Molecular mechanism of genistein action in terms of its potential use in the treatment of psoriasis

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Psoriasis (Ps) is a common immune-mediated chronic inflammatory disease that affects approximately 1 - 3% of the European and US population. The symptoms of psoriasis include redness, scaling, flaking, pruritus, skin tightness, pain, and bleeding, which can all have a significantly negative impact on patients' physical and mental functioning, and often lead to disability.

Skin lesion are a result of hiperproliferation of epidermis and of the shortening of the transposition time of keratinocytes from basal to corneal layer of epidermis. Primarily psoriasis was considered as a phenomenon of an epidermal imbalance between proliferation and differentiation. The large, silvery scales observed in the lesions are a consequence of altered differentiation (hyper- and parakeratosis), whereas thickening of the epidermis is due to a strongly increased pool of proliferating keratinocytes. Altered differentiation of psoriatic keratinocytes is characterized by down-regulation of the late keratinocyte differentiation markers (such as filaggrin and loricrin) and up-regulation of the early differentiation markers (involucrin, keratin 16, and keratin 17). Differentiation of epidermal cells is controlled by transcription factors. One of the pathogeneses of psoriasis is genetic components, and many genes are associated with it, but it is unclear how those genes work together. Currently the hypothesis takes the psoriasis as being an innate and adaptive immune-mediated disorder. T cells become active, migrate to the dermis, and trigger the release of cytokines which cause inflammation and the rapid production of skin cells. Over recent years, there have been great advances on Th1 and Th17 cells and their roles in the inflammatory and autoimmune diseases, among them in psoriasis. These immune cells move from the dermis to the epidermis and secrete inflammatory cytokines such as tumor necrosis factor α (TNF-α), interferon γ (IFN-γ) and interleukin (IL): IL-6, IL-8, IL-17, IL-19, IL-23. These secreted inflammatory signals stimulate the keratinocytes to proliferate and differentiate [1].

Psoriasis can occur at any age, however two main peaks: type I (early) and type II (late) are selected. The early manifestation of the disease (usually at the age of 15 to 40 years) is associated with a positive family history, more severe course and numerous relapses. 80% cases of this type are associated with the presence of HLA-Cw6 *locus*. The second type of psoriasis is most common in people aged 50-70 years.

Numerous studies have reported the coexistence of psoriasis with other serious systemic diseases, most often mentioned are cardiovascular diseases, metabolic syndrome,

including hypertension, dyslipidemia and diabetes mellitus, and Crohn's disease. In addition to the skin, psoriasis can be associated with an inflammatory arthritis known as psoriatic arthritis, which involves the joints of the spine and other joints. A review of the literature showed that psoriatic arthritis affects between 5% and 30% of patients diagnosed with psoriasis.

Various topical medications, phototherapy, and systemic drugs are available to treat patients with psoriasis of varying disease severity. However, many studies are constantly performed to detect new therapeutic anti-psoriatic substances, which are verified in preclinical laboratory tests and clinical phase trials. The data demonstrate, that natural compounds may prove to be very important in the treatment of psoriasis. Their anti-inflammatory and antiproliferative effects may be complementary to systemic treatment, but also a substantive therapy for milder forms of the disease. Among the natural compounds, great attention is paid to flavonoids. They are applied worldwide as alternative medicaments among others for psoriasis because of their perceived beneficial impact on the skin state. Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is the principal isoflavone found predominantly in soy beans and has attracted considerable attention due to its potential effects on some of the degenerative diseases, such as cardiovascular disease, osteoporosis, and hormone-related cancers. Interest in genistein as a potential therapeutic agent for Ps has recently risen, as studies have shown that genistein (Glyteer) exerts evident anti-inflammatory properties on a psoriatic model in mice. Importantly, genistein is a potent inhibitor of the production of pro-inflammatory factors, such as IL-1, IL-6, and TNF-α by modulating the nuclear factor kappa B (NF-κB) and phosphoinositide 3 kinase/protein kinase B (PI3K/Akt) pathways in macrophages and endothelial cells. However, detailed molecular mechanisms of the anti-inflammatory effects of genistein are still elusive.

The objective of my PhD thesis was to describe the mechanism of action of genistein in terms of their modulatory effect on cellular processes with emphasis on application in the treatment of psoriasis. In my research, I worked on an *in vitro* model by using spontaneously immortalized human keratinocyte line (HaCaT). Moreover, my experimental work was also based on a clinical trial in the first phase. Patients with mild to moderate chronic plaque psoriasis were treated with oral film-coated tablets in one of two treatment regimens: genistein 75 mg or 150 mg per day or placebo 56 days.

