Immunology
The Journal of cells, molecules, systems and technologies
British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Varying expression of four genes sharing a common regulatory sequence may differentiate rheumatoid arthritis from ageing effects on the CD4$^+$ lymphocytes

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doi:10.1111/j.1365-2567.2010.03341.x
Received 22 April 2010; revised 9 July 2010; accepted 15 July 2010.
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Summary

The *CD28* gene is similarly down-regulated in CD4$^+$ lymphocytes from both healthy elderly people and patients with rheumatoid arthritis (RA) because of impaired protein-binding activity of the 'α' sequence in its promoter region. Other genes important for the CD4$^+$ cell function may share that sequence and may be similarly regulated and affected. We searched GenBank for possible 'α' homologues and then compared transcriptional activities of the respective genes in the CD4$^+$ cells of young and older healthy individuals and those with RA by real-time PCR. We show here that genes encoding one of the zinc finger proteins (ZNF334), the 'aging hormone' Klotho, the retinoid acid receptor β2 (RARβ2) and the T-cell adapter protein GRAP-2, contain sequences with various (exceeding 70%) degrees of homology to the 'α' sequence near their promoters. These genes are transcribed in human CD4$^+$ lymphocytes; the expressions of *RARβ2*, *KLOTHO* and *ZNF334* are significantly decreased in a correlated manner in the cells of patients with RA compared with those of healthy individuals. In RA patients, the extremely reduced expression of *ZNF334* does not depend on the individual's age, apparently constituting a disease-related phenomenon; whereas that of *RARβ2* and *KLOTHO* occurs mostly in the cells of relatively younger patients, making them similar to the lymphocytes of healthy elderly in this aspect.

Keywords: aging; cell-signalling molecules; genomics; klotho; retinoic acid receptors; rheumatoid arthritis; T cells; zinc finger protein

Introduction

Impaired function of the CD4$^+$ lymphocytes in patients with rheumatoid arthritis (RA) is thought to affect function of the synovium inducing its proliferative inflammation, and to influence other functions of the immune system.[1–3] Peripheral blood CD4$^+$ cells of patients with RA exhibit decreased proliferation in response to stimulation,[1,4,5] which is associated with their significantly shorter telomeres.[6] They also contain lower proportions of early thymic emigrant cells retaining the T-cell receptor gene rearrangement excision circles than those of age-matched healthy people[6] and their T-cell receptor repertoire is reduced.[4,7,8] Furthermore, the CD4$^+$ lymphocytes of patients with RA exhibit partial or total loss of surface CD28 molecule, also described for the cells of healthy elderly individuals.[1,9,10] Taken together, the above-mentioned features of the peripheral blood CD4$^+$ lymphocytes of patients with RA 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.[5,8,11,12] Expression of the *CD28* gene is controlled by specific sequences (dubbed 'α' and 'β') in its promoter, that bind certain not fully known, presumably regulatory nuclear proteins, whose expression in turn is reduced by tumour necrosis factor (TNF), present in elevated amounts in the sera of both patients with RA and at least some healthy elderly. The decrease of CD28 expression on the surface of CD4$^+$ lymphocytes of both healthy elderly and the patients with RA results from the action of TNF.[9,10,13] The 'α' sequence of the CD28 promoter exhibits a strong homology (difference in just three out of 15 base pairs) to the initiator portion of retinoic acid receptor β2 (*RARβ2*) gene promoter,[14] which so far was not reported to be expressed in human T cells, known to exhibit other RAR receptors on

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their surface; their engagement might affect proliferation of these cells.[15] Therefore, it is conceivable that elevated amounts of TNF in patients with RA would affect the *RAR* expression also on the T cells of these individuals.

We have recently shown that the gene coding for the β-glucuronidase Klotho, sometimes called alternatively 'aging hormone' or 'youth fountain', contains the 'α'-homologous sequence in the vicinity of its promoter and that the expression of this gene is extremely reduced in the $CD4^+$ lymphocytes of patients with RA.[16] We hypothesize that 'α'-homologous sequences may be present also in the promoter or other regulatory regions of other genes expressed in human T cells and susceptible to similar regulation as *KLOTHO* and *CD28*, and that expression of these genes (including *RARβ2*) may be affected in RA and in healthy elderly individuals, possibly participating in the impaired function of these lymphocytes.

