Role of CD40-Dependent Signal in Induction of Recombinase RAG-1 Expression in Peripheral T Cells of Patients with Autoimmune Diabetes Mellitus

E. M. Kuklina, O. L. Gorbunova, and E. N. Smirnova*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 152, No. 9, pp. 306-309, September, 2011
Original article submitted February 24, 2011

We studied the mechanisms of induction of recombinase activity in peripheral T cells of patients with autoimmune type 1 diabetes mellitus. It was shown that the presence of CD40 on T cell membrane (not typical of these cells) is crucial for this process: expression of recombinase RAG-1 in diabetic patients was detected primarily in $\alpha\beta TCR^{+}CD40^{+}$ lymphocytes; targeted CD40-dependent activation of intact T cells *in vitro* increases, while blockade of CD40 signal in the culture of stimulated T cells abolishes recombinase expression.

Key Words: *T cells; TCR gene reaggangement; autoimmunity; type 1 diabetes mellitus*

The search for mechanisms of induction of autoimmune diseases is still an urgent medical problem. Differentiation of immunocompetent cells outside the central immune organs, *e.g.* extrathymic differentiation of T cells, can be a solution [1]. The assumption stems from the absence of conditions for effective clonal selection providing elimination of autospecific T cell clones in peripheral organs, in contrast to the thymus; therefore, the formation of T lymphocytes outside the thymus should inevitably lead to the appearance of autoreactive cells and creates prerequisites for the development of autoimmune processes.

Our recent studies confirmed this assumption and demonstrated induction of rearrangement of antigen receptor genes (T cell receptor, TCR) in T cells, a key event of T cell differentiation, in mature peripheral T cells in autoimmune diabetes mellitus (DM). For instance, we detected expression of the main markers of gene rearrangement, recombinases RAG-1 and RAG-2 (recombination activating genes) in αβT lymphocytes from patients with type 1 DM (DM1) at both mRNA (RAG-1 and RAG-2) and protein levels (RAG-1) [2].

These findings raised a question on factors responsible for induction of recombination activity in peripheral T cells. Published data suggest that membrane molecule CD40 can be involved in this process: first, signal from CD40 in mature B cells triggers the expression of RAG-1 and RAG-2 recombinases regulating switching between different classes of immunoglobulins [6]; second, in NOD mice predisposed to spontaneous autoimmune diabetes up to 60% peripheral T lymphocytes carry this atypical marker [13].

Here we studied the expression of CD40 on the membrane of peripheral αβT lymphocytes in patients with autoimmune diabetes and evaluated the contribution of CD40-dependent signal into induction of recombinase activity in these cells.

MATERIALS AND METHODS

The experiments were performed in peripheral blood lymphocytes from patients with first diagnosed DM1 (<8 weeks) or with DM1 history up to 20 years (patients' age 18-47 years). Lymphocytes from healthy donors (19-37 years) and patients with non-autoimmune DM (type 2 DM, DM2) served as the control;

Institute of Ecology and Genetics of Microorganisms, Ural Division of Russian Academy of Sciences, Perm'; *E. A. Vagner Perm' State Medical Academy, Federal Agency for Health Care and Social Development, Russia. ***Address for correspondence:*** ibis_07@mail.ru. E. M. Kuklina

0007-4888/12/1523-0336

patients' age was 29-63 years and DM2 history was 1-12 years. DM1 was confirmed by tests for antibodies to the major diabetes-associated autoantigens, insulin and glutamate decarboxylase (ORGENTEC).

Lymphocytes were isolated from the peripheral blood by centrifugation in Ficoll-verografin density gradient (ρ=1.077 g/cm^3). For *ex vivo* cytometric analysis, non-fractionated lymphocyte suspension was used; for culturing, monocytes and B cells were successively removed from this suspension by adhesion to glass and immunomagnetic separation using monoclonal antibodies (MAB) to CD19 (Invitrogen). Fractionated T cells (10^6 cell/ml) were cultured in RPMI-1640 (Sigma) supplemented with 10% FCS (Serva), 300 μg/ml L-glutamine (Serva), 0.01 M HEPES (Sigma), and 100 μg/ml gentamicin (Pharmacia) at 37°C and 5% CO_2 for 18 h. MAB to CD3 (Biolegend) and/or CD40 labeled with biotin (Invitrogen) ensuring receptor cross-linking by adding streptavidin to the culture (Invitrogen). For inhibition of CD40-dependent signal, MAB to CD40 ligand (eBioscience) were added to the culture in a concentration of 20 μg/ml 1 h before addition of stimulants.

