Effect of VEGF on Mouse Thymocyte Proliferation and Apoptosis *In Vitro*

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Vascular endothelial growth factor is not only angiogenic, but also immunoregulatory factor. For evaluation of the possibility of its direct interaction with mouse thymocytes we studied the effect of vascular endothelial growth factor on proliferation and apoptosis of thymocytes and expression of genes for the corresponding receptor on these cells. Vascular endothelial growth factor modulated mitogen-induced proliferation of thymocytes and stimulated spontaneous apoptosis in intact thymus cells. Thymocytes express mRNA of type 2, but not type 1 vascular endothelial growth factor receptors.

Key Words: *vascular endothelial growth factor; thymocytes; apoptosis; proliferation; type 2 vascular endothelial growth factor*

Vascular endothelial growth factor (VEGF) is one of the main angiogenic factors; it plays an important role in normal wound healing and many pathological processes associated with the formation of new vessels [6], but its immunoregulatory role is little studied. VEGF suppresses differentiation of dendritic cell [7] and stimulates monocyte chemotaxis [2]. Few reports describe the effects of VEGF on peripheral T cells [8,13]. Long-term treatment with VEGF caused thymus atrophy in experimental animals [10].

We studied the possibility of direct *in vitro* effect of VEGF on proliferative activity and apoptosis of mouse thymocytes and detected VEGF receptors (VEGFR) on these cells.

MATERIALS AND METHODS

The study was carried out on female BALB/c mice (16-18 g) from Harlan Inc. Breeding Center (Indianapolis, USA) and from Rappolovo Breeding Center, Russian Academy of Medical Sciences. The animals were sacrificed by cervical dislocation, the thymus was removed; cell suspension was prepared by crushing the thymus in RPMI-1640 with 10% FCS, the stroma was removed by filtration through a nylon filter. Thymocytes were incubated with or without VEGF (R&D) for further evaluation of the proliferative activity or apoptosis.

Proliferative activity of thymocytes was evaluated by ^{3}H-thymidine incorporation into cells after 72-h incubation with or without Con A (0.5-5.0 μg/ml, Sigma). Mouse recombinant IL-4 (R&D) served as the positive control.

Thymocyte apoptosis was evaluated after 24-h incubation at 37°C by staining with propidium iodide for detecting cells with hypodiploid DNA by the pre-G_0/G_1 peak as described previously [9]. The results were recorded on a flow cytofluorimeter (Beckton Dickinson).

The expression of VEGFR mRNA in isolated thymocytes and in thymus and lung tissues was determined by reverse transcription polymerase chain reaction (RT-PCR). To this end, summary RNA was isolated using guanidine thiocyanate (Sigma) [3]. Summary RNA (2 μg) was used for reverse transcription, which was carried out with oligo(dT)15 primers and M-MLV reverse transcriptase (Promega) in accordance with manufacturer's protocol. PCR was carried out using specific primers selected so that the lengths of the product during the reaction for cDNA and nuclear

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DNA were different. Primers for VEGFR1 (Flt-1): direct 5"GAAGCGGTTCACCTGGACTGAGACC-3' and reverse 5'-GGCTTTGCTGGGGGGATTTCTCT AA-3' (product size 432 n. p.). Primers for VEGFR2 (KDR/Flk-1): direct 5'-ACAGACAGTGGGATGGT CCTTGCAT-3' and reverse 5'-AAACAGGAGGTG AGCTGCAGTGTGG-3' (272 n. p.). Primers for β-actin: direct 5'-ATGGATGACGATATCGCT-3' and reverse 5'-ATGAGGTAGTCTGTCAGGT-3' (568 n. p.). The level of β-actin mRNA expression was determined for evaluation of the efficiency of RT and was used as the internal reference value. The results were visualized by gel electrophoresis followed by ethidium bromide staining.

The data were statistically processed using Student's *t* test.

RESULTS

VEGF in concentrations of 50 and 100 ng/ml suppressed spontaneous proliferation of thymocytes (Fig. 1, *a*) and modulated this parameter in the presence of Con A in different doses (Fig. 1, *b*).

Con A in doses of 0.5 to 5.0 μg/ml without addition of VEGF increased the response; the peak of response shifted to 1 μg/ml Con A in the presence of the cytokine (Fig. 1, *b*). Addition of 50 ng/ml VEGF to the least dose of Con A (0.5 μg/ml) caused a costimulatory effect, addition to the highest dose (5 μg/ml) resulted in a significant suppression of label incorporation, and addition of VEGF to the intermediate dose of Con A (1 μg/ml) did not change proliferative activity of thymocytes.