Materials and methods

Patients

Altogether, 16 patients with RA aged 21–98 years (average 41·5 ± 19·8 years) and 16 age-matched and sex-matched healthy people (average age 40·6 ± 17·7, range 29–76 years) took part in the study. Healthy people above the age of 65 years conformed to the SENIEUR Protocol criteria.[17] For some analysis the healthy individuals and those with RA were divided into 'young' [< 40 years of age, average 28·7 ± 3·9 years (healthy), 24·5 ± 4·3 years (RA); $n = 8$ for each group] and 'older' [$\geq$ 40, average 58·6 ± 15·5 years (healthy), 58·3 ± 16·1 years (RA); $n = 8$ for each group] age groups. Average disease duration was 3·3 years with the range 1–6 years. Patients were treated according to standard protocol with low doses of methotrexate. No biological treatment was applied to any of them. 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 Gdansk. Diagnosis of RA was based on American College of Rheumatology criteria.[18]

GenBank search for the genes containing homologues of the 'α' sequence

We used the online GenBank (BLAST-n) database search facility (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The target sequence of the 'α' site used was: 5′-GTTATATC-CTGTGTG-3′.[9,10,19]

Cells

Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque™ (Sigma Aldrich, St Louis, MO) flotation from venous blood; they were counted and their viability (accepted at least 95%) was estimated with trypan blue exclusion test.

Preparation of purified CD4+ lymphocytes for reverse transcription–PCR analysis

Resting $CD4^+$ lymphocytes were purified with the $CD4^+$ Negative Isolation Kit™ (Dynal Biotech, Oslo, Norway), and the Miltenyi (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) magnet according to the manufacturer's protocol. Purity of the $CD4^+$ cells obtained that way assessed by FACS analysis was always better than 97%. Enriched $CD4^+$ cells were flash-frozen in liquid nitrogen and stored at − 80° until further processing.

Real-time PCR estimation of RARβ2, ZNF334, GRAP-2 and KLOTHO *gene expression*

Total RNA was isolated from enriched $CD4^+$ cells of healthy individuals and patients with RA using TriReagent™ (Sigma Aldrich, St Louis, MO) and following the manufacturer's protocol. Complementary DNA was prepared using oligo-dT as starters and the avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). The following pairs of primers were used for detection of the product of expression of the genes of interest: for *RARβ2* sense: 5′-GGGTCAATCCACTGAAGCAT-3′, antisense: 5′-CTGGGAAAAAGACCAACAGC-3′; for the T-cell adapter protein gene *GRAP-2* sense: 5′-CACTTCAAG-GTCATGCGAGA-3′, antisense: 5′-TTCATCGAAGGTCG-GATTTC-3′; for the zinc finger protein gene *ZNF334* sense: 5′-AGGAAAGCCAGCAATCAAAA-3′, antisense: 5′-TCCAGTGAGGCTTGTCTTCA-3′, for *KLOTHO* sense: 5′-GCTTTCCTGGATTGACCTTG-3′, antisense: 5′-TGT-AACCTCTGTGCCACTCG-3′[16] and for β-actin-, sense: '5′-CACCTTCACCGTTCCAGTTT-3, antisense: 5′-GTC-CACCTTCCAGCAGATGT-3′. Products of *RARβ2*, *GRAP-2*, *ZNF334*, *KLOTHO* and β-actin genes were amplified by PCR using the same sets of primers to prepare the standard curves for the quantifications. Amplification of the products was performed using the Eppendorf Personal Mastercycler™ (Eppendorf AG, Hamburg, Germany) and the following reaction conditions: initial denaturation 94° for 10 min; 30 amplification cycles comprising melting for 30 seconds at 94°, annealing for 30 seconds at 55° and elongation for 30 seconds at 72°; after last cycle termination for 10 min at 72° followed by cooling and storage at 4°. LightCycler™ and FastStart DNA Master SYBR Green I Kit (both Roche Diagnostics, Roche Diagnostics Polska, Warsaw, Poland) were used for real-time PCR reaction, and analysis of raw data was performed using the LIGHTCYCLER SOFTWARE™ 4.05. The reaction was performed using 10 min activation at 95°, followed by 40 cycles of 10 seconds at 95°; 5 seconds at 55° and 10 seconds at 72° each,

followed by 30 seconds of cooling at 40°. Results were presented as the proportion of the amount of accumulated gene products to that of β-actin gene product.

Comparison of the 'α' and homologue sequences ability to bind T cell nuclear proteins by electrophoretic mobility shift assay

Oligonucleotide sequences identical to 'α' and its four homologues were synthesized, ^{32}P-tagged and confronted with the nuclear protein extracts.[16,19,20] The sequences were as follows: for the α site: 5′-ACG TTA TAT CCT GTG TGA AAT-3′, for *ZNF334*: 5′-TCC TTA TAT CCT GTG TGA TTC-3′, for *KLOTHO*[16]: 5′-GCT CTA TAT CCT GTG TCC ACA-3′, for *RARβ2*: 5′-ATC TTT CAT TCT GTG TGA CAG-3′, and for *GRAP-2*: 5′-TTG TGC ACT CCT GTG TGT ATA-3′.