The first step of research was the selection of concentrations of genistein and assessment of their cytotoxic and antiproliferative activity in cultured keratinocytes [2].

Genistein was used in concentration of 30, 60 and 100 μM, dissolved in 100% dimethyl sulfoxide (DMSO) in order to achieve the final concentration of 0,05%. The cytotoxic effect of tested isoflavone was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) which depends upon a mitochondrial activity. To this end, cells were treated with various concentrations of genistein for 24 hours, 48 hours and 7 days. No cytotoxicity was observed when the cells were exposed to genistein for 24 and 48 hours. The concentrations lethal to 25, 50 and 75% of cells (LC25, LC50 and LC75, respectively) were estimated after 7 days of treatment. No remarkable cytotoxicity features of genistein were observed in the range of tested conditions, while inhibition of proliferation of keratinocytes in a dose-dependent manner was detected [2].

In order to investigate more extensively the effects of genistein in keratinocytes cells culture, DNA microarrays Illumina's Human HT-12 v3 and v4 Expression BeadChips were used to assess the global effects of these compound on gene expression in HaCaT. Analyses were performed on keratinocytes after 24 and 48 hour treatment with genistein of 30, 60 and 100 μM. Microarray results were interpreted by calculation the signal ratio for samples treated with genistein and for vehicle (0.05% DMSO). The estimated ratios were significant when the values were below or equal 0.7 and $\geq$ 1.3. Testing the effects of genistein on human HaCaT transcriptome via the microarray analysis, we found that this compound induced significant dose- and time-dependent alterations in profiles of hundreds of transcripts. As discovered in three independent assays, in total 4039 transcripts for 24 hours and 4186 for 48 hours handling with 100 μM genistein were affected [2].

Moreover, modulated metabolism pathways were defined according to the KEGG annotation (Kyoto Encyclopedia of Genes and Genomes) and AmiGO. Gene Ontology analysis and data visualization were performed on the up-regulated and down-regulated gene lists using the web tools GOrilla (Gene Ontology enRIchment anaLysis and visuaLizAtion tool) and REViGO (REduce + VIsualize Gene Ontology) restricting the output to biological process and cell compartment. Interestingly, the analysis of "Cellular Compartment" as well as "Biological Processes" terms showed that enrichments for categories related to intracellular, cytoplasmic and membrane-bounded organelles were among the significant ones with positively modulated expression of genistein treatment [2]. Gene Set Enrichment Analysis (GSEA) performed on the up-regulated gene lists has shown various metabolic pathways, like the peroxisome and peroxisome proliferator-activated receptor (PPAR) signaling pathway (important in psoriasis and keratinocyte homeostasis). Among the down-

regulated gene sets, genes belonging to a wide range of pathways involved in nucleotide-binding oligomerization domain-like (NOD-like) receptor signaling were detected [2].

To provide independent validation of microarray data and to examine in more detail the expression patterns of psoriasis-related genes involved, a real-time qRT-PCR was used. Both microarray and real-time qRT-PCR analyses indicated that genistein influence expression of several psoriasis-associated genes [1]. Particularly noteworthy is the observed in the case of genistein-treated keratinocytes, a decrease in the level of *PI3* gene expression (peptidase inhibitor 3) and *SERPINB8* (serpin family B member 8), which are markers of the induction of psoriatic process in epidermis [1, 2].

The importance in the development of psoriatic lesions is also attributed to the immune mechanisms. Keratinocytes have a unique biology and thus are widely used for experiments to study the activity of oncogenes in epithelial neoplasias, and the molecular mechanisms implicated in warts and other skin associated disorders. In addition, several *in vitro* skin models have been developed that accurately mimic the epidermis making it possible to study the skin in a physiologically relevant context. While these cells are extremely useful in the laboratory, they are notoriously difficult to isolate and culture. Published data showed that keratinocytes produce a vast repertoire of cytokines, including interleukins, growth factors, colony stimulating factors, and chemokines. Under normal conditions, most of them are not synthesized or remain in the cytoplasm, but external stimuli, such as trauma, bacterial infections, chemical substances, or ultraviolet irradiation induce the production and release of these cytokines from keratinocytes. Keratinocyte-derived cytokines regulate the immune and inflammatory responses through their receptors on keratinocytes, dermal fibroblasts and endothelial cells, and infiltrating T-cells [1]. Preliminary analysis of the HaCaT transcriptome, based on oligonucleotide microarray technology, showed that the culture conditions used so far cause little changes in the activity of inflammatory genes.