IL-4 activating both spontaneous and Con A-induced proliferation of thymocytes in both cytokine concentrations (10 and 50 ng/ml) served as positive control (Fig. 1, *a*, *c*). IL-4 acts as an inductor of T cell and thymocyte proliferation [1].

In contrast to IL-4, VEGF is not a mitogen for thymocytes, because it does not stimulate their spontaneous proliferation (Fig. 1, *a*), but exhibited a costimulatory effect when used together with Con A.

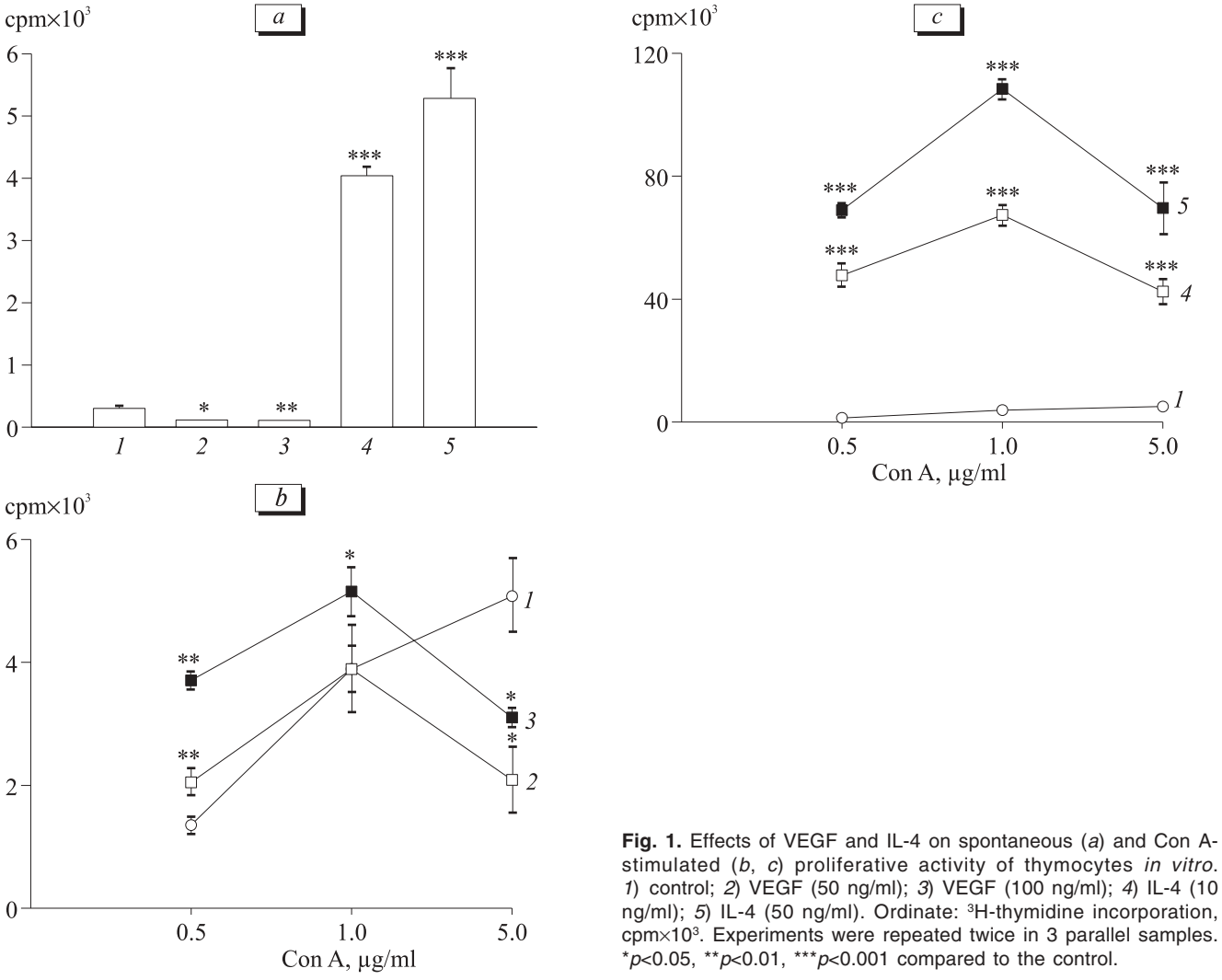

Fig. 1. Effects of VEGF and IL-4 on spontaneous (*a*) and Con A-stimulated (*b*, *c*) proliferative activity of thymocytes *in vitro*. *1*) control; *2*) VEGF (50 ng/ml); *3*) VEGF (100 ng/ml); *4*) IL-4 (10 ng/ml); *5*) IL-4 (50 ng/ml). Ordinate: ^{3}H-thymidine incorporation, cpm×10^3. Experiments were repeated twice in 3 parallel samples. **p*<0.05, ***p*<0.01, ****p*<0.001 compared to the control.

