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Klotho—a Common Link in Physiological and Rheumatoid Arthritis-Related Aging of Human CD4⁺ Lymphocytes

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Human CD4⁺ T lymphocytes undergo aging-related changes leading to decreased immunity to infections and neoplasms, and to increased frequency of autoimmune diseases including rheumatoid arthritis (RA). Certain changes, observed in the CD4⁺ cells of RA patients, resemble those observed during physiological aging, but occur at earlier age. Underlying cellular mechanism(s) of these similarities are so far largely unknown. Here we show that KLOTHO, a β-glucuronidase gene whose activity changes are associated with aging phenotype, is down-regulated at the mRNA, protein, and enzymatic (β-glucuronidase) activity levels both in the healthy elderly and especially in RA CD4⁺ lymphocytes. Although the exact role of Klotho activity for CD4⁺ cell function is unknown, we propose here that it might be involved in anti-inflammatory processes occurring in the young and healthy individuals, but reduced in both healthy elderly and RA patients. To support this hypothesis, we show here that the reduction of Klotho expression and activity in both elderly and patients’ lymphocytes occurs in concert with the down-regulation of T cell costimulatory molecule CD28, the latter known to be dependent on increased levels of TNF-α. Thus, a common mechanism of KLOTHO down-regulation, but executed at various times in life, may underlie both physiological and disease-related T cell aging. Klotho activity might become a target of anti-RA drug development as well as a tool to help increase the immune system efficiency in the elderly. The Journal of Immunology, 2007, 178: 771–777.

Impaired KLOTHO gene activity is apparently associated with aging process (1, 2). Klotho knockout mice age faster than their wild siblings and their phenotype shows multiple features of accelerated aging, including multiorgan failures, greying, and osteoporosis (1). People exhibiting a polymorphic variant of KLOTHO gene are more prone to osteoporosis, arterial hypertension, atherosclerosis, emphysema and cognitive impairments, and might exhibit reduced lifespan (3–7), which directly implicates Klotho in the mechanisms of human aging, coining it a name of “aging hormone” (8, 9). It was recently shown that the KLOTHO gene product possesses β-glucuronidase activity (10), although its precise role in the regulation of intra- and extracellular processes, and especially participation (if any) of Klotho in the immune cell function, is still unknown. The “helper” CD4⁺ T lymphocytes of mammals and man undergo physiological aging, considered a major factor leading to increased loss and/or aberration of the immune system function with advanced age (11, 12). Characteristic features of the aging peripheral blood CD4⁺ lymphocytes include reduced proliferative capabilities, changed cytokine secretion patterns and lower proportion of “new emigrants” from the thymus retaining circular DNA fragments resulting from TCR gene rearrangement (TCR rearrangement excision circles) (13–15). Telomere shortening, reduced TCR repertoire (ability to recognize new Ags) and accumulation of memory (CD4⁺CD45RO⁺) lymphocytes and of the CD4⁺ cells that lack CD28, the main costimulatory molecule required for adequate activation of these lymphocytes upon Ag challenge (CD4⁺CD28⁻), with profound changes in the signal transduction mechanisms, are the cellular background for the above (14, 16–20). One of the immune system aberrations associated with advanced age is an increasing frequency of autoimmune diseases, including rheumatoid arthritis (RA)—a chronic, inflammatory disease with very high impact on the quality of life of a huge proportion (~1%) of world population, permanently crippling ~30% of sufferers (21). Estimated lifespan of RA patients is 10–15 years shorter than that of healthy age-matched cohort (21). The disease manifests itself as proliferative inflammation of the intraarticular synovial tissue and at its advanced stages is usually involving progressive destruction of multiple symmetric joints and adjacent bones. Systemic changes including aggravated osteoporosis, pleuritis and pericarditis, inflammation of heart and blood vessels, decreased hematopoiesis, and amyloidosis, may accompany joint destruction and related symptoms (22). Immunological etiology of RA comprises mainly of impaired function of the CD4⁺ cells, thought to affect function of synovium inducing its inflammation (23, 24). Changes are observed both in the peripheral blood T cells of RA patients as well as in their counterparts residing in the inflamed synovium; in fact, CD4⁺ lymphocytes were shown to traffic between the blood, synovium and lymph node compartments (25). Phenotypic changes observed in the peripheral blood CD4⁺ cell compartment of RA patients include accumulation of both memory CD4⁺CD45RO⁺ lymphocytes and of the CD4⁺ cells lacking CD28 (CD4⁺CD28⁻) (23, 26). Average number of CD28 molecules per cell is significantly decreased also in those CD4⁺ cells of RA patients that did not