Expression of intracellular protein RAG-1 and membrane molecules αβTCR (antigen receptor) and CD40 was assayed by flow cytometry using the corresponding MAB: anti-RAG-1+antimouse IgG1*PerCP (Becton Dickinson), anti-αβTCR*FITC (Invitrogen), and anti-CD40*PE (Invitrogen) by three-parameter or single-parameter (only for RAG-1) method for T cell culture. Intracellular staining for RAG-1 was performed using a set of fixattion/permeabilization buffers (Biolegend) according to manufacturer's instructions. The samples were analyzed on a FACScalibur flow cytofluorometer (Becton Dickinson) the results were processed using CellQuest software.

The use of RAG-1 as the marker of TCR gene rearrangement was dictated by the fact that recombinase expression can be detected only at the stage of genetic recombination and is irreversibly suppressed after its completion [12]. Of the two recombinases involved in the process, RAG-1 possessing nuclease activity plays a key role in rearrangement [1,5], while the second recombinase RAG-2 is presumably required for stabilization of RAG-1 binding with DNA [3] and is not obligatory condition for rearrangement [1,5]; therefore, detection of RAG-1 is enough for making conclusion about activation of gene rearrangement.

The data were processed statistically using Student *t* test.

RESULTS

Phenotypical analysis showed that mature peripheral αβT lymphocytes from DM1 patients carry CD40, an atypical marker for these cells (15.5±1.89% αβTCR$^+$ cells; n=17), in contrast to cells from healthy donors (0.510±0.037% αβTCR$^+$ cells; n=16) and DM2 patients (0.770±0.059% αβTCR$^+$ cells; n=12). The expression of antigen receptor in the subpopulation of αβTCR$^+$CD40$^+$ cells was lower than in the main T cell pool. It should be noted that T cells normally do not express CD40, but carry a membrane ligand for CD40, CD154; co-stimulating signal from CD154 upon antigen binding is necessary for adequate activation of T lymphocyte.

The pattern of RAG-1 distribution in different lymphocytes depending on the presence of CD40 molecules on the membrane attests to the participation of this molecule in induction of recombinase activity: RAG-1 was detected in trace amounts in the main CD40$^-$ population of αβT lymphocytes (1.81±0.49%) and in more that half CD40$^+$ αβT lymphocytes (65.10±3.37%).

Analysis of the role of CD40-dependent signal in activation of RAG-1 expression was carried out in 18-h culture of fractionated T cells from DM1 patients. The following regularities were revealed: culturing of intact T cells did not change the level of RAG-

TABLE 1. Expression of RAG-1 Protein by Fractionated Peripheral Blood αβT Lymphocytes from DM1 Patients in 18-h Culture in Response of Cross-Linking of CD40 on the Membrane ($M\pm m$; n=14)

Experimental conditions	Percent of RAG-1$^+$ cells		
	before culturing	after culturing	
		without CD40-dependent activation	against the background of CD40-dependent activation
Intact culture	10.10±1.59	8.70±1.03	18.9±3.1*$^+$
Stimulated culture (anti-CD3 antibodies)		17.80±2.54*$^+$	20.3±2.76*

Note. p<0.05 compared to corresponding parameter: *before culturing, $^+$in intact cells.

TABLE 2. Expression of RAG-1 Protein by Cultured Peripheral Blood αβT Lymphocytes from DM1 Patients against the Background of Blockade of CD40-Dependent Signal (*M*±*n*; *n*=14)

Experimental conditions	Percent of RAG-1$^+$ cells		
	before culturing	after culturing	
		CD40-dependent activation	blockade of CD40-dependent activation
Stimulated culture (anti-CD3 antibodies)	11.20±1.56	9.40±4.11*$^+$	9.00±1.75

Note. $p<0.05$ compared to corresponding parameter: *before culturing, $^+$under conditions of blockade.

1$^+$ αβT lymphocytes, but CD40 cross-linking on the cell membrane significantly increased this parameter (Table 1). In case of T cell stimulation via antigen receptor (anti-CD3-dependent stimulation), culturing by itself increased the level of RAG-1$^+$ αβT lymphocytes; CD40-dependent co-stimulation did not contribute to this process (Table 1). These findings can be explained as follows: in stimulated culture, CD40-dependent signal is effectively provided by other T cells normally carrying ligand for CD40, co-stimulating molecule CD154, and additional cell activation via CD40 does not play a role.