Estimation of CD28 expression on CD4$^+$ lymphocytes

The PBMC were isolated as above and stained with a cocktail of FITC-conjugated anti-CD3, phycoerythrin-conjugated anti-CD28 and peridinin chlorophyll protein-conjugated anti-CD4 monoclonal antibodies for 30 min in the dark, washed and analysed by flow cytometry on a FACScan (Becton Dickinson, Sunnyvale, CA) with the help of WinMDI™ version 2.9 (J. Trotter, La Jolla, CA). Mean fluorescence intensity (MFI) of the phycoerythrin-conjugated anti-CD28 antibody bound to the CD3$^+$ CD4$^+$ lymphocytes identified by appropriate gating served as a measure of surface expression of the CD28 molecule.

Statistical analysis

Statistical description, regression analysis and calculations of significance of observed differences were performed with the STATISTICA data analysis software system, version 7.1 (StatSoft, Inc. 2005; http://www.statsoft.com). The results were presented either as means ± standard deviation (SD) or as medians ± 25th and 75th percentiles, as indicated in the figure legends, depending on the normality (or lack of it) of data distribution. Accordingly, Student's *t*-test for independent data or Mann–Whitney *U*-tests were used for significance assessment of differences.

Results

'α'-homologous sequences can be found in or near the promoter regions of many genes

The GenBank search confirmed that the promoter of the gene for RARβ-2 (retinoic acid receptor β2) contains an 'α'-homologous sequence.[14] In addition to *RARβ2* and previously described *KLOTHO*[16] we have also found that the gene *GRAP-2* (a lymphocyte-specific adapter molecule participating in signal transduction from the T-cell receptor–CD3 complex, known also under various synonymic names, including: Gads, GRAPL, Mona, Grf40 or Grid[21–24]) also contains the 'α'-homologue sequence in its promoter. The 'α' homologue site, TATA box and starting codons of the *GRAP-2* gene are located within a stretch of DNA about 100 bp long. Finally, we have also found that the promoter region of a member of the vast family of zinc finger regulatory proteins containing the Kruppel domain (KRAB), designated *ZNF334*, also contains the 'α'-homologous sequences (of varying degrees of homology) near its promoter. The level of homology of its sequence to the target 'α' sequence form of the CD28 promoter was the greatest (93%, even greater than that of 'α'-homologous sequence of *KLOTHO*, which we had reported earlier[16]; see below).

It is worth mentioning that sequences with at least 50% homology with the target sequence of 'α' were found in 759 human genes. However, we have limited the study group to just a few by excluding the genes in which the 'α-like' sequences were present within the transcribed exons or in the introns, and all those with level of homology to the target 'α' sequence below 70%. The homologies found (for the 20-nucleotide DNA stretches containing the target sequence) are shown below (target sequence in the 'α' and its homologues are marked in boldface, non-homologous bases are omitted for clarity and marked with '_'):

'α' site: 5′-AC**GTTATATCCTGTGTG**AAA-3′
ZNF334 5′-_ C_ **TTATATCCTGTGTG**A_ _-3′ (93% target sequence homology)
KLOTHO 5′-_ C_ **_TATATCCTGTGT_** _A_ -3′ (80% target sequence homology)
RARβ2: 5′-_ _ _ **TT_ _AT_ CTGTGTG**A_A-3′ (73% target sequence homology)
GRAP-2: 5′-_ _ **GT_ _ _ _TCCTGTGTG_** A_-3′ (73% target sequence homology).

As shown above, the sequence derived from the *ZNF334* gene shows the highest level of homology with that of α site, followed by these of *KLOTHO*, *RARβ2* and *GRAP-2*. Homology of the target 'α' sequence is reported to be important for binding of the TNF-dependent proteins[9]; (boldface above) and the corresponding region of *ZNF334* reaches 93%, that of *KLOTHO* reaches 80%, while those of *RARβ2* and *GRAP-2* are both 73%. As the reported, consensus target sequence is 15 nucleotides long[9], we arbitrarily accepted identity for at least 10 out of 15 nucleotides (67%) as the lowest acceptable homology level for our purpose. As shown above, we only found sequences with at least 73% homology. Interestingly, most differences between the homologous sequences in the 'α', *ZNF334*, *KLOTHO*, *RARβ2* and *GRAP-2* occur in the 5′ half-region of the sequence (and for the latter reaching more than 70% difference level),

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while the 3′ half-region was much more homologous and in fact did not differ from that seen in the 'α' site by more than one nucleotide (for *RARβ2* and *KLOTHO*).

