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Ph.D. thesis

Gene expressional studies of TL1A/VEGI

in differentiating chicken chondrocytes and in

human autoimmune processes

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Background

Identification of members of the Tumor Necrosis Factor Superfamily

Discovery of the TNF was done in 1893-94, when William Coley, a surgeon in New York described that when patients suffering from tumour diseases received bacterial infection, their cancer has been disappeared by haemorrhagic necrosis. Later, by the result of research, other proteins having structural relations to TNF has been discovered, which can be sorted into a superfamily. These proteins have several biological activities, and so they are engaged in most of the processes of the life. Assuming that TNF exists in fish, our aim was to identify the gene of the TNF in chicken, because the orgin of these gene(s) is still mysterious. It was described earlier, that macrophages of chicken origin producing TNF-like bioactivity with a 17 kDa molecular mass, after bacterial LPS treatment. The TNF superfamily could have been occurred by a gene duplication process.

TL1A, the closest homologue of TNFα

Human TL1A (TL1A= TNF Like factor 1…, VEGI=Vascular Endothelial Growth Inhibitor) is a member of Tumor Necrosis Factor SuperFamily (TNFSF), called TNFSF15, according to the nomenclature. Besides structural relationship between the members, in the case of TL1A the similarity is supported by biological effects, like the ability of inducing apoptosis in L929 mouse fibroblast cells. Important feature of the members is that they exert their activities in a homotrimerized form through their cognate receptorr, which are trimerized, either. Receptor for TL1A is DR3, the closest structural relative of TNFR.1. Other receptor is the circulating DcR3 receptor, which binds to two other TNF superfamily members LIGHT and FasL, too. DcR3 can be detected in tumours causing the immunoprivileged status of tumor cells, and also has a role in differentiation of oscteoclast cells from macrophages. DcR3 was described in chicken, too.

Biological effects of TL1A/VEGI

The gene for TL1A located on the long arm of chromosome 9 (9q32), spanning 16.8 kilobasepairs and consists of 4 exons. There are 3 isoforms translated from the gene driven by akternative promoters, and important to note, that it has a posttranslational modification. Biological activities of the isoforms are the inhibitory effect on vascular endothelial cell growth

(174 aminoacid long isoform, VEGI174), tumor growth inhibitory effect (192 amino acid long isoform). The latter one activating the antigen presentation feature of naive dendritic cells (DC), which is a very important step in immunological processes. The most abundantly produced isoform has a T cell coactivating effect (251 amino acid long isoform, TL1A). TL1A was described in Crohn disease and it is produced at an increased level in the layer of lamina propria of the inlamed gut.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the small joints caused by a not yet determined agent. TL1A production in dendritic cells and macrophages is increased by Fc parts of IgG1 immunoglobulins. There are reports describing SNPs in the gene of DR3, which are cumulated in patients with RA. Also, there is a gene duplication of DR3 gene, which spans only to a part of the gene, resulting a DcR3-like protein.

Multiple sclerosis (MS) is an autoimmune neurological disorder, where neuronal fibers attacked at the periphery, by auto-reactive T cells. Experimental encephalomyelitis, an accepted model of MS is induced at a level of 50% lower in TL1A knock out mice.

Psoriasis is a T cell mediated autoimmune disease of the skin, where inflammatory citokines produced by activated T cells. These mediators are TNFα and IFNγ which has important role in generating and maintaining the symptoms. Keratinocytes have certain immunological behavior, so they presenting receptors for several citokines and upon engagement of cognate ligand they reflecting with change in gene expressional pattern.

All the three Th1 mediated diseases are treated by anti-TNF therapies, successfully, in spite of adverse effects, like opportunistic infections.

Dermal expression of cytokines

Dermis is one of the most important organ in protecting the body from external injuries, infectious agents and UV irradiation. Keratinocytes (KC) having immunocyte behaviours are important members of the defense system. Beside KCs other elements of the adaptive immune system also can be found in the skin. These are the dendritic cells (DC) of myeloid origin with antigen presenting capability to T cells, which causes T cell recruitment to the organ/tissue originating the immune-signal. Upon activation these T cells will be the sources of above mentioned cytokines, also with effects on KCs. According to recently published results, epidermal keratinocytes change their gene expressional pattern upon TNF induction resulting the modulation of 1300 genes.

Transcriptional factors in psoriasis

Transcriptional activating factors which are important in generation of psoriasis are the Interferon Regulated Factors (IRF), the Signal Transducer and Activator of Transcripton (STAT) and NF-kB as master regulator thought to be mentioned. Detailed description will be given on IRF1 and IRF2 factors, as their protein level ratio in psoriatic keratinocytes is changed, according to published studies.

There are two levels of response given to IFNγ induction of KCs in their gene expressional pattern. The „First Level of Genes“ consist of ones having activator binding elements in their promoter for STATs, for example IRFs. The „Second Level of Genes“ groupped as they have binding elements for IRFs, like iNOS (inducible nitric oxide synthase) and IL-8, whose role in psoriasis are also well-studied.

