

RESEARCH ARTICLE

Effects of TNF Secreting HEK Cells on B Lymphocytes' Apoptosis in Human Chronic Lymphocytic Leukemias

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Abstract

Background: Tumor necrosis factor (TNF) related apoptosis-inducing ligand (TRAIL) is an antitumor candidate in cancer therapy. This study focused on effects of TRAIL, as a proapoptotic ligand that causes apoptosis, in B-CELL chronic lymphocytic leukemia cells (B-CLL). **Materials and Methods:** A population of HEK 293 cells was transduced by lentivirus that these achieved ability for producing the TRAIL protein and then HEK 293 cells transduced were placed in the vicinity of CLL cells. After 24 hours of co-culture, apoptosis of CLL cells was assessed by annexin V staining. **Results:** The amount of Apoptosis was examined separately in four groups: 293 HEK TRAIL (16.17±1.04%); 293 HEK GFP (2.7±0.57%); WT 293 HEK (2±2.6%); and CLL cells (0.01±0.01%). Among the groups studied, the maximum amount of apoptosis was in the group that the vector encoding TRAIL was transduced. In this group, the mean level of soluble TRAIL in the culture medium was 253pg/ml; also flow cytometry analyzes showed that proapoptosis in this group was 32.8±1.6%, which was higher than the other groups. **Conclusions:** In this study, we have demonstrated that TNF secreted from HEK 293 cells are effective in death of CLL cells.

Keywords: B-CELL chronic lymphocytic leukemia - tumor necrosis factor-related apoptosis-inducing ligand

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Introduction

B-CELL chronic lymphocytic leukemia (B-CLL) is one of the common leukemia in the western countries that men are more affected with (Weirnik et al., 1991). In this type of leukemia, apoptosis of cells become deficient and long life; and the proliferation rate of 0.1% to greater than 1% per day. In final, some colonies of B-cells are formed in peripheral blood and lymphoid organs and bone marrow (Lagneaux et al., 1998; Kay et al., 2007).

Today, Leukemias are treated with surgery, chemotherapy and radiotherapy but response to these treatments is very weak and metastatic cancers remain in the body; so, looking out the new treatments are necessary (Loebinger et al., 2009).

Nowadays, Tumor necrosis factor (TNF) -related apoptosis-inducing ligand (TRAIL) is a candidate for cancer therapy (Ashkenazi et al., 1999) that as a death ligand, without affecting the normal cells, may cause apoptosis in a variety of human tumors; in fact, this ligand is a part of the immune system that helps prevent the formation and spread of tumors (Wiley et al., 1995; Mahalingam et al., 2009). By binding to the death receptors (DR) particularly on TRAIL-R1/DR4

and TRAIL-R2/DR5 this ligand causes to form the death complex, and ultimately apoptosis (Sprick et al., 2000).

In this study, The HEK 293 cells were transduced with a vector that encodes the human TRAIL gene and the green fluorescent protein (MIGR1-TRAIL-GFP). The studies suggest that the modification of HEK 293 cells with a vector that encodes TRAIL gene, express a protein that is released naturally as a soluble ligand into the environment (Wiley et al., 1995) and environmental factors in the adjacent of CLL cells affect the survival of them (Panayiotidis et al., 1996; Lagneaux, et al., 1998). In this study, the effect of Hek cells new

Materials and Methods

To produce viral particles, 24 hours before transfection, HEK 293 cells were cultured in a 10 cm plate 2.5×10^6 , when confluency of cells reached approximately 60-70% lentiviral plasmids containing transgene of the full-length human TRAIL with fluorescent proteins (MIGR1-TRAIL-GFP) were transduced with a rate of 10µg along with packaging plasmid psPAX2 and envelope plasmid pMD2 each were mixed with Lipofectamine with quantities of 5µg and according to the protocol of lipofectamin 2000

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(Invitrogen) were transfected into the HEK 293 cells. About 24 and 48 hours after transfection the green points as a sign expressing GFP was observed by fluorescent inverted microscope (Olympus IX 71) and after 72 hours the supernatant containing lentivirus particles was fully collected and filtered through a 0.45µM filter and stored, in a temperature of -70°C in the refrigerator.

Cell collection

After the consent was obtained from the patients diagnosed definitely as B-CLL, 5cc of peripheral blood was drawn, Peripheral blood mononuclear cells (PBMCs) were isolated using cold ficoll with density gradient and were positive by flow cytometry markers of CD19+/CD5 +/CD23 + in the cells (median: 95.0%).

For isolating B-CLL cells from other cells the magnetic bead activated cell sorting (MACS) using the B Cell Isolation Kit (B -CLL) was performed according to the protocol of the manufacturer (MiltenyiBiotec) (Seiffert et al., 2010; NwaboKamdje et al., 2012).