Transcriptional activity of the *RARβ2*, *GRAP-2* and *ZNF334* genes can be detected in resting human CD4$^+$ lymphocytes and differs between healthy individuals and those with RA

All three genes: *ZNF334*, *RARβ2* and *GRAP-2*, were transcriptionally active in the purified resting CD4$^+$ lymphocytes of both healthy individuals and those with RA (Fig. 1), as is the *KLOTHO* gene.[16] With the exception of *GRAP-2*, the expression in RA cells was lower than in the healthy CD4$^+$ lymphocytes (Fig. 1 panels a,c,e,g); however, the difference was significant only for *ZNF334*, possibly because of extensively broad dispersion of the results of quantification of *RARβ2* expression (Fig. 1c,e). In contrast, analysis of *GRAP-2* expression in CD4$^+$ lymphocytes did not show any differences between examined groups, whether healthy or with RA (Fig. 1g). It is worth noting here that the apparent level of expression of *GRAP-2* seemed to be similar to that of *β*-actin, whereas expression of *RARβ2*, *KLOTHO* and *ZNF334* were respectively 10, 100 and 1000 times lower (Fig. 1). Interestingly, the two oldest RA patients (respectively 98 and 76 years of age) exhibited virtually no detectable expression of any of the four genes, including *GRAP-2*. As their clinical history indicated that they differed from the rest of the patients with RA in that they had developed the disease late in life (so called elderly-onset rheumatoid arthritis), they were excluded from further correlations.

Knowing from our previous work that variability of such results may be the resulst of the varying age of the individuals tested,[16] we decided to re-analyse the expression of *ZNF334*, *RARβ2*, *GRAP-2* and *KLOTHO* again after dividing the healthy individuals and the patients with RA each into 'young' (younger than 40 years of age) and 'older' (40 years and above) age groups. This approach yielded interesting and diverse results. With the exception of *GRAP-2*, the expression of genes of interest in RA material is significantly lower than in the cells of healthy individuals, regardless of the age of individual (Fig. 1b,d,f,h). However, whereas expression of *RARβ2* and *KLOTHO* is significantly decreased in the CD4$^+$ cells of healthy older people (Fig. 1b,f), it is not so for *ZNF334* (Fig. 1d). Significant differences were shown in *RARβ2* expression between the CD4$^+$ cells of young healthy individuals and young patients with RA, but not between the cells from older individuals (Fig. 1f). For *KLOTHO* and *ZNF334* expression (Fig. 1b,d, respectively) the differences between healthy individuals and those with RA were significant for both young and older people. Finally, for *GRAP-2*, also after dividing the subjects into age groups, we observed only a non-significant tendency to reduced expression in the CD4$^+$ cells of healthy older people compared with the cells of healthy young individuals. Interestingly in this case the transcriptional activity remained at the high level also in the older RA patients (Fig. 1h).

Benefiting from the relatively broad ranges of expression levels for the four genes of interest, and hypothesizing that they might be co-regulated, we checked if their respective expressions correlated with each other. Significant positive correlations were found for each of six combinatorial pairs possible (Fig. 2). Also, when the activities of the four genes were assessed in relation to the surface expression of *CD28* on the CD4$^+$ cells from each tested individual, significant correlations were found between the levels of expressions of *CD28* and *KLOTHO* or *ZNF334* ($r^2 = 0{\cdot}356$, $P = 0{\cdot}003$, and $r^2 = 0{\cdot}358$, $P = 0{\cdot}007$, respectively). No such correlations were observed for either *CD28*/*RARβ2* ($r^2 = 0{\cdot}008$, $P = 0{\cdot}725$), or *CD28*/*GRAP-2* ($r^2 = 0{\cdot}061$, $P = 0{\cdot}295$). Significant correlations were found even if the actual difference between surface expression of CD28 expressed as MFI was only marginally significant ($P = 0{\cdot}08$ by Mann–Whitney *U*-test). For the healthy group, the expression was 211·4 MFI units (191 and 245 as 25th and 75th percentile values respectively, $n = 14$) and for the RA group it was 189·0 MFI units (181 and 198 as 25th and 75th percentile values respectively, $n = 14$).

'α' homologous sites in the *GRAP-2*, and *ZNF334*, but not *RARβ2* genes bind protein complexes apparently similar to those bound by 'α' in the CD4$^+$ T-cell nuclei

To compare the capabilities of the sequences containing the 'α' homologues from the vicinities of *ZNF334*, *KLOTHO*, *RARβ2* and *GRAP-2* genes to bind T-cell nuclear proteins and to check if they may bind to the protein repertoire similar to that bound by 'α' we used the electrophoretic shift mobility assay technique (Fig. 3). It can be seen that all four sequences bound some T-cell nuclear proteins; however, the pattern of bands containing protein-bound radioactive probes was heterogeneous and different for various sequences. All four apparently bound some higher molecular weight protein complex, unrelated to the 'α' (open solid arrowhead in Fig. 3), whereas only the sequences corresponding to *ZNF334* and *KLOTHO* seemed to be binding nuclear proteins similarly to the 'α' (filled solid arrowhead in Fig. 3). The sequence corresponding to *RARβ2* did not seem to bind detectable amounts of the protein(s) bound by 'α'. Finally, only *ZNF334* and *RARβ2* seemed to share yet another ability to bind an even higher molecular mass T-cell nuclear protein complex (Fig. 3, dashed arrowhead).