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3 Abbreviations used in this paper: RA, rheumatoid arthritis; CQ, chloroquine; DAS, disease activity score; DSL, di-saccharide lactone; KAAL, Klotho-associated α-like sequence; MPI, mean fluorescence intensity.

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loose it completely, due to increased amount of proinflammatory cytokine TNF shown to reduce the expression of the CD28 gene (27). Peripheral blood CD4⁺ cells of RA patients exhibit decreased proliferation in response to in vitro stimulation (26, 28), which is associated with their significantly shorter telomeres (29). They also contain lower proportion of cells retaining TCR rearrangement excision circles than those of age-matched healthy people (29) and the TCR repertoire is reduced in this cohort (28, 30).

Taken together, the abovementioned features of the peripheral blood CD4⁺ lymphocytes of RA patients closely resemble those described during physiological aging, a fact which lead to a suggestion that RA might be related to or even a cause of accelerated aging of the T cell population (26, 28, 30). In this work we attempt to investigate the participation of KLOTHO—a gene deeply involved in human aging, but until now not associated with the immune system aging—in physiological and disease (RA)-related aging of human CD4⁺ lymphocytes.

Materials and Methods

Patients

Altogether, 14 RA patients aged 21–98 years (average 41.5 ± 19.8 years) and 15 age- and sex-matched healthy people (average age 41.4 ± 20.4, range 29–76 years) took part in the study. Healthy people above the age of 65 years conformed to the SENIEUR Protocol criteria (31). All participants were informed about the purpose of the tests and gave their written informed consent; the project was approved by the Local Committee for Biomedical Research Ethics at the Medical University of Gdańsk. Diagnosis of RA was based on American College of Rheumatology criteria (32). Average RA disease activity score DAS28 (32) was 4.5 ± 1.3 (range 2.4–6.6; 14.3% exhibited low, 64.3%—moderate and 21.4%—high level of disease activity at the time of study), while average period of disease duration since diagnosis was 5.3 ± 5.9 years (range 0–20 years). Half (7 of 14) of the RA patients were tested at diagnosis and received no specific treatment at the time of study; another half received specific treatment consisting of typical doses of corticosteroid alone (2 of 14) or in combination with methotrexate (3 of 14), methotrexate alone (1 of 14) or arecine (1 of 14) for variable times before the study. Both the patients and healthy individuals reported sporadic usage of NSAID medication within a month preceding the study; the frequencies of their use were similar for both groups.

Peripheral blood mononuclear cell isolation, immunomagnetic purification of CD4⁺ lymphocytes, flow cytometry analysis, and quantification of CD28 on peripheral blood CD4⁺ cells

For estimation of relative level of expression of CD28 on CD4⁺ lymphocytes, PBMC isolated by flotation over Histopaque (Sigma-Aldrich) cushion were mixed with PerCys-anti-human CD4 (DakoCytomation) and R-Phycoerythrin labeled anti-CD4 (BD Biosciences) at 1 µg of Ab per 10⁵ cells for 30 min at room temperature in the dark, washed with PBS and analyzed by flow cytometry (FACScan; BD Biosciences). CD4⁺ lymphocytes were immunomagnetically purified using the Dynal CD4 Negative Isolation Kit (Invitrogen Life Technologies) according to the manufacturer’s instructions; samples of such purified cells were stained with PerCys-anti-human CD4 plus RPE-anti-human CD8 (both DakoCytomation) mAbs for estimation of purity, using analogous staining conditions. Raw data were acquired with dedicated CellQuest software (BD Biosciences) and analyzed with WinMDI 2.9 (J. Trotter; The Scripps Institute). The mean fluorescence intensity (MFI) of cell-bound PE-anti CD28 Ab was obtained upon appropriate gating of the CD4⁺ population within the PBMC and adopted as a measure of CD28 expression.