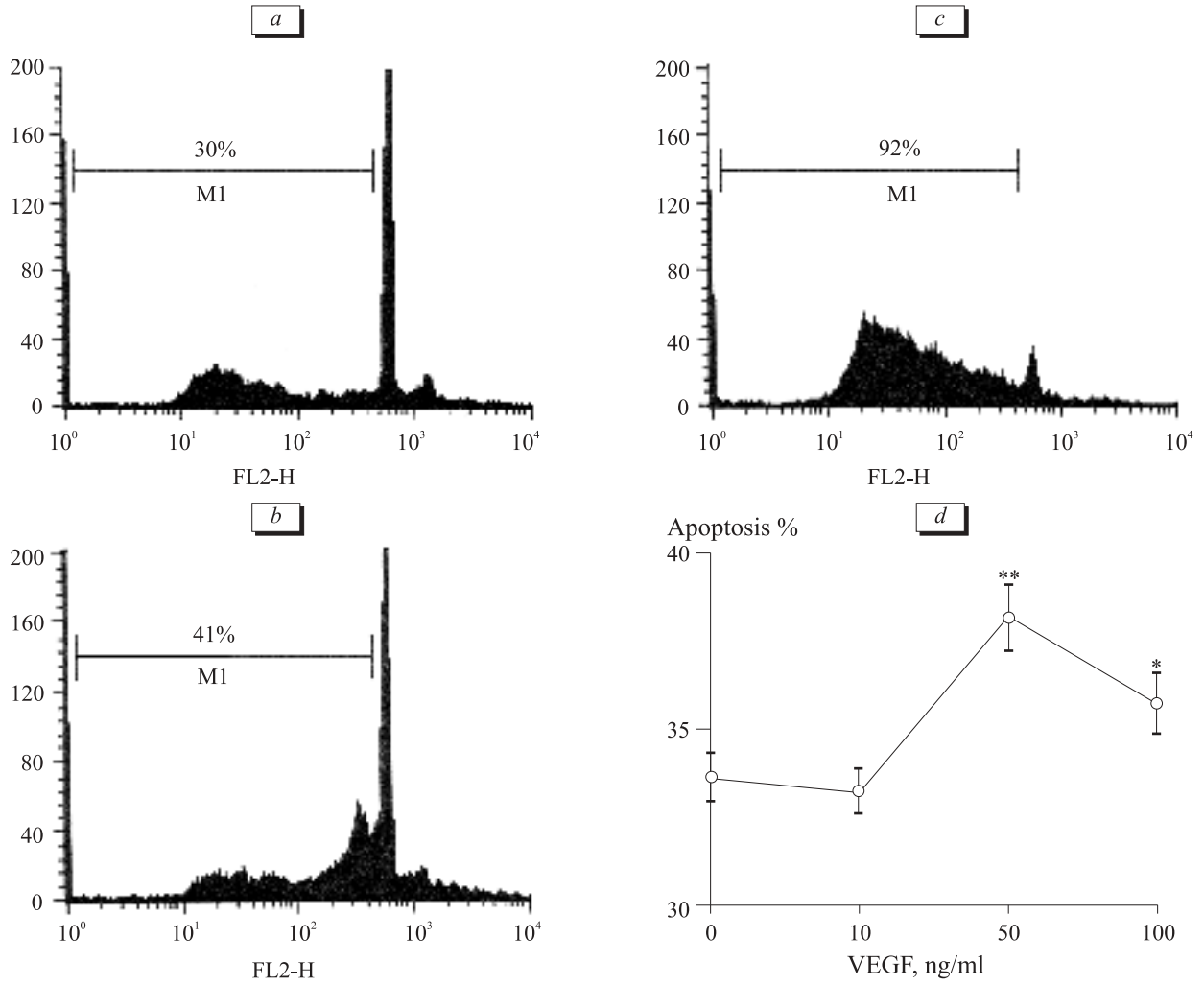

Fig. 2. Effects of VEGF on thymocyte apoptosis *in vitro* evaluated by propidium iodide staining (pre-G_0/G_1 DNA peak). *a*) spontaneous apoptosis without VEGF; *b*) spontaneous apoptosis in the presence of 50 ng/ml VEGF; *c*) apoptosis induced by 1 μM dexamethasone; *d*) spontaneous apoptosis in the presence of different doses of VEGF. Each point presents the parameters from 10 intact animals. *$p<0.05$, **$p<0.01$ compared to the control.