Discussion

Our results suggest that DNA sequences significantly homologous to that contained in the minimal promoter of

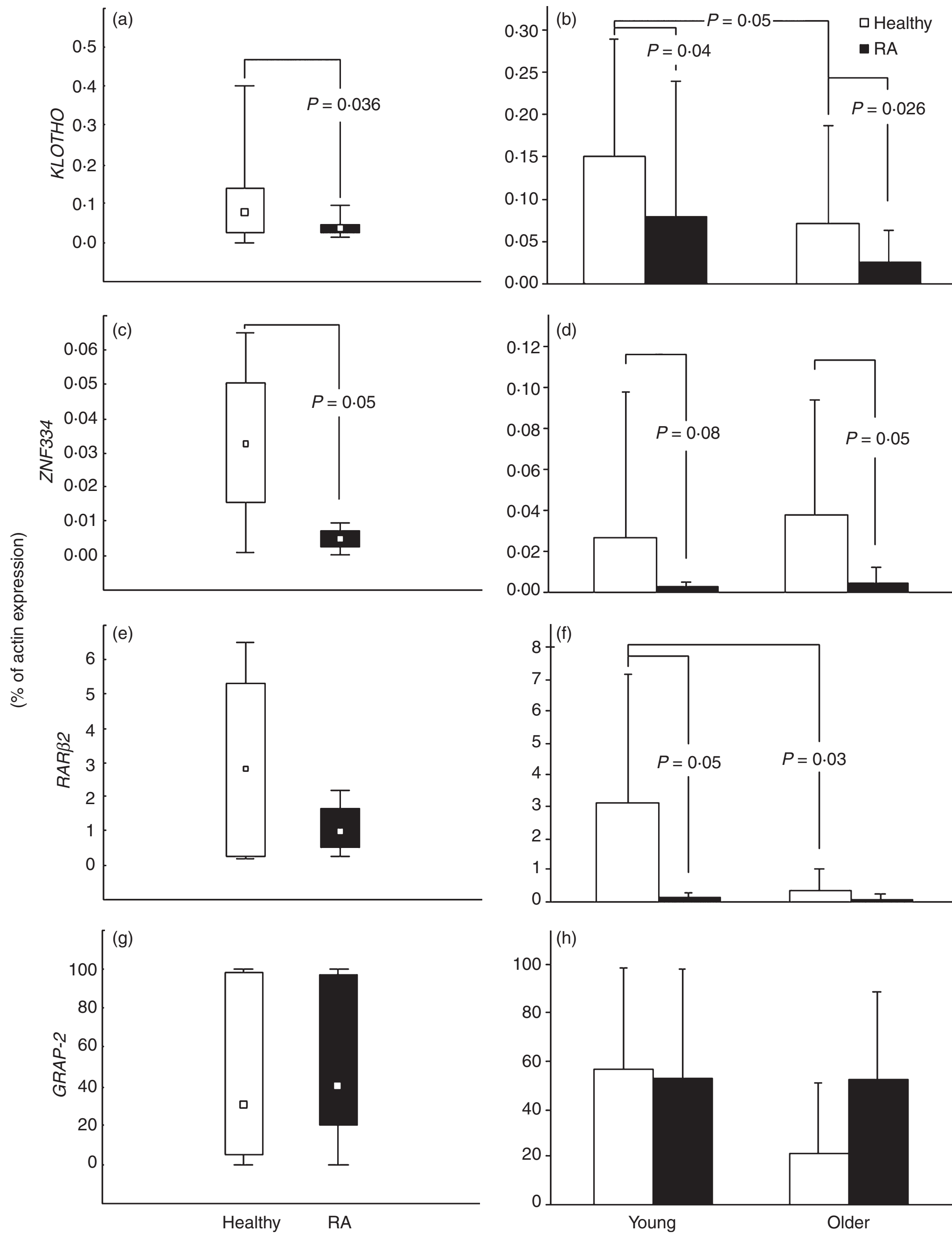

Figure 1 Transcriptional activities of *KLOTHO*, *RARβ2*, *ZNF334* and *GRAP-2* genes in the CD4+ cells vary depending on age and healthy/rheumatoid arthritis (RA) status. The genes' activity was assessed by real-time PCR relative to that of the *β*-actin gene. Comparison of the expression levels between healthy individuals and those with RA is shown in the left column (a, c, e, g; medians, 25th and 75th percentiles, and the minimum and maximum values obtained from 14 paired experiments; statistical significance was assessed by Mann–Whitney's *U*-test). Comparison of the expression levels between young and older healthy individuals and those with RA is shown in the right column (b, d, f, h; bars depict means ± SD; unpaired Student's *t*-test used for statistical comparison).