RT-PCR estimation of KLOTHO gene expression

Total RNA was isolated from 2 × 10⁵ immunomagnetically purified CD4⁺ lymphocytes per sample using TriReagent (Sigma-Aldrich) and manufacturer’s protocol, and immediately converted to cDNA, using the Improm-II Reverse Transcription System (Promega). PCRs to detect the products of KLOTHO and β-actin genes were performed using the PCR Core System (Promega) reagents and thermostable Taq polymerase. Following pairs of primers were used for KLOTHO: sense 5'-GCTTTCGATGACCTTG-3' and antisense 5'-TGGTAACTCTGGCACCCTG-3', and for β-actin: sense 5'-CACTGTCCAGCCTTTGATTT-3' and antisense 5'-GGTCACTTACAGCAGATGT-3'. Amplification of both products was performed using the Eppendorf Personal Mastercycler and following reaction conditions:

- For estimation of relative level of expression of CD28 on CD4⁺ lymphocytes:
  - Primer for human KLOTHO (GenBank accession no. H11032): sense 5'-GCTTTCCTGGATTGACCT-3' and anti-sense 5'-GCTTTCCTGGATTGACCT-3'.
  - Primer for housekeeping gene β-actin:
    - Forward: 5'-GTCCACCTTCGCTTGAGAATG-3'
    - Reverse: 5'-ACCTTGACACTTCGGCCACTG-3'.

- For estimation of relative level of expression of β-actin gene product:
  - Forward: 5'-GTCCACCTTCGCTTGAGAATG-3'
  - Reverse: 5'-ACCTTGACACTTCGGCCACTG-3'.

- Conditions for amplification:
  - 45 cycles of 10 s at 95°C; 30 s at 55°C, and 1 min at 72°C.

- Quantification of PCR products was performed using the LightCycler Software 4.05. The reaction was performed using 10-min activation at 95°C, followed by 40 cycles composed of 10 s at 95°C; 5 s at 55°C and 10 s at 72°C each, followed by 30-s cooling at 40°C. Results for KLOTHO gene product quantitation were presented as proportion of the amount of accumulated gene product to that of β-actin gene product.

Western blot analysis of expression of Klotho protein in isolated CD4⁺ lymphocytes

Protein composition of lysates from two million immunomagnetically purified CD4⁺ lymphocytes per sample was resolved using standard SDS-PAGE technique according to Laemmli (34). Proteins were transferred to nitrocellulose membrane using the Trans-Blot SD SemiDry Transfer Cell (Bio-Rad), the membrane blocked with 5% no-fat milk and probed for Klotho using rabbit anti-Klotho affinity pure Ab (Gentaur) at 2 µg/mL used for actin (gel loading control) using mouse mAb to β-actin (Abcam) diluted 1/1000.

- Appropriate peroxidase-conjugated anti-Ig Abs and ECL system (Super Signal West Pico Chemiluminescent Substrate; Pierce) were used to detect and visualize the proteins of interest. The bands were recorded on x-ray film, digitized using the GDS-8000 instrument and quantified using the LabWorks software (both from Ultra-Violet Products).
- Relative amounts of Klotho protein were expressed as arbitrary densitometric units after standardization vs actin content.