IL-4 15-20-fold stimulated mitogen-induced proliferation of thymocytes, while VEGF increased the values 1.5-2.5 times. The curves reflecting combined use of the mitogen with each of the cytokines were similar (Fig. 1, *b*, *c*), which suggests that VEGF produced a weak activating effect on thymocytes.

This assumption is in line with published data on the stimulatory effect of this factor on peripheral T cells. VEGF stimulates proliferation of mitogen-induced human blood mononuclears [13] and the production of IFN-γ [8] and metalloproteinase [11] by animal T cells.

Since addition of VEGF to thymocyte cultures not stimulated with Con A significantly decreased their proliferative activity (Fig. 1, *a*), we tried to clear out whether VEGF was an apoptosis inductor. The study of spontaneous apoptosis of thymocytes revealed a dose-dependent effect and stimulation of thymocyte apoptosis in the presence of 50 and 100 ng/ml VEGF (Fig. 2, *d*; representative experiment: Fig. 2, *b*). Dexamethasone (1 μM) served as the standard stimulator; it caused apoptosis of 90-92% thymocytes (Fig. 2, *c*). VEGF increased thymocyte apoptosis by no more than 15% of the control level and hence, cannot be regarded as an apoptosis inductor. Presumably, this effect was due to activating influence of VEGF on thymocytes.

The expression of VEGFR1 and VEGFR2 mRNA in mouse thymocytes was studied for the first time. Mouse lung tissue with rich vascular network was used as a positive control (both receptors were detected in endothelial cells) [6]. VEGFR1 and VEGFR2 mRNA were expressed in the lung and intact thymus tissues, while isolated thymocytes expressed only VEGFR2 mRNA, but not VEGFR1 (Fig. 3). Similar data were obtained for T cells from rat lymph nodes [8].

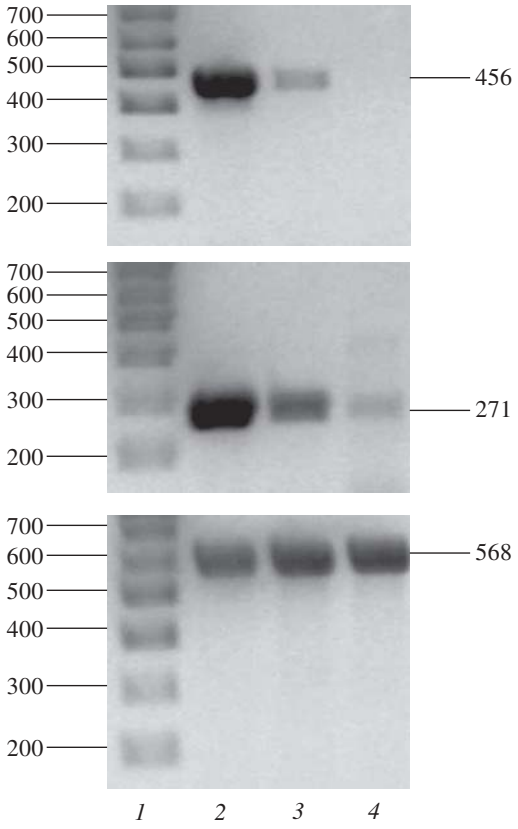

Fig. 3. Expression of VEGF receptor mRNA. *1*) molecular weight marker (left: number of nucleotide pairs); *2*) lung; *3*) intact thymus; *4*) suspension of isolated thymocytes. Right: size of amplification product (number of nucleotide pairs). β-Actin was used as the internal standard. *a*) VEGFR1; *b*) VEGFR2; *c*) β-actin.

VEGFR2 is the main receptor triggering a cascade of activating signals in endothelial cells [12]. Presumably, it plays an important role in thymocyte differentiation, as was shown for c-kit and flk-2, which, similarly to VEGFR2, are thyroxine kinase receptors [5]. VEGFR2 were detected on early prehemopoietic precursors originating from the embryonal stem cell capable of differentiating into T cells *in vitro* [4].

Hence, the expression of VEGFR gene in mouse thymocytes was detected for the first time, which confirms the possibility of direct interactions between this factor and thymic cells. VEGF is not a mitogen or apoptosis inductor for thymocytes. On the other hand, it can act as a co-stimulator of mitogen-induced proliferation of these cells and increases their sensitivity to spontaneous apoptosis. Presumably, VEGF can modulate maturation of thymocytes in the body by modifying their reactivity to other stimuli. For instance, injection of VEGF to animals followed by appreciable increase in blood concentration of this factor leads to thymus involution [10]. These data are very important for understanding the interactions between angiogenic factors and immune system.

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