the *CD28* gene (called 'α'), whose activity is regulated by TNF,[9,10,13] can be found in the vicinity of the promoter of *KLOTHO*[16], and of *ZNF334*, *RARβ2* and *GRAP-2* genes. All these genes are transcribed (as we show) in the resting peripheral blood CD4+ lymphocytes. Transcriptional activity of these genes is reduced in the resting cells of patients with RA (with the exception of *GRAP-2*-related homologue, possibly because of its homology level being the lowest). The activity of *RARβ2* and *ZNF334* genes in human CD4+ cells was not reported before. The products of all four genes under study may be important for the T-cell biology and RA-related pathology.

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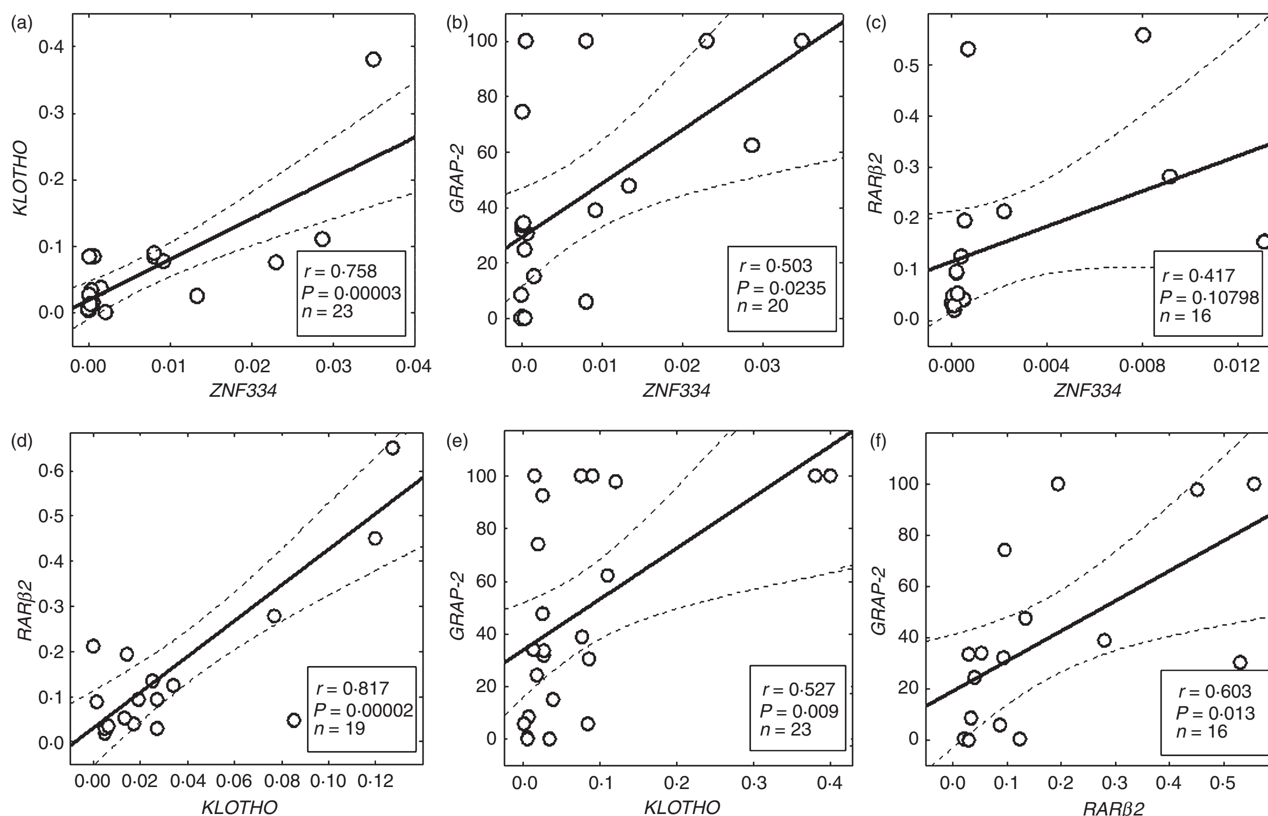

Figure 2 Transcriptional activities of *KLOTHO*, *RARβ2*, *ZNF334* and *GRAP-2* genes in the $CD4^+$ lymphocytes are variably correlated. The genes' activity in the lymphocytes isolated as described in the Materials and methods was assessed by real-time PCR relative to that of the *β*-actin gene. Correlation coefficients for each combinatorial pair [*KLOTHO* vs. *ZNF334* (a), *GRAP-2* vs. *ZNF334* (b), *RARb2* vs. *ZNF334* (c), *RARb2* vs. *KLOTHO* (d), *GRAP-2* vs. *KLOTHO* (e) and *GRAP-2* vs. *RARb2* (f)] and their statistical powers are shown in the inserts.