Estimation of β-glucuronidase activity associated with Klotho product

Flow cytometric assay to detect and compare Klotho-associated β-glucuronidase activity was performed according to Refs. 10 and 35. Briefly, 2 × 10⁵ of the immunomagnetically purified CD4⁺ lymphocytes suspended in biotin- and flavin-free medium containing 11 mM glucose and 4% FCS in PBS were incubated with 100 µM fluorescein-di-β-D-glucuronide for 60 min at 37°C. The reaction was stopped by fourfold dilution in ice-cold staining medium, then the cells were immediately washed in ice-cold substrate-free staining medium and stored on ice until FACS analysis. In separate samples, Klotho-associated β-glucuronidase activity was inhibited by preincubation of cells with 5 mM 1,4-di-saccharolactone (DSL), and total β-glucuronidase activity by treatment of the cells with 0.3 mM chloroquine (CQ) (35) for 30 min before addition of fluorescent substrate and throughout the reaction time. MFI of cells containing converted substrate was measured after exclusion of dead cells by propidium iodide staining and subtraction of background fluorescence of substrate-free cells. The DSL-sensitive (Klotho-associated) activity of β-glucuronidase was expressed as arbitrary units, calculated as a difference between the background-adjusted MFI corresponding to total (CQ-inhibited) β-glucuronidase activity and that obtained for cells pretreated with DSL.

Database search for the sequence homologous to a site of the minimal promoter of CD28 gene in the vicinity of KLOTHO gene

Human KLOTHO gene is contained within the 13th chromosome (GenBank accession no. Z92540). The target α sequence of CD28 minimal promoter (5'-ACGTATATCTGGTGTAAGAT-3') was taken from (27, 36, 37) and inserted in the National Center for Biotechnology Information BLAST-n online service (38) which was used for online GenBank database search and identification of the sequences. A 66 bp homologous sequence 5'-GCTCTATATCTGGTGTAAGAT-3' (Klotho-associated α-like, KAAL) was found at 46 kilobase pairs 5' from the initial ATG sequence of the KLOTHO gene.

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initial denaturation 94°C, 10 min; 30 amplification cycles containing melting for 30 s at 94°C, annealing 30 s at 55°C, and elongation 30 s at 72°C; after last cycle termination 10 min at 72°C followed by cooling and storage at 4°C. PCR products were resolved in standard 2% agarose gel with ethidium bromide and band fluorescence visualized using the GDS-8000 Image Acquisition and Analysis System and LabWorks software (both from Ultra-Violet Products). Densitometric data obtained for KLOTHO product bands were standardized using the level of expression of β-actin as housekeeping gene.

Real-time PCR quantification of KLOTHO expression

The same primer pairs as used for RT-PCR were also used for the real-time PCR. Products of KLOTHO and β-actin genes were amplified by PCR as above to prepare the standard curves for both quantifications. For obtaining enough KLOTHO gene product, total RNA was isolated from samples of healthy human kidney tissue (33) obtained during elective surgery. LightCycler and FastStart DNA Master SYBR Green I Kit (Roche Diagnostics) were used for real time PCR. RT-PCR was performed using 10-min activation at 95°C, followed with 40 cycles composed of 10 s at 95°C; 5 s at 55°C and 10 s at 72°C each, followed by 30-s cooling at 40°C. Results for KLOTHO gene product quantitation were presented as proportion of the amount of accumulated gene product to that of β-actin gene product.
EMSAs comparison of T cell nuclear protein binding to the α and α-homologous Klotho (KAAL) sequences

The procedure was performed according to Refs. 36 and 39. The nucleotide sequences identical to the α and to KAAL were synthesized, 32P-tagged and applied for EMSA test, using T cell nuclear proteins isolated according to Refs. 36 and 39. For competition experiments, “cold” nonradioactive oligonucleotides identical with the α, KAAL and irrelevant, nonhomologous sequence corresponding to the Ig switch region recognized by a B cell specific transcription factor LR1 (40, 41) (5'-CCTGGGTCAGCGCGGCTAGA-3’) were synthesized. These were incubated with the nuclear proteins at concentrations 20, 100, and 300 times exceeding these of radioactive probes, for 1 h before the exposure to 32P probes, according to Refs. 36 and 39.