Transcriptional binding elements for NF-kB and IRF exists in several genes, with close vicinity to each other. These two factors in their activated form synergistically increase the expression of the target gene. Formerly the promoter of TL1A was studied in detail and results are in accordance with its inducibility with different cytokines.

Important fact in the study of psoriasis mechanism that $IRF2^{-/-}$ mouse has spontaneous psoriatic lesions. Also, psoriasis susceptibility locus PSORS3 contains the gene for IRF2.

In psoriais the level of activated STAT3 is increased. Also, in a mouse model where a constitutively activated form of STAT3 overexpressed epidermally resembles symptoms of psoriasis.

Aims

Originally our aims were to ***(1)*** identify TNF in the chicken *(Gallus gallus)* genome, or ***(2)*** prove the existence of TNF related genes, at least. Based on the existence of TNF in fish, it was predictable the same in birds. Later we modified our aims, so we decided ***(3)*** to study the expression of TL1A gene in proliferating chondrocytes of limb growth discs of a 14.5 days old chicken embryo. Also, we aimed to study human TL1A expression in human inflammatory diseases, like psoriasis and rheumatoid arthritis.

Materials and methods

In silico steps

A tblastx search was done on a publicly available EST library data bank through the internet, by using protein sequences of human and rainbow trout TNF. Promoter analysis was made by aligning a 3000 basepairs long upstream activator region of TL1A gene from six species, including chicken.

Cells, cell lines and tissue samples

Chondrocytes suspensions was made from 14.5 days old embrional chicken limb growth discs, then was used for cDNA preparation. Monolayer of chondrosarcoma cell line SW1353 of human origin and psoriatic skin samples – according to the instruction of the local ethical commity – was used for gene expression studies of the human TL1A.

Biological assays

Induction experiments were done by applying recombinant IFNγ, IL-1β and TNFα for 24 hours. Biological activities were approved against commercially available sources.

cDNA preparation

Total RNA was prepared from cell lysates according to standard protocols based on guanidium-isothiocyanate. Extracted RNA quality and quantity was checked by denaturating gelelectrophoresis and UV spectrophotometry. cDNA production was made by using point mutant RNaseH minus reverse transcriptase, then applied to real-time PCR experiments.

PCR primers and PCR method

After an *in silico* search of EST sequences with homologue stretches a 470 basepairs (bp) long fragment was cloned by PCR and proved by sequencing. Further, a 670 bp longer sequence of the same EST – which encodes the biological activity – was cloned into bacterial and baculoviral expressional plasmids.

Chicken DcR3 expression was proved by PCR cloning of a 370 basepairs long amplicon which designed according to an existing EST sequence of the same database. Plasmid miniprep DNAs were sequenced and proved to be identical to desired one.

Real-time PCR

Gene specific TaqMan probes for human TL1A, IRF1 and IRF2 cDNA were designed by using commercially available (Applied Biosystems and Roche Applied Science) kits and instructions of the manufacturers. PCR reaction was done on a BioRad iCycler according standard TaqMan assay conditions.

Expression of chicken TL1A protein in bacterial and baculoviral systems

PCR fragments generated by primers with restriction enzyme *BamHI-XhoI* cutting sites were cloned into pET28a and pFastBacHTB plasmids. After checking DNA sequences at important sites of the plasmids, correct recombinants were transformed into suitable E. coli strains by standard methods for further expression.

Affinity chromatography

Recombinant clones were grown up to stationary phase (bacterial) or to a high virus titer (Sf9 insect cell baculovirus host). Cells were collected and disrupted by ultrasonication. Cell debris were removed by centrifugation and clarified cell lysates were applied to a PBS buffer equilibrated Co^{2+}-chelating Talon column. Column was washed with several volumes of loading buffer, then His_6-tag containing proteins were eluted with a linear imidazole gradient, according to the instructions of the manufacturer. Fractions were analyzed on a SDS-polyacrilamide gelelectrophoresis.

Western blotting

Western blotting experiment were done on nitrocellulose membrane using baculoviral cell extracts and fractions from affinity chromatography, according to standard methods. A polyclonal, multispecies-specific goat anti-VEGI antibody as „primary“, and a horseradish peroxidase-conjugated anti-goat secondary antibody was used for the detection of TL1A protein, using a commercially available chemiluminescent kit.

Immunohistochemistry

The same primary and secondary antibody asembly was used for the immunohistochemical detection of chicken TL1A in paraffin embedded sections of the embrional chondrogen tissue.

Results

Identification of TL1A in the chicken genome

Our original aim was to identify TNF in the chicken genome, because traces of TNF-like biological activities has been reported by others. First we have tried to amplify TNF-related sequences from chicken genomic DNA by using degenerated PCR primers with no success! Fragments of these amplification experiments were not identical, not even related to known sequences of the TNF superfamily. In the second approach we searched an EST library sequence databank from the chicken with sequences of known TNF proteins and identified chicken TL1A, from a clone derived from embrional limb growth disc chondrocytes. This EST sequence has been cloned, sequenced and proved to be the chicken orthologue of mammalian TL1A. Biologically active part of the human TL1A spans from aminoacid 72 to 251. We identified the putative active part of the chicken protein by aligning it with the human orthologue, then PCR primers were designed to clone a 670 basepairs long fragment of the chicken TL1A cDNA into expression vectors.