The $CD4^+$ lymphocytes of healthy elderly individuals and those with RA share a common feature of accumulating a subpopulation that does not express CD28 ($CD4^+$ $CD28^-$) or in which the expression of this co-stimulatory molecule per cell is significantly decreased ($CD4^+$ $CD28^{low}$).[10,13,25,26] Reduced expression of CD28 depends at least in part on the activity of TNF, elevated both in the patients with RA and in at least some of the otherwise healthy elderly individuals, and involves TNF-dependent suppression of expression of the 'α'-binding proteins.[9,10,27,28] Our working hypothesis was therefore that the genes containing 'α' homologues in or near their promoter regions should experience a similar reduction of expression in the cells of healthy older people and of patients with RA. We believe that this hypothesis is hereby confirmed in by the following. First, we demonstrate that the level of reduction of expression of the gene in question in the T cells of either healthy older people or patients with RA or both is associated with the degree of homology between the gene-associated homologue of the 'α' sequence and the 'α' itself. It is therefore much more pronounced for *ZNF334* and *KLOTHO* (with the 'α' homology levels 93% and 80%, respectively) than for *RARβ2* and *GRAP-2* sharing weaker (just 73%) homology. Interestingly enough, it seems that it is the maintenance of homology in the 3′ region of 'α' and 'α'-like sequences that decides their functional similarities; this notion would of course have to be verified in further, more detailed molecular studies. Second, we show that the levels of expression of all four genes are correlated with each other and with the expression of *CD28*; again the degrees of these correlations depend at least in part on the levels of homology. Third, we show by the electrophoretic mobility shift experiments that sequences derived from the two genes that share the highest levels of homology with 'alpha' (*ZNF334* and *KLOTHO*) are in fact those that apparently bind protein complexes similar to those bound by 'α', which in our opinion makes them more likely to be susceptible to the factors controlling the 'α' itself (especially the TNF). Notwithstanding our hypothesis of direct controlling effect of increased TNF levels on the expression of genes under study, we did not attempt to correlate their expression with the serum levels of TNF, as it was earlier shown to be broadly variable and even undetectable in

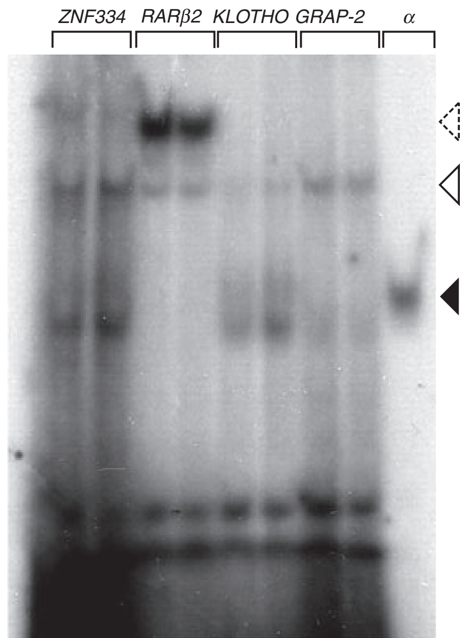

Figure 3 'α' homologous sites in the *ZNF334*, *RARβ2*, *KLOTHO* and *GRAP-2* genes bind with distinctly similar pattern of $CD4^+$ T-cell nuclear proteins. For the detailed description of the electrophoretic moblity shift assay experiment see the Materials and methods. ^{32}P-tagged oligonucleotide probes for *ZNF334*, *KLOTHO*, *GRAP-2* (but not *RARβ2*) bind proteins similar to those bound by the 'α' sequence (filled solid arrowhead). All four sequences share the ability to bind another nuclear protein complex (open solid arrowhead) from lymphocyte nuclear lysates, whereas *RARβ2* and *ZNF334* sequences preferentially bind yet another complex of higher molecular weight (dashed arrowhead). Data from one out of three representative experiments yielding similar results are shown.

patients' samples.[10,13,27,28] Still we believe that our results are strongly suggestive of the common mechanism decreasing the expression of CD28 and genes sharing high homology of the 'α-like' sequences in their promoters, i.e. TNF-dependent reduction of binding of nuclear proteins to these regions leading to decreased activity of the promoters.[9,10,27,28]