Statistical analysis

Statistical description, regression analysis and calculations of significance of observed differences were performed with the help of StatSoft. STATISTICA (data analysis software system), version 7.1 (www.statsoft.com). Depending on the normality (or lack of it) of data distribution, averaged values were presented either as means ± SD or as medians ± 25th and 75th percentile, as indicated in the figure legends. Accordingly, Student t test for independent data or Mann-Whitney U test were used for assessment of significance of differences.

Results

Reduction of KLOTHO gene expression in CD4+ cells of elderly and RA patients

When assessed by RT-PCR, KLOTHO gene is transcriptionally active in the purified resting CD4+ lymphocytes of healthy individuals, and apparently inactive in the same cell type of RA patients (Fig. 1A). It has to be stressed that the amount of the KLOTHO gene product is rather small even in the cells of healthy people (~1/10th of the product of simultaneously assessed reference gene—β-actin). Yet even triplication of the amount of cDNA isolated from the RA CD4+ cells in the PCR mixture did not result in the appearance of visible KLOTHO gene product bands (data not shown).

To check if apparent lack of the KLOTHO gene product in the lymphocytes of RA patients was genuine (i.e., the gene was turned off), or the level of its transcription was below the sensitivity threshold of the RT-PCR method, we used the real time PCR to quantify the KLOTHO gene activities in the CD4+ lymphocytes isolated from both healthy and RA individuals. We have found that by real time PCR the amount of Klotho mRNA standardized to that of β-actin mRNA is extremely low even in CD4+ cells of healthy subjects; however, some transcriptional activity of the KLOTHO gene in the CD4+ lymphocytes of RA patients could in fact be demonstrated. It was significantly lower than that recorded for the cells of healthy people (Fig. 1B). We have observed a huge variation in the amount of real time PCR product among the healthy individuals and, when analyzed as a function of age, a significant negative correlation could be demonstrated (Fig. 2). No such correlation could be shown between the age and amount of KLOTHO gene product for RA patients, where even for young subjects the amount of product was very low (Fig. 2). Although the RA patients differed with respect to the disease activity (DAS28 score) and their time from initial diagnosis varied significantly, neither disease activity nor its duration correlated with the levels of KLOTHO expression assessed by either RT-PCR or real time PCR. Similarly,

![FIGURE 1](image1.png)

**FIGURE 1.** Transcriptional activity of KLOTHO gene is significantly reduced in the CD4+ cells of RA patients. A. By RT-PCR, strong bands of reaction product can be seen in healthy (upper panel) but not in RA material (lower panel). Results for RT-PCR for β-actin given as sample uniformity control. Representative results from 4 of 12 pairs (1 to 4, healthy and RA each) of subjects tested pairwise in separate experiments. B. By real-time PCR, KLOTHO transcriptional activity relative to that of β-actin gene is significantly reduced in RA material (bars: median ± 25th and 75th percentile; *, p = 0.021 by Mann-Whitney U test, n = 14 for RA and 15 for healthy group).

![FIGURE 2](image2.png)

**FIGURE 2.** KLOTHO expression in the CD4+ lymphocytes of healthy individuals inversely correlates with age. Relative KLOTHO expression in the CD4+ lymphocytes assessed by real-time PCR is significantly, inversely correlated with age for healthy (black symbols, r = −0.51, p = 0.025) but not for RA subjects (open symbols, r = 0.05, p = 0.87).