Transduction of the HEK 293

To transduce again the HEK 293 cells at a rate of 35×10^3 in 24-well plates in culture medium DMEM (Sigma Aldrich) with high glucose containing pyruvate and L-glutamine (GIBCO), 10% serum inactivated FBS by heat, 3.7g/L sodium bicarbonate (Sigma Aldrich), non-essential amino acid 1% (Sigma Aldrich) were cultured without antibiotics. Culture of cells was considered as a zero day. The cells were incubated in an incubator with a temperature of 37°C and 5% CO₂ for 24 hours. On day one i.e. 24 hours after seed, cells adhered to the floor of the container and 70% of the plate was filled by cell. At this time 200 µl of lentiviruses (previously produced) transduced to the HEK 293 cell and incubated in an incubator 37°C and 5% CO₂ for one hour. After one hour the supernatant was collected and was discarded and a perfect medium was added. In this study, four groups were considered:

- (1) 293H cells that were transduced with vector coding for TRAIL (MIGR1-TRAIL-GFP)
- (2) GFP vector that were transduced with GFP vector only (MIGR1- GFP)
- (3) Plate seeded with 293 H(WT HEK)
- (4) CLL cells were cultured alone

Co-culture of CLL cells with HEK 293

The next day (day two) the HEK 293 cells transduced by confluent monolayer were observed by fluorescent inverted microscope (Olympus IX 71) and after confirmation of GFP expression, these cells were co-cultured with B-CLL cells. Before co-culture, the live CLL cells that were isolated the same time (by a method described above) were detected using Trypan blue dye and counted by hemocytometer and in the second day were added to the studied groups in a certain proportion (with three repeats per each group).

The ratio of HEK cells to the cancer cells was approximately 1 to 5, which ability of HEK cell transduced for the death of CLL cell was examined during 24 hours of co- culture. The amount of the TRAIL molecule in

the form of solution ligand in the culture medium was assessed in the days of two and three. In the third day the CLL cells floating in the medium were collected but the most of the CLL cells adhered strictly to the cells on the floor of the plate (HEK cells). The CLL cells were isolated with pipetting gently and exactly by ice-cold phosphate-buffered saline (NwaboKamdje et al., 2012).

ELISA.

An amount (with an amount of 100µl) of supernatant of the cell culture was removed in the second and third days and amount of the solution TRAIL in the culture medium was examined.

Samples were performed with human quantikine enzyme-linked immune sorbent assay (ELISA) kit as described by the manufacturer. Briefly, the ELISA plates were incubated with samples for one hour at 37°C, washed with wash buffer, and subsequently they were incubated with Detector antibody for one hour washed in the wash buffer. After addition of Streptavidin- HRP conjugate for 30 min, washed with wash buffer, dispense substrate and incubate in the dark for 30 minutes and colorimetric changes were measured at 450nm/630nm.

Apoptotic assay

Therefore, briefly HEK 293 cells were cultured in the 24-home plate (the day of zero). It is necessary to mention that in the fourth group only medium was added and then in the upcoming 24 hours (the first day) HEK 293 cells were transduced. On the second day after isolating the cancer cells, the co- culture with HEK 293 cells transduced and untransduced with the same ratio that was mentioned, was conducted. In the fourth group also CLL cells were cultured alone. After 24 hours of the co- culture, by collecting the cancer cells in the third day, apoptosis rate was assessed by annexin V (eBioscience) staining by fluorescence- activated cell sorting (FACS).

Statistical analysis

Data were tested using the Kruskal-Wallis and one-way analysis of variance test and Post Hoc Tukey test to compare multiple groups as well as internal comparison between multiple groups; in addition, data were analyzed using the EXCELL 2010 and SPSS 19 and P value with ≤ 0.05 was considered statistically significant.

Results

The HEK cells were transduced by lentiviruses (as described above), and these cells are capable for expressing the human TRAIL (MIGR1-TRAIL-GFP) after 24 hours. The HEK cells, which their genes were modified by TRAIL-encoding vector, are able to produce the related protein in the form of the solution ligand in the culture medium. The amount of the solution TRAIL in the culture medium in the second day in all groups, whether in a group that has been transduced (HEK TRAIL and HEK GFP) or not transduced (WT HEK, CLL), is extremely low (12.3 ± 0.5 pg/ml); however, on day 3 in the HEK TRAIL group (253 ± 25.1 pg /ml) significantly increased but the rest of the group had no significant change on day 3 (Figure 1).