The absence of a specific profile that defines the psoriatic phenotype necessitates the use of physiologically relevant and reliable *in vitro* models to investigate this disorder and develop more effective treatments. Monolayer keratinocyte cultures have been widely used for biological and pharmacological studies and screening of anti-psoriatic drugs because they provide an easily reproducible first step model system. To mimic the clinical situation as closely as possible, an evident source is needed to present keratinocytes derived from psoriatic plaques, although psoriatic keratinocytes are difficult to culture. Ps-associated features are induced by controlled addition of selected cytokines in order to mimic disease pathogenesis mechanisms. The most commonly studied cytokines in this context are strongly

associated with either innate (IL-1, IL-6, and TNF-α) or adaptive immune responses (IL-17A). The choice of this specific set of cytokines in my work was made on the basis of information reported by several research groups, addressing cytokine stimulation of keratinocytes. For selection of the best proinflammatory mix to create a Ps model, a detailed evaluation of the individual and possible synergistic effect of selected cytokines (IL-1, IL-17A, IL-22, oncostatin-M (OsM), TNF-α, and IFN-γ) on the expression of Ps-associated genes was made. IL-1 and TNF-α exhibit analogous regulation patterns, particularly with respect to the expression of genes encoding cytokines and chemokines. In addition, IL-1 isoforms are present in psoriatic skin. OsM is a potent keratinocyte activator that induces similar effects as TNF-α, IL-1, IL-17, and IL-22, and regulates many genes related to innate immunity, angiogenesis, adhesion, and motility. The combined effects of all of these selected cytokines appeared to mimic some features of psoriasis. Recent data has demonstrated that cytokine-treated keratinocyte cells represent a comparable system in which inflammatory markers are up-regulated. These markers include antimicrobial peptides, cytokines, and chemokines, as well as the major histocompatibility complex (MHC) molecules [1, 2].

To mimic the inflammatory process in keratinocytes, a set of proinflammatory cytokines that are important regulators of the immune response *in vitro* and *in vivo*, were tested in the course of my study. The choice of this specific set of cytokines (IL-1A, IL-17A, IL-22, OsM, TNF-α and INF-γ) was based on previous reports addressing cytokine stimulation of keratinocytes [1]. As output parameters, the expression of the marker genes *S100A7* (S100 calcium-binding protein A7) and *S100A9* (S100 calcium-binding protein A9), which encode antimicrobial peptides and are highly up-regulated under hyperproliferative and inflammatory skin conditions were tested. Furthermore, early keratinocyte differentiation markers genes were verified, *KRT10*, highly up-regulated under hyperproliferative and inflammatory skin conditions, and *LOR* (ang. *loricrin*), down-regulated in psoriasis skin [1, 2].

Modulated pathways were defined according to the KEGG annotation (Kyoto Encyclopedia of Genes and Genomes) and AmiGO. Conducted a detailed analysis of the expression level of these genes whose products are involved in the mechanism of psoriatic - Panel 1 [2, 3] and pathways involved in the pathogenesis of psoriasis, whose dysfunction is responsible for the occurrence of inflammation in the skin – Panel 2 [2, 3], The transcriptomic profiling with the use of real-time qRT-PCR on “psoriasis-like” HaCaT cells treated with and without genisteinMeasurements determined by the two transcript assessment systems, Panel 1 and Panel 2, showed various clusters of gene activity. Except for a set of genes with

unchanged expression for both panels (i.e., 20 transcripts of Panel 1 representative of psoriasis-related genes and 7 transcripts of Panel 2 representative of inflammation-immune axis regulation genes), were observed the occurrence of four main profiles of gene responses composing particular clusters, common for both panels. The first and second cluster, respectively, cover genes of their reduced activity or of their increased expression in response to both "cytokine mix" stimulation and proinflammatory "cytokine mix" stimulation plus genistein treatment. The third group includes genes of decreased expression after "cytokine mix" stimulation, while enhanced activity as a result of exposure to the "cytokine mix" plus genistein. The fourth cluster genes of increased activity after "cytokine mix" activation and its reduction after incubation with the "cytokine mix" plus genistein. Additionally, in the case of Panel 2 with inflammation-immune axis regulation genes, was detected the existence of an additional cluster, the fifth one, with genes of their reduced activity in response to "cytokine mix" stimulation, followed by maintenance at the same level of activity after incubation with the "cytokine mix" plus genistein [2].