![FIGURE 3](image3.png)

**FIGURE 3.** Klotho protein amount in the CD4+ lymphocytes of RA patients is significantly reduced compared with healthy individuals. A. Representative (of three experiments yielding similar results) Western blot detection of Klotho and actin (lane loading control) proteins in CD4+ samples from a healthy and two RA individuals. B. Statistical analysis of the results illustrated in A. The amount of Klotho protein per 1 million CD4+ cells is given as arbitrary densitometric units corrected for actin contents. Note significant decrease of Klotho protein expression in the cells of RA patients compared with those of healthy ones (bars: mean ± SD; p = 0.033 by two-tailed Student t test for independent data, n = 3 for each group). Results for kidney Klotho expression serve as positive control.
there was no observable influence of pharmacological treatment. RA patients who were tested at diagnosis, before any specific treatment was started, exhibited the same negligibly low levels of the gene’s expression by RT-PCR as those methotrexate and/or corticosteroid treated. This notion was corroborated by finding that the data variability was actually less for the RA samples than for healthy ones (Figs. 1–3). Also, when the results of real-time PCR were grouped according to “pharmacologically treated” or “untreated” status the difference between both groups was numerically negligible and statistically insignificant, while that between either group and healthy remained significant (not shown).

Decreased amount of Klotho protein in RA lymphocytes
At the protein level, we have shown by western blotting that the amount of Klotho protein in the CD4$^+$ lymphocytes of healthy subjects was at least three to five times lower than in the same quantity of cellular protein from human kidney, known to contain detectable amounts of Klotho protein (33) (Fig. 3). In the CD4$^+$ cells of RA patients amount of Klotho protein is very low and significantly decreased in comparison to that seen in the cells of healthy individuals (Fig. 3). No significant correlations of the amount of Klotho protein with age of donors could be seen. Interestingly, a second band at ~58 kDa molecular mass could be seen in the samples obtained from both kidney and healthy CD4$^+$ cells; this band, probably corresponding to the secretory form of Klotho (42), was absent from the RA samples (Fig. 3A).

Klotho-associated $\beta$-glucuronidase activity is reduced in the CD4$^+$ cells of both healthy elderly and RA individuals
It was recently shown that Klotho might exhibit an enzymatic activity of $\beta$-glucuronidase (10). Therefore, we have compared both total (CQ-sensitive) and Klotho-dependent (DSL-sensitive) $\beta$-glucuronidase (10, 35) activities in the purified CD4$^+$ lymphocytes of healthy and RA individuals, using flow cytometry. We have found that both total and Klotho-dependent activities of $\beta$-glucuronidase are significantly decreased in the CD4$^+$ lymphocytes of RA patients compared with healthy individuals (Fig. 4). The proportion of the latter (putative Klotho-associated $\beta$-glucuronidase activity) in the total activity of the enzyme in CD4$^+$ lymphocytes was also significantly reduced in the patients’ cells (0.38 ± 0.16 vs 0.66 ± 0.13 in the healthy, $p = 0.05, n = 12$ for each group; Student $t$ test for independent groups). Klotho-associated activity of $\beta$-glucuronidase was significantly correlated with the amount of KLOTHO gene mRNA product ($r = 0.596, p = 0.04$) but only for CD4$^+$ lymphocytes of healthy subjects; no such correlation could be demonstrated for RA cells. Interestingly, both the total and Klotho-associated $\beta$-glucuronidase activities were significantly, negatively correlated with age, but only in the group of healthy individuals; again, no such correlation could be seen for the cells of RA patients (Fig. 5).
Expression levels of Klotho and CD28 are correlated

Next we attempted to assess the possibility of any relation between the levels of KLOTHO gene expression and Klotho-associated β-glucuronidase activity and the level of CD28 expression on CD4+ lymphocytes. The amount of KLOTHO gene product as assessed by real time PCR strongly correlated with the relative level of expression of CD28 (measured as MFI of PE-antiCD28—stained CD4+ cells determined by flow cytometry. Activity of KLOTHO and expression of CD28 are significantly correlated for CD4+ cells of RA (open symbols, r = 0.64, *, p = 0.018) but not healthy subjects (black symbols, r = 0.47, p = 0.14).

