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Possible role of granulysin in pathogenesis of osteoarthritis

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ABSTRACT

Increased presence of immune mediator and cytotoxic/apoptotic molecule granulysin was noticed in different tissues during pathological processes with the domination of Th1 over Th2 mediated immunity. Beside granulysin expression in T and NKT cells, activated NK cells are thought to be the major source of chemotactic 15 kDa and cytotoxic 9 kDa granulysin *in vivo*. As NK cells are the principal joint's tissue-infiltrating lymphocyte subset, we hypothesized that granulysin mediated human cell death (apoptosis) could be responsible for the relatively silent damage of the joint's tissue without clinically notable signs of systemic inflammation in the patients with osteoarthritis (OA). The analyzes of the presence and frequency of granulysin expressing lymphocytes at protein and gene levels in peripheral blood and synovial samples and/or the samples of joint's tissue after the joint replacement therapy in patients with OA could give the initial insight to evaluate our hypothesis. It would be of the particular interest to differentiate the expression of 9 kDa and 15 kDa granulysin forms in the effector cells, since only the shorter form exhibits cytotoxic properties. The measurement of granulysin mediated early apoptosis in human NK sensitive K562 cells could be suitable *in vitro* model for evaluating granulysin activity. Furthermore, disturbed balance of pro-inflammatory and anti-inflammatory cytokines in OA patients, could influence the level of the granulysin expression. Having in mind that the granulysin and its regulation is still unknown in the pathogenesis of OA, it could be worth to explore this important pro-inflammatory, cytotoxic/apoptotic mediator.

Introduction

It has been considered that OA is induced by a mechanical stress, which causes cartilage destruction without significant participation of the immune response, particularly when compared with rheumatoid arthritis (RA) [1,2]. However, there were no statistical differences between the OA and RA patients regarding the percentages of T cells (T helper and T cytotoxic), B cells and natural killer (NK) cells in the peripheral blood, and the peripheral CD4/CD8 ratio, reflecting the similarities in the immune cell profiles of RA and OA patients [2,3]. Immune cells were also found in the synovial fluids and synovial membrane. T cells and their Th1-oriented $CD4^+$ subset were much less numerous in the joints of patients with OA than in the RA joints, but they showed a similar expression of activation HLA-DR and CD69 markers and profile of IFN-gamma cytokine secretion [3]. The predominance of Th1 cells were confirmed in both OA and RA joints [4]. Only the frequency of Th17 cells tended to be lower in the joint of patients with OA, than with RA [4]. Locally present $CD4^+$ T lymphocytes increased concentration of macrophage inflammatory protein-1 gamma in homogenized synovial tissue in an anterior cruciate ligament-transection model of OA in wild-type B6 mice [5]. The macrophage inflammatory protein-1 gamma, subsequently supported osteoclast formation, enhanced macrophage infiltration and matrix metalloproteinase-9 expression, which are all responsible for tis-

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sue destruction in mice model of OA [5]. Similarly, $CD8^+$ cells in the synovial membrane in OA patients correlated positively with increased matrix metalloproteinase-1 [6]. Several more studies in animal models [7] and human tissue [8] illustrate the potential importance of cytokine balance in local tissue changes and the development of OA. Thus the counteraction of the main pro-inflammatory interleukin (IL)-1 cytokine can prevent the progression of joint's structural changes in mice with OA [7]. The concentration of interferon-γ inducible protein-10 is low in the plasma and synovial fluid of OA patients and it inversely correlates with radiographic severity of the disease [8]. Conversely, IL-15 is elevated in the synovial fluid and the synovial membranes in early knee OA and positively correlated with IL-6 concentration [6], suggesting that these cytokines are the local factors responsible for the differentiation and the proliferation of T cells [9] and B cells [10] *in situ*, and activation of an innate immune response, mediated primarily by NK cells [6]. IL-15 is an effective chemoattractant for resting and activated T and NK cells [11]. It regulates the cytokine production and the cytotoxic potential of NK cells [12], as well as the mRNA expressions of perforin, Fas ligand [13], and granulysin [14]. However, little is known about the role of the cell-mediated immune response and its cytotoxic/apoptotic mechanisms, particularly perforin and granulysin in the pathogenesis of OA at the local and systemic levels. The presence of cytotoxic protein – perforin within the cytoplasm granules of the $CD4^+$, $CD8^+$, $CD56^+$, $CD16^+$, and $CD25^+$ lymphocyte subsets from the synovial fluid and the synovial membrane of OA patients, was identified using flow cytometry [3]. Perforin expression suggests the role of activated cytotoxic immune T and NK cells in the pathogenesis of OA [3], as perforin correlates with cell mediated cytotoxic potential of T cell and NK cells in different tissues [15–17].