Genestructure and chromosomal localisation

Chicken genome sequences has been published in 2004. Chromosomal localisation of the TL1A gene has been identified by BLAST analysis with the sequence of the EST clone originated from the chondrocyte library. Chicken TL1A is located on the chromosome No. 17, on a 13 kilobasepairs long segment. Members of the superfamily could have been occured by gene duplication processes, because they are located in clusters in a vicinity to each other. In chicken the situation is in accordance with this rule. Exon-intron structure of the chicken is closely related to mammalian orthologues, as well.

Results of promoter analysis

Besides other transcriptional activator regions, we presume the existence of an IRF binding site, with almost full identity with published consensus sequence! An alignment analysis has been conducted with upstream sequences of the TL1A gene from six different species. Also, it has been compared to databanks with specialisation to all the known transcriptional activator factors. The fact, that a binding site of an immunologically important activator exists shows its importance on the evolutional scale, because IRFs also exists in these species. Also, it is

noteworthy that IRF1 is an important regulator of the immune system in chicken. Multiple IRF sites exists in the promoter region of the human IL-4, their functionality has been proved!

Chicken TL1A expression in proliferating chondrocytes

At the beginning of our work antibody against chicken TL1A was not available (it was not discovered, yet). So we used a multispecies-specific antibody, as it was proved on three species by the manufacturer (peptide-antigen sequence however was not given!). To show whether this antibody is good for chicken TL1A protein detection, we applied it to sections from embrional chicken limb growth discs, with a great success! To validate immunohistochemical results we had to clone and express the protein bacterially, wich was encoded by the EST, and its gene originating from.

Proving the expression of chicken DcR3 in chondrocytes

cDNA of DcR3, a soluble receptor of TL1A could be expressed in chondrocytes, which could give help in the study of TL1A. First we checked the same EST library database and surprisingly found that DcR3 gene is expressed in this tissue. We designed PCR primers based on the published databank and proved to be expressed and identical by PCR-cloning and sequencing.

Increased expression of TL1A in psoriasis

Based on the increased TL1A expression in proliferating cells we sought that it could happen in other proliferative situations, like psoriasis. Keratinocytes are proliferating in this autoimmune disease and these cells have certain immunological behaviours. So we decided to study TL1A expression in samples taken from patients suffering from psoriasis. Expression level of transcriptional activator factors IRF1 and IRF2 were also studied in cytokine treated keratinocytes.

The results of these expresiments shows that TL1A expression is elevated to more than 70 times in psoriatic lesions over the level of the normal skin. Also, cytokine inducibility of TL1A and IRF factors is in congruance with published results of other studies.

Summary of Results

1. Members of the TNF superfamily exists in clusters on chromosms. Chicken TL1A localisation is in congruance with this rule.
2. TL1A is expressed in the 14.5 days old chiocken embrional limb growth disc cartilage tissue.
3. Transcriptional activator IRF binding site exists in the promoter of TL1A gene of several species.
4. It was succesfully shown – the inducible expression of TL1A in human chondrosarcoma cell line SW21353, and
5. – in lesions of the psoriatic skin, and that the
6. – ratio of cDNA levels of IRF1 and IRF2 factors are altered in layers of psoriatic skin.

Discussion

Our results are suggesting the possibility that highly expressed TL1A in psoriatic skin is a potential modulating factor, which has two effects: first is to coactivate recruited T cells in lesions of the psoriatic dermis, second is to activate dendritic cells, which makes the afferent immune signal to the nodes, causing T cell proliferation and migration.

Existing targeted therapeutical protocols against T cell mediated diseases are the anti-TNF therapies, which has high effectivity besides several adverse effects.

Unmet need on the pharmaceutical market is the development of locally administered, but effective therapy, which can be a neutralizing and humanized anti-TL1A antibody.

Published results of the thesis with first authority

Tubak, V., E. Határvölgyi, L. Krenács, É. Korpos, E. Kúsz, E. Duda, É. Monostori "Novel function of immunoregulatory TL1A in chicken chondrocyte differentiation" Can. J. Vet. Immun. Accepted for publication!

Other papers, not related to the thesis

Lukacs, J., **V. Tubak**, J. Mester, S. David, Z. Bartfai, T. Kubica, S. Niemann and A. Somoskovi (2004). "Conventional and molecular epidemiology of tuberculosis in homeless patients in Budapest, Hungary." J Clin Microbiol **42**(12): 5931-4.

Praznovszky, T., J. Kereso, **V. Tubak**, I. Cserpan, K. Fatyol and G. Hadlaczky (1991). "De novo chromosome formation in rodent cells." Proc Natl Acad Sci U S A **88**(24): 11042-6.

Hadlaczky, G., T. Praznovszky, I. Cserpan, J. Kereso, M. Peterfy, I. Kelemen, E. Atalay, A. Szeles, J. Szelei, **V. Tubak** and et al. (1991). "Centromere formation in mouse cells cotransformed with human DNA and a dominant marker gene." Proc Natl Acad Sci U S A **88**(18): 8106-10.