In general, the altered expression signatures point to stimulation of mainly (i) the AMP-activated protein kinase (AMPK) signaling pathway (known to limit inflammation), (ii) Fatty acid metabolism, (iii) Forkhead box O (FoxO) signaling pathway, Tight junction and Longevity regulating pathway, while to inhibition of the (iv) Cell division cycle and (v) Metabolic signaling pathways as important targets for genistein [2, 4].

The next purpose of my dissertation was to establish, which cellular mechanisms are involved in modulation of observed changes in phenotype of cells treated with "cytokine mix" or their mixtures with isoflavone. NF-κB is a transcriptional regulator that plays a central role in responses to inflammatory signaling in psoriasis. Phosphorylation of NF-κB p65 is an important step for its transcriptional activity. One of the immediate effects of IL-1 is the degradation of IκB kinase (IκB) and consequent nuclear translocation and activation of the transcription factor NFκB [1]. IκB degradation begins within minutes of the addition of IL-1 and persists for the duration of the treatment. Consequently, NFκB is activated and translocated into the nucleus. In order to study the effect of genistein on nuclear translocation of the NF-κB p65 subunit, laser scanning indirect immunofluorescence confocal microscopy using an antibody to the p65 molecule was performed. The immunofluorescence staining pattern showed clearly the translocation of p65 into the nucleus of human keratinocytes after activation with a cytokine cocktail. Similarly and as expected, the p65 molecule remained in the cytoplasm of stimulated keratinocytes with a combination of proinflammatory "cytokine mix" and treated with genistein. Additionally, to assess activation of the NF-κB p65 subunit

following TNF-α stimulation plus genistein exposure, p65 molecule nuclear translocation in keratinocytes treated with these compounds was examined. The obtained results pointed to the effect of genistein on NF-κB p65 nuclear translocation when cells were activated with TNF-α alone instead of a cytokine cocktail, as the p65 molecule remained in the cytoplasm of cells. Based on these results, it was concluded that genistein suppresses either "cytokine mix" or TNF-α-induced nuclear factor κB p65 subunit translocation into nucleus [2].

The translocation of the NF-κB p65 subunit into the nucleus is an important step for its transcriptional activity, which in turn seems to be mediated by PI3K signaling. Previous work has reported the importance of the PI3K pathway as an important regulator of growth and inflammation in inflammation-mediated diseases such as psoriasis. To this end, in further experiments the activity of phosphatidylinositol-3-kinase was investigated by using the analogous procedure with MUSE® Cell Analyzer. The experiments revealed no statistically significant differences in the activity of this kinase in keratinocytes stimulated with the cytokine cocktail versus the unstimulated one, similarly with no effect of genistein on this phenomenon. Interestingly, at the same time, modulation of *PI3K* gene expression in such conditions was found. Furthermore, genistein prevented "cytokine mix" as well as TNF-α-induced NF-κB translocation. Thus, it was concluded at lack of PI3K pathway involvement in NF-κB activation in our experimental design, which is not surprising, as it seems to be a cell- and tissue-specific event.

Next, to elucidate the mechanism of genistein-induced inhibition of NF-κB nuclear translocation, experiments to determine the effects of the tested isoflavone compound on intracellular ROS accumulation and levels of cytokines in supernatant were performed. Attenuation of ROS levels by genistein was examined by confocal fluorescence microscopy and quantitative measurements of ROS was acquired with MUSE® Cell Analyzer. Both results regarding intracellular ROS accumulation showed that stimulation of keratinocytes with TNF-α and LPS, but surprisingly not with the "cytokine mix", increases ROS levels, which on the other hand was effectively reduced by genistein. These data indicated that the tested isoflavone could attenuate TNF-α- and LPS-induced inflammatory responses in HaCaT by suppressing ROS activation. This is consistent with the antioxidant properties of genistein and reports of others, where this agent has been shown to protect cells against ROS by scavenging free radicals, enhancing activity of antioxidant enzymes and reducing production of hydrogen peroxide. In addition, it was found that, regardless of the type of cell activation used, the levels of three (IL-8, IL-20 and CCL2) out of five cytokines tested were decreased in the keratinocyte supernatant in response to the isoflavone. It is worth emphasizing, too, that

the same trend (i.e., first an increased expression in response to stimulation, followed by reduction after genistein treatment) was observed in both RNA and protein levels of these three cytokines [2]. Based on these results, it was concluded that genistein may suppress inflammatory cytokine production, at least partly, by inhibiting the ROS/NF-κB pathway in activated HaCaT cells. There is a hypothesis that genistein attenuates ROS-mediated NF-κB activation and subsequent inflammatory cytokine production in "psoriasis-like" keratinocytes. These data provide new insight into the anti-inflammatory mechanism and antipsoriatic activity of genistein, giving scientific support for its use in the treatment of Ps.