For verification of this assumption, we performed an inhibitory analysis with the use of CD40-dependent signal blocker, MAB to CD40 ligand. It was shown that this blockade abolished induction of the expression of RAG-1 in cultured anti-CD3-stimulated T cells (Table 2).

These findings suggest that DM1 is associated with the expression of CD40, an atypical molecule for T cells, on peripheral αβT lymphocytes; one of the functions of this molecule is induction of recombinase in the cell. This is confirmed by the following findings: 1) the majority of CD40$^+$ T lymphocytes expressed recombinase RAG-1; 2) directed CD40-dependent activation of T cells from patients (DM1) *in vitro* increased the percent of RAG-1$^+$ cells in the culture; 3) blockade of CD40-dependent signal abolished induction of RAG-1 expression in T cells.

Membrane molecule CD40 has long ago became the focus of studies of autoimmunity problems. Some studies showed that CD40-dependent signal contributed to the development of autoimmune pathologies [10,11], but this marker has been thought to act as a co-stimulator during activation of T lymphocytes by antigen-presenting cells normally expressing CD40 [7]. Even in few studies where CD40 was detected on T cells [13,14], the role of this molecule was interpreted as additional stimulation of other T cells, both CD4$^+$ and CD8$^+$ [9]. The co-stimulating signal via CD40 will potentiate the immune response, including the response to autoantigens, but specific role of this molecule in the development of immune pathologies can be realized only when its expression is related to primarily to autoreactive T cell clones. This phenomenon was also demonstrated on the model of autoimmune DM in NOD mice predisposed to this pathology [13].

There are published data explaining the relation between CD40-dependent signal with autoimmune process, rather than general immune reactivity of the organism. It was shown that CD40-dependent activation enhances maturation of the thymic medullary epithelium responsible for clonal selection and elimination of autospecific T cell precursors in the thymus [4]. Moreover, aberrant expression of CD40 on non-immune cells, *e.g.* the presence of this molecule in endocrine tissues in some types of autoimmune thyroiditis can also contribute to the development of autoimmune pathologies [8].

CD40 was never considered as a signal molecule triggering gene rearrangement in peripheral T cells and modulating the antigen-presenting repertoire of these cells, although experiments on NOD mice clearly indicated this possibility [13]. At the same time, this process can be a trigger in induction of autoimmune pathologies leading to the appearance of potentially reactive T cell clones at the periphery.

The study was supported by Program "Fundamental Sciences to Medicine" established by Presidium of Russian Academy of Sciences (No. 21).

REFERENCES

1. E. M. Kuklina, *Biokhimiya*, **71**, 1013-1020 (2006).
2. E. M. Kuklina, V. A. Lopatina, O. L. Gorbunova, *et al., Dokl. Akad. Nauk*, **435**, 557-560 (2010).
3. Y. Akamatsu and M. A. Oettinger, *Mol. Cell Biol.*, **18**, No. 8, 4670-4678 (1998).
4. T. Akiyama, Y. Shimo, H. Yanai, *et al., Immunity*, **29**, No. 3, 423-437 (2008).

5. M. J. Difilippantonio, C. J. McMahan, Q. M. Eastman, *et al., Cell*, **87**, No. 2, 253-262 (1996).
6. I. S. Grewal and R. A. Flawell, *Annu. Rev. Immunol*., **16**, 111-135 (1998).
7. G. Iezzi, I. Sonderegger, F. Ampenberger, *et al., Proc. Natl. Acad. Sci. USA*., **106**, No. 3, 876-881 (2009).
8. E. M. Jakobson, A. K. Huber, N. Akeno, *et al., Genes Immun.*, **8**, No. 3, 205-214 (2007).
9. M. E. Munroe, *Semin. Immunol*., **21**, No. 5, 283-288 (2009).
10. U. Schonbeck and P. Libby, *Cell. Mol. Life Sci.*, **58**, No. 1, 4-43 (2001).
11. E. Toubi and Y. Shoenfeld, *Autoimmunity,* **37**, Nos. 6-7, 457-464 (2004).
12. L. Turka, D. Schatz, M. Oettinger, *et al., Science*, **253**, No. 5, 778-781 (1991).
13. D. M. Waid, G. M. Vaitaitis, D. H. Wagner Jr., *Eur. J. Immunol.*, **34**, No. 5, 1488-1497 (2004).
14. D. M. Waid, R. J. Wagner, A. Putman, *et al., Clin. Immunol.,* **124**, No. 2, 138-148 (2007).