KLOTHO and CD28 genes might be coregulated

It has been recently demonstrated, that the decrease in the expression of CD28 on RA lymphocytes is related to the action of TNF on the initiator/regulatory element (called the α site) in the CD28 gene promoter (27, 37). Using the GenBank database we have found a DNA sequence homologous to the α site (Klotho-associated α-like, KAAL) at 46-kb pairs 5’ from the initial ATG sequence of the KLOTHO gene. The sequences share 66.6% overall homology, with 100% identity of central 12-nucleotide fragments (5’-TATATCTGTGTT-3’), identified for the α site to be the most important protein-binding part that presumably influence the activity of CD28 promoter (27, 36, 37). To check if the KAAL is capable of binding any T cell nuclear proteins and if α and KAAL sequences might bind to similar protein repertoire we have performed the EMSA and competitive EMSA experiments. The result of a representative EMSA experiment is shown in Fig. 7A. It can be seen that both α and KAAL sequence bind T cell nuclear proteins of apparently the same molecular mass, although KAAL is also binding protein(s) or their complexes with lower molecular weights. There is also some degree of competition for the nuclear proteins between both sequences, as can be assumed from the results of competitive EMSA experiments in which preincubation with “cold” KAAL oligonucleotide reduces binding of T cell nuclear proteins to the α sequence (Fig. 7B).

Discussion

Growing amount of data is pointing toward the possibility that RA is associated with (or even stronger, a cause or consequence of) accelerated, precocious aging of the CD4+ T lymphocytes (26, 28). The mechanisms of T cell aging itself, despite extensive effort of multiple groups, are still largely elusive (reviewed in Refs. 43–45). Thus, observed parallelisms between the behavior of CD4+ lymphocytes in healthy elderly and in the RA patients are mostly descriptive and not answering to the question of underlying—possibly common—cause(s). Specifically, although the number of genes possibly involved in the process of aging has been variably described and not answering to the question of underlying—possibly common—cause(s). Specifically, although the number of genes possibly involved in the process of aging has been variably reported as hundreds or even thousands (46), there are only very few of them for which a direct participation in aging mechanisms had been demonstrated. One of these few is KLOTHO, known to participate in the aging-related phenomena in both mice and men (1–7), whose protein product had been recently named the “aging hormone” (8, 9). Data on possible participation of KLOTHO in the immune cell function are so far at most fragmentary and concerning murine immune system only; thus, it was shown that spleen and other immune organs of KLOTHO knockout kl-kl mice are underdeveloped and that B cell development and differentiation is impaired (1, 47). Currently, there are no published data on Klotho expression and/or involvement in the function of either murine or human T lymphocytes. In this work we have demonstrated that Klotho expression at the mRNA level significantly decreases in resting human CD4+ lymphocytes proportionally to advancing age and that it is heavily suppressed in the CD4+ cells of RA patients regardless of the patients’ age. We have also shown the reduction of Klotho protein level and, finally, the decrease of Klotho-associated β-glucuronidase activity in the RA patients’ lymphocytes. Neither the KLOTHO gene transcriptional activity nor the enzymatic activity of Klotho-associated β-glucuronidase in the patients’ lymphocytes was influenced by (sometimes prolonged)
pharmacological treatment or by the level of activity of the disease assessed with the DAS-28 score. In our opinion these finding indicate that the phenomenon of reduction of Klotho expression and associated enzymatic activity is characteristic for the disease itself and thus possibly (causatively?) involved in its mechanism, rather than being the disease’s consequence. In the cells of healthy subjects, the activity of DSL-inhibited β-glucuronidase and the amount of Klotho mRNA were significantly correlated, additionally (indirectly) confirming the identity of the enzymatic activity as associated with Klotho protein. Our observations suggest that the reduction of Klotho-associated enzymatic activity is at least partially dependent on the reduction of KLOTHO transcriptional activity and availability of Klotho RNA for translation; however, lack of significant correlation between this activity and Klotho mRNA level in RA cells points to the possibility of another—yet unknown—factor(s) affecting the enzyme, at least in the patients’ cells.