When observing the HEK cells by florescent inverted microscope in the groups one and two, which were transduced by vector encoding the TRAILGFP gene, due to the GFP expression the green points were seen in these two groups that these points were in more and more specific rates (Figure 2). On the third day after collecting cancer cells the annexin V staining was used for examining the apoptosis rate in these cells. After analyzing the flow

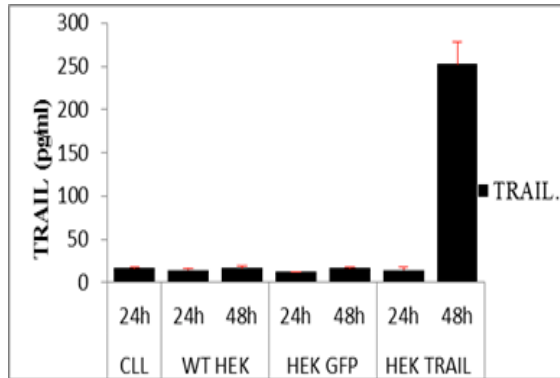


Figure 1. The Mean TRAIL in the Cell Culture Medium in the Different Groups During 24 hours (the Second Day) and 48 hours (the third day) After Transduction Using an ELISA kit

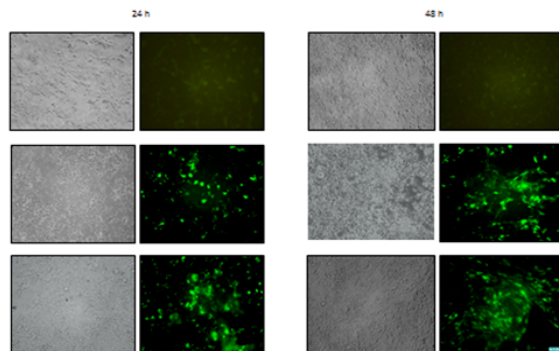


Figure 2. *In vitro* Cultures of CLL Cells with WT HEK Alone (top), CLL with HEK GFP (middle), and HEK TRAIL (Bottom) Visualized by Both Phase-Contrast and GFP Filter Fluorescence Microscopy at 1:5 T:E Ratio. Scale bar, 500 μ m. The percentages of fluorescent cells at day 2 (left) are even more prominent at day 3 (right)

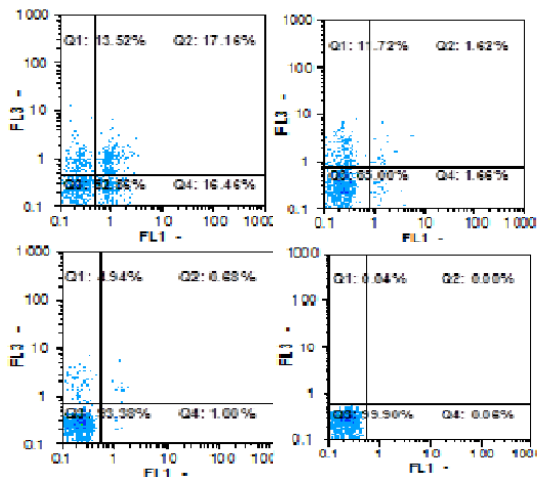


Figure 3. FACS Analyses of HEK TRAIL (Top, Right), HEK GFP (Top, Left), WT HEK (Bottom, Right), CLL (Bottom, Left). FL1 (Annexin V), FL3 (PI)

Table 1. Mean Cell Changes in Different Groups (%)

Variable	Changes Cells (x) (%)			
	G1	G2	G3	G4
Proapoptosis	32.8	8.6	2.1	0.04
Necrotic	29	11	9.5	0.07
Apoptotic	16.6	2.6	2	0.01

*Abbreviation: G1) HEK TRAIL group; G2) HEK GFP group; G3) WT HEK group; G4) CLL group

Table 2. A Comparison between the Cellular Changes in HEK TRAIL Group with Others Groups

Variable	Mean	"Std. Error"	Sig	Lower Bound	Upper Bound
Proapoptosis					
HEK GFP	24.1	3	0.00	14.4	33.8
WT HEK	30.6	3	0.00	20.9	40.3
CLL	32.7	3	0.00	23	42.4
Necrotic					
HEK GFP	18	2.4	0.00	10	25.9
WT HEK	19.5	2.4	0.00	11.5	27.4
CLL	28.9	2.4	0	20.9	36.9
Apoptotic					
HEK GFP	13.5	1.1	0.00	9.7	17.2
WT HEK	14.1	1.1	0.00	10.3	17.9
CLL	16.1	1.10	0.00	12.3	19.9

*Abbreviation: The mean difference is significant ($p \leq 0.05$)

Table 3. The Mean Proapoptosis and Apoptosis in the Homogeneous Groups

Variable	Proapoptotic	Apoptotic
HEK TRAIL	32.83	16.16
HEK GFP	8.66	2.60
WT HEK	2.16	2.00
CLL	0.04	0.01
Sig	0.08	1.00

*Abbreviation: Uses Harmonic Mean Sample Size=3.000

cytometry data the rate of apoptosis and proapoptosis in these groups was determined (Figure 3). PI-positive staining cells in co-culture are necrosis, cells that were positive by annexin V, were affected by proapoptosis and cancer cells that were positive by annexin V and PI, were apoptosis.

