocyanate-labeled rabbit antibodies (Cedarlane) to murine immunoglobulin showed that virtually 100% of cells isolated by positive penning were B-lymphocytes.

In the suppressor test, activated lymphocytes ($2-3\times10^5$/well) were cultured with LPS (12 μg/ml) in the presence of the above-mentioned soluble additives or without them in the control in a round-bottom 96-well BDSL plate for 48 h. The level of cell proliferation was assessed by incorporation of ^{3}H-thymidine added into all wells in a dose of 1 μCi 6 h before the end of incubation. The percentage of suppression was estimated using the following formula: count in experiment/count in control×100. Each test variant was represented by 3-4 parallel cultures.

Oligonucleotides used in this study were synthesized at the Institute of Bioorganic Chemistry of Siberian Division of the Russian Academy of Sciences. The sequence of antisense oligonucleotide to mRNA TGF-β was (5'-3')-GGGAGGCGGCGCCCCACGGP. For better penetration into the cell, oligonucleotides were modified by cholesterol. For control, oligonucleotides with a random sequence of 20 nucleotides were used. The oligonucleotides were stored at -20°C before experiment.

Anti-TGF-β monoclonal antibodies (Genzyme) neutralizing 0.1-0.5 ng/ml total TGF-β in a concentration of 20-30 μg/ml were stored in liquid nitrogen at -196°C.

The results were statistically processed using Student's *t* test. The differences were significant ($p<0.05$).

RESULTS

Previous reports indicate that NEC can secrete a factor capable of suppressing B-cell proliferation [10]. Figure 1 shows that culture medium conditioned by NEC exerts a direct dose-dependent suppressive effect on LPS-stimulated proliferation of activated B-lymphocytes.

We revealed the expression of the TGF-β mRNA in NEC [13]. Together with the published data [7,11] on the involvement of TGF-β in immunosuppression mediated by natural bone marrow suppressors, these results prompted us to investigate the probable role of TGF-β in NEC-mediated suppression of B-cell blastogenesis. Our experimental approach was based on specific binding of the TGF-β mRNA by antisense oligonucleotides. Figure 2 shows that NEC culturing in serum-free medium in the presence of antisense but not control (random) oligonucleotides was associated with a marked decrease of suppressor activity of culture medium conditioned by NEC. Thus, our results confirm that secretion of TGF-β

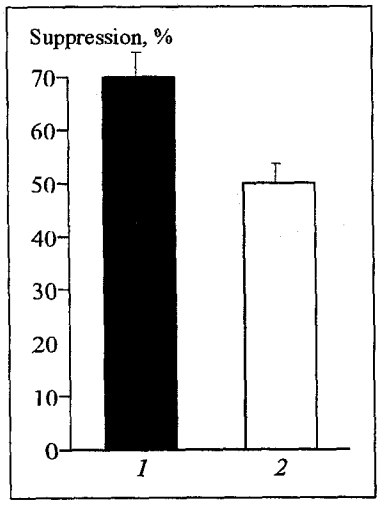

Fig. 3. Effect of anti-TGF-β antibodies on suppression of B-cell blastogenesis mediated by erythroid soluble products. Proliferative response of activated B-cells was suppressed by culture supernatant (30%) from nuclear erythroid cells in the presence of control IgG (*1*) or anti-TGF-β antibodies (*2*). Data of 4 independent experiments are shown.

contributes to NEC-mediated immunoregulation. One more argument in favor of this hypothesis is the data demonstrating that the addition of neutralizing anti-TGF-β antibodies (but not control IgG) in a final concentration of 2.5 μg/ml to B-cell cultures appreciably decreases the suppression of B-cell blastogenesis induced by erythroid soluble products (Fig. 3). Further increase in the concentration of anti-TGF-β antibodies in the cultures to 25 μg/ml decreases the observed suppression (data not shown). It is noteworthy that the blocking of TGF-β production or functional activity led to partial but not complete reversion of the studied suppression. This probably indicates that along with TGF-β, other soluble NEC products can be involved in suppression of B-cell blastogenesis. Recent identification of suppressor activity associated with a NEC-produced soluble factor differing from TGF-β by physicochemical properties confirms this hypothesis (data not presented). This factor is now investigated in our laboratory.

TGF-β was shown to inhibit NEC growth [5,6]. In addition, this cytokine is one of the main inductors of NEC differentiation into erythrocytes [8]. Together with our results, these data outline the possible role of NEC-produced TGF-β in the regulation of the balance between erythro- and lymphopoiesis. On the one hand, TGF-β might suppress lymphopoiesis, on the other, it might decrease the count of suppressor NEC by inhibiting their growth and differentiating them into erythrocytes. Therefore, production of TGF-β by NEC in immunogenesis and under conditions of emergency erythropoiesis might prevent the imbalance in the hemopoietic process and eventually normalize it.

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