The final stage of my PhD work was a comprehensive evaluation of the results obtained during the clinical trial of monotherapy in patients with mild to moderate plaque psoriasis [3] The primary objective of this study was to determine the safety and tolerability of genistein in cohorts treated with this agent or placebo. Although genistein has previously been seen to be able to modulate the anti-psoriatic members and anti-inflammatory mediators of inflammation, in this work it showed mixed success. In certain cases, some evidence of genistein-dependent improvement for the psoriasis clinical severity scores was found, such as for the Physician's Global Assessment (PGA) score in two doses of 75 and 150 mg genistein groups and placebo on Day 56, which was close to statistical significance. In turn, in the case of four of the 34 enrolled subjects, named u.09, 11, 12 and 15, for patient u.12 a reduction of both factors, Psoriasis Area and Severity Index (PASI) and Body Surface Area (BSA), but not PGA was inferred. What is interesting, is that a certain regression of the psoriatic phenotype was visible for this patient, based on photo-documented psoriatic lesions evaluation. The isoflavone was well tolerated by 85% of patients and no serious adverse events or discontinuations of treatment occurred. At the same time, no dose-limiting toxicities were reported and no substantial changes in pharmacodynamic parameters were observed. The clinical efficacy was rather poor in this test, which may in turn be related to the fact that high doses of the agent do not result in better effects than low doses due to the limited absorption of isoflavone preparations. In addition, frequent and modest intakes of genistein over the day rather than as a single high dose per day could be more efficient [3].

Furthermore, genistein impact on expression of genes when treating psoriasis patients was studied. Testing the effects of this isoflavone on transcript levels in both skin specimens and peripheral blood cells of four psoriatic subjects, I found that this compound modulated activities of genes coding for anti-psoriatic members and anti-inflammatory mediators of inflammation. The comparison of gene activity in the skin specimens and peripheral blood cells (PBCs) of u.09, 11, 12 and 15 patients participating in the genistein test is also

noteworthy. It was possible to produce a refined list of genes that were consistently differentially expressed in both inflamed skin and PBCs derived from all three patients treated with genistein, while not for placebo. Within this list, there are several genes that were deregulated; for patient u.09 were found 4 down-regulated transcripts (*CCL4*, *IL10*, *NFKB1* and *STAT3*), for subject u.12 there were 2 decreased (*CXCL10* and *IL6*) and 1 stimulated in activity (*IL1RN*), and in the case of patient u.15, there was one with increased (*IL8*) and one reduced (*IL10*) mRNA level [3]. Moreover, taking into consideration previous results on the use of "psoriasis-like" HaCaT keratinocytes exposed to isoflavone genistein, it was found that the 10 mRNAs (i.e. *CCL4*, *CXCL1*, *CXCL10*, *IL1A*, *IL6*, *IL8*, *KRT6B*, *PI3*, *SIGIRR* and *ZNF483)* studied in psoriatic skin patients were among the genes which were deregulated in the genistein-treated "psoriasis-like" epithelial cell line HaCaT [2, 3]. It impairs the activity of certain genes which are overexpressed in psoriasis, while stimulating the expression of other transcripts that are repressed in dermatosis.

In order to accomplish scientific purposes comprising my dissertation, the mechanism of action of genistein was proposed. The obtained results complement the existing knowledge on this subject, and are the basis for further research. It is worth emphasizing that, for the first time, the inhibitory nature of genistein in the ROS/NF-κB signaling pathway in keratinocytes mimicking the inflammatory process in psoriasis *in vitro* was shown. These studies form the foundations for basic research on potential pharmaceuticals using the developed *in vitro* model. Another important issue is the fact that, in the first phase of the clinical trial, the benefits of genistein in the treatment of psoriasis relate primarily to its safety. Therapeutically, this isoflavone represents a promising option, as it is well-tolerated and no blood test or imaging exam is mandatory during treatment. Another important advantage of this agent is the route of administration and the possibility of it being self-administered. Genistein may offer an oral treatment option for those patients who discontinue treatments because of ineffectiveness, intolerability, or ineligibility to the currently available drugs. As it is nowadays increasingly observed, another aspect of the medical use of genistein is its contribution to a combination therapy. Certainly, monotherapy with systemic agents is effective for many patients with psoriasis; however, some of them require combination approaches. For these reasons, clinical and therapeutic aspects related to the use of genistein still need to be investigated and studied in more depth.

Literature:

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