Table 1 shows the mean proapoptosis and necrosis apoptosis rate in each four groups separately. As it can be seen, a considerable difference exists between the HEKTRAIL group with other groups in proapoptosis and apoptosis rate. A significant difference exists in cancer cells ($p < 0.05$) between the HEKTRAIL group with other groups (Table 2). The results show that no significant difference exists between three groups that have not modified by TRAIL gene (Table 3).

Discussion

Cancer is the main cause of death throughout the world so that during 2010 in USA 25% of the deaths in all ages is related to the cancer (1).

So, the cancer necessitates new treatments. Today lentiviruses vectors, which are prepared by an immune deficient virus, are used for gene therapy. This virus is a strong tool for providing gene to the different cells. Nowadays lentiviruses vectors are used for providing gene in the laboratory environment and in the human body. The 293T HEK cells are able to produce lentiviruses vectors; in

this study these cells have been used. By gene modification of these cells the TRAIL is made and released in a soluble form. Today, Adipose-derived mesenchymal stem cells (AD-MSD) are transduced by lentiviruses and used as a constant source for producing the soluble TRAIL. The TRAIL has this feature that can be used as an antitumor factor on the tumor cells and their metastases (Loebinger et al., 2009), oral cancer (Chen et al., 2013), glioma (Kim et al., 2009), cervical carcinoma (Grisendi et al., 2010). Because it causes the death selectively only in the tumor cells not in the normal cells (Walczak et al., 1999; and Duiker et al., 2006). Recently this antitumor ligand has been used in the studies that results of all these studies show an antitumor effect of the TRAIL: oral cancer (Chen et al., 2013), glioma (Kim et al., 2009), cervical carcinoma (Grisendi et al., 2010).

Since no certain treatment exists yet for CLL disease, in this study we used TRAIL for apoptosis of CLL cells. This molecule in the form of the solution releases into the environment. On the third day, really 48 hours after transduction, the amount of this ligand in the culture medium has had a considerable increase. The past studies have reported that the secretion of TRAIL begins six hours after the transduction and its rate of the release in 12, 24 and 48 hours after the transduction is constant that it is more than 366.4pg/ml (Grisendi et al., 2010). Compared with the results of other studies, the amount of soluble TRAIL in our study is lower, which can be due to lack of virus titration; in addition, titration and the concentration of the virus by doing transduction the performance increases and more cells would be enforced to release TRAIL. In GFP Vector and other groups at all times the amount of soluble TRAIL has been the non-measurable (Grisendi G et al., 2010) but our data on average (12.3 ± 0.5 pg/ml) has shown perhaps all the cells release a very slight amount of the TRAIL.

In this study, the data suggest that CLL cells are sensitive to TRAIL so that even with the optical microscope it can be observed that with the passage of time more debris will be more evident in the environment so that the flow cytometry analysis also have confirmed apoptosis of CLL cells. In this study, the cancer cells have been only 24 hours in the vicinity of the TRAIL, and among the groups, the HEK TRAIL group has had a higher proapoptosis and apoptosis. Of course, this difference can be justified due to the higher concentration of the TRAIL in the cell culture environment.

On the other hand, compared to study conducted by Cho SA et al., 2011, the low level of TRAIL effect in CLL cells against brain stem Glioma can be due to the low concentration of the TRAIL in the cell culture medium that is not enough to stimulate the cancer cell death Or it can be due to the slight time of adjacency of the cancer cells to TRAIL or a change in the ratio of the HEK cells to the cancer cells, meaning that perhaps the number of HEK cells is not enough to feed the tumor. With the increase in HEK cells compared to cancer cells cancer cells may be placed in the vicinity of more TRAIL. As can be observed in table 1, in the HEK TRAIL group the percentage of cells that are in proapoptotic phase 32.8% is higher apoptosis 16.6%. More time may be needed until the cells have

ample opportunity to enter into a phase of apoptosis.

The TRAIL can be used also in combination with effective factors in inducing the apoptosis in the treatments; thus the efficiency of TRAIL can be increased (Zhao B et al., 2011 Jiang et al., 2013; HengXu et al., 2014).

In this study, not only as an anti-proliferative effect or even as a powerful inhibitory the TRAIL ligand is able to affect on CLL cells. With consideration of the impact of TRAIL on CLL cells and due to lower durability HEK cell, in future Adipose-derived mesenchymal stem cells (AD-MSD) will be used for delivering TRAIL.

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