As we have shown, reduced Klotho mRNA levels are correlated with the level of CD28 expression on the CD4+ lymphocytes in both RA and (to a lesser extent) healthy lymphocytes. Lower level of correlation seen for healthy individuals might be due to lesser variability of CD28 expression on CD4+ cells in this population, even in advanced age (48).

We have found a sequence (KAAL) homologous to the α site controlling the activity of CD28 gene promoter in the vicinity of the KLOTHO gene and shown by EMSA and competitive EMSA experiment that the two sequences might share common, as yet uncharacterized, regulatory proteins. It is worth noting that the gene regulation (enhancing or silencing) had been reported over even much higher distances than that found between the KAAL sequence and the KLOTHO gene promoter, even up to >300 kbp (reviewed by Cook (49)). Klotho promoter itself seems to belong to TATA-less, CAAT-less promoters, heavily regulated by their ability to bind factors like the Sp-1(50). Thus, these observations may suggest a possibility that the two genes, KLOTHO and CD28, are coregulated in the CD4+ lymphocytes, possibly by the level of or the degree of reaction to TNF, already shown to regulate the promoter of CD28 by affecting the α (27). Recently two nuclear proteins, nucleolin and hnRNP-D0, were identified among those uncharacterized, regulatory proteins. It is worth noting that the gene regulation (enhancing or silencing) had been reported over even much higher distances than that found between the KAAL sequence and the KLOTHO gene promoter, even up to >300 kbp (reviewed by Cook (49)). Klotho promoter itself seems to belong to TATA-less, CAAT-less promoters, heavily regulated by their ability to bind factors like the Sp-1(50). Thus, these observations may suggest a possibility that the two genes, KLOTHO and CD28, are coregulated in the CD4+ lymphocytes, possibly by the level of or the degree of reaction to TNF, already shown to regulate the promoter of CD28 by affecting the α (27). Recently two nuclear proteins, nucleolin and hnRNP-D0, were identified among those uncharacterized, regulatory proteins. It is worth noting that the gene regulation (enhancing or silencing) had been reported over even much higher distances than that found between the KAAL sequence and the KLOTHO gene promoter, even up to >300 kbp (reviewed by Cook (49)). Klotho promoter itself seems to belong to TATA-less, CAAT-less promoters, heavily regulated by their ability to bind factors like the Sp-1(50). Thus, these observations may suggest a possibility that the two genes, KLOTHO and CD28, are coregulated in the CD4+ lymphocytes, possibly by the level of or the degree of reaction to TNF, already shown to regulate the promoter of CD28 by affecting the α (27). Recently two nuclear proteins, nucleolin and hnRNP-D0, were identified among those uncharacterized, regulatory proteins. It is worth noting that the gene regulation (enhancing or silencing) had been reported over even much higher distances than that found between the KAAL sequence and the KLOTHO gene promoter, even up to >300 kbp (reviewed by Cook (49)). Klotho promoter itself seems to belong to TATA-less, CAAT-less promoters, heavily regulated by their ability to bind factors like the Sp-1(50). Thus, these observations may suggest a possibility that the two genes, KLOTHO and CD28, are coregulated in the CD4+ lymphocytes, possibly by the level of or the degree of reaction to TNF, already shown to regulate the promoter of CD28 by affecting the α (27). Recently two nuclear proteins, nucleolin and hnRNP-D0, were identified among those uncharacterized, regulatory proteins. It is worth noting that the gene regulation (enhancing or silencing) had been reported over even much higher distances than that found between the KAAL sequence and the KLOTHO gene promoter, even up to >300 kbp (reviewed by Cook (49)). Klotho promoter itself seems to belong to TATA-less, CAAT-less promoters, heavily regulated by their ability to bind factors like the Sp-1(50). Thus, these observations may suggest a possibility that the two genes, KLOTHO and CD28, are coregulated in the CD4+ lymphocytes, possibly by the level of or the degree of reaction to TNF, already shown to regulate the promoter of CD28 by affecting the α (27). Recently two nuclear proteins, nucleolin and hnRNP-D0, were identified among those uncharacterized, regulatory proteins.