Reduced expression of the *RARβ2* gene may be of direct relevance for the reported impaired proliferative behaviour of $CD4^+$ lymphocytes in both healthy older individuals and patients with RA. Our pilot experiments where anti-CD3-stimulated PBMC of healthy controls and patients with RA were exposed *in vitro* to all-*trans*-retinoic acid demonstrated that this treatment increases the cell cycle length and extends the G0→G1 time of the $CD4^+$ cells of the patients with RA to the values observed for cells of healthy people. Some reports show a relative lack of retinoids in the blood of patienst with RA.[29] On the other hand, retinoids up-regulate transcription of their receptors genes (including the *RARβ2*),[30] so the down-regulation of expression of the *RARβ2* gene observed in patients with RA may be the result of relative retinoid deficiency rather than of the action of TNF via an 'α' homologue. This may be true especially in the light of our demonstration of rather lower homology between the 'α' sequence from the *CD28* promoter and its *RARβ2* simile and lack of appreciable binding between the *RARβ2* sequence and T-cell nuclear protein complexes similar to those binding the 'α' (Fig. 3).

Decreased average expression levels of the *RARβ2* gene in the lymphocytes of patients with RA compared with those from the healthy cohort are apparently the result of their extreme reduction in the cells from young patients (Fig 2f), whereas the expression of the gene in the lymphocytes of older people (both healthy and those with RA) is equally low. In our opinion, this again supports the hypothesis of accelerated aging of the $CD4^+$ lymphocytes in individuals with RA.[13,16] Retinoids are suggested to act *inter alia* by inducing cyclin D1 proteolysis.[31] We were the first to show cyclin D1 (in addition to other forms of cyclin D) to be significantly over-expressed in the proliferating $CD4^+$ lymphocytes of healthy elderly people.[25] Confronted with the current notion of RA as a form of accelerated aging of the $CD4^+$ lymphocytes, these may open new avenues for further studies.

Observation of the expression of a new zinc finger protein, the ZNF334, in the human $CD4^+$ lymphocytes, and especially of its down-regulation in the cells of patients with RA (irrespective of their age) deserves attention, as this zinc-dependent protein was not reported before to be expressed in human lymphocytes. Zinc finger proteins containing the KRAB (Kruppel) domains are considered very important regulators of many genes[32] and are variably involved in the regulation of cellular proliferation.[33,34] Decreased ZNF334 activity may therefore be partially responsible for the proliferative impairment of RA lymphocytes possibly by allowing the increased activity of the genes controlling the length of the cell cycle. Cyclin D1 was recently shown to be regulated by another member of zinc finger protein family, the KLF13.[35] The importance of zinc finger proteins for the proper proliferative behaviour of the lymphocytes[34] directs our attention also to zinc as a possible usable factor in modulation of the function of T cells in RA. In fact, zinc was recently proposed to modulate T-cell function in RA,[36] which stresses the possible role of zinc finger proteins (including ZNF334) in its pathogenesis.

The GRAP-2/Gads protein, a member of the SH2 and SH3 domain-containing Grb2 family of adaptor proteins highly specific to T cells and monocytes/macrophages[37–39] has been reported to interact with SH2 domain-containing protein 76 and the linker for activation of T cells to regulate nuclear factor of activated T-cell (NF-AT) activation[23,40] and with the CD28.[24] It works by down-regulating the signal relay through the Ras/Erk pathway, and so negatively influences the mitogenic responses of

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lymphocytes.[41] All of the above stress the high level of importance of *GRAP-2* expression for proper T-cell functions. However, apparently it has no involvement in the pathology of RA or in the aging-related impairment of T-cell function. This notion seems additionally supported by our finding of unchanged expression of the gene in the CD4+ lymphocytes of patients with RA.

Finally we show that the homologue sequences in question are in fact binding some, as yet unknown, T-cell nuclear proteins or their complexes, sharing the ability to bind some of them. This observation may suggest common mechanisms of regulation of these genes, including, but potentially not limited to, the one depending on the binding of TNF-regulated proteins to the 'α' homologue sequence.

In conclusion, the emerging picture is as follows: decreased expression of *KLOTHO* and *RARβ2* genes depends both on the individual age and succumbing to RA, while that of *ZNF334* is apparently related to the disease only, and not to the patient's aging. Finally, expression of *GRAP-2* is susceptible to neither age-related nor RA-related changes. Summarizing, observation of decreased expression of either *KLOTHO* or *RARβ2* in the CD4+ lymphocytes of patients with RA would be a possible sign of their premature aging, whereas that of *ZNF334* would be a potential age-independent marker of RA disease.

Acknowledgements

We thank Ms Anna Budzyńska for excellent technical help. This work was supported by a Polish State Committee for Scientific Research Grant P05B 039 22 (to J.M.W.).

Disclosures

None.

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