In the activated NK effector cells, perforin is stored in dense vesicles together with pro-apoptotic mediators, such as granzymes, Fas ligand, and cytotoxic 9 kDa granulysin form [17,18]. Longer, 15 kDa form of granulysin is stored in distinct granules, situated directly beneath the cell membrane and it can be processed to the shorter, cytotoxic 9 kDa granulysin form [18]. The longer 15 kDa granulysin shows predominantly regulatory properties as alarmin, due to the recruitment of immune cells to the site of inflammation [17,19]. It strongly chemoattracts monocytes, $CD4^+$ and $CD8^+$ memory T cells, NK cells and mature dendritic cells [20] and induce differentiation of monocyte into dendritic cells [18]. Both granulysin containing granules are released from the effector cells on demand, in accordance with cell stimulation [17]. However, 9 kDa form is released after the receptor mediated granule exocytosis pathway, depending of current expression of activation and inhibitory NK cell receptors and their ligands on target cells [18]. Longer 15 kDa form of granulysin is released constantly by IL-15 activated NK cells mostly [18]. Granulysin uses multiple mechanisms to enter the cells and kill targets. As granulysin is kationic molecule of saposin-like family of proteins, it possesses structural features allowing him to associate with lipids [21]. 9 kDa granulysin is endocytosed by the infected eukaryotic cells via lipid rafts [21]. In the cells 9 kDa granulysin kills bacteria efficiently within minutes [20,22], because it forms defects in negatively charged, cholesterol-free membranes of prokaryotic bacterial cells, but leave the eukaryotic cell intact [21]. It is believed that cytotoxic activity of granulysin against eukaryotic cells is due to binding of granulysin for negatively charged mitochondrial membrane [23], after the quick cytoplasmic access by perforin pores [24,25]. Mitochondrial cell damage supports the quick release of apoptosis-inducing factors and mitochondrial cytochrome C, which then induces DNA fragmentation in a caspase-dependent or caspase-independent manner [23,26,27]. Granulysin can also mediate apoptosis by nuclear accumulation [28]. A slower mechanism of granulysin-mediated apoptosis seems to be conducted by ceramide generation in the target cell membrane, likely without perforin support [27].

Hypothesis

We hypothesized that cytolytic immune mediator granulysin participates in cartilage destruction in patients with OA. In OA patients, granulysin could increase lymphocyte infiltration in synovial tissue and synovial fluid, as well as in the plasma and peripheral blood lymphocyte subpopulations. NK cells might be the main source of granulysin in OA patients, since activated NK cells has been shown as the major source of serum granulysin *in vivo* [18]. Moreover, NK cells are the principal tissue-infiltrating lymphocyte subset in patients with OA, comprising nearly 30% of the $CD45^+$ mononuclear cell infiltrates in the synovial tissue obtained from patients undergoing total joint replacement surgery [29]. Increased presence of granulysin has been noticed in different tissues during pathological processes with the domination of Th1 over Th2 mediated immunity. For example, the frequency of granulysin expressing NK cells is enhanced in peripheral blood of patients with preeclampsia [30] and psoriatic arthritis [31]. Skeletal muscles and the hearth are shown to be infiltrated with granulysin positive cells during polymyosytis [32] or acute infarction [33], respectively. Granulysin concentration in human serum and broad cytotoxic activity of granulysin against virally infected cells, tumors, and transplanted cells [28] indeed classify this mediator as a human marker of the Th1 immune response [20,26,30,34]. Thus the dynamic changes in the expression of granulysin in patients with OA could be the reflection of disturbed balance of humoral pro-inflammatory and anti-inflammatory factors, at least at the local level. And finally, granulysin mediated cell death supporting apoptosis could be responsible for the relatively silent damage of the joint's tissue without clinically notable, eclatant systemic inflammatory reaction.

Evaluation of the hypothesis

To estimate the granulysin-mediated cytotoxic potential of lymphocytes in patients with OA, it is essential to form homogenous group of patients with OA and healthy control. The inclusive criteria could be set up according to the recommendations of the American College of Rheumatology for diagnosis of knee [35], hand [36] or hip [37] OA.

The exclusion criteria for the group of patients with OA and control group should also be defined precisely, particularly in terms of other possible systemic immune diseases. The therapy of participants deserves particular attention and should not interfere with the immunological examinations.

The estimation of the presence and frequency of granulysin expressing lymphocyte subpopulations in freshly isolated peripheral blood samples of patients with OA at protein and gene levels will give the first approach to evaluate our hypothesis. Our preliminary data show that granulysin positive cells in peripheral blood samples of OA patients counts about 30% and it was approximately twice higher, when compared with healthy control. It was due to higher granulysin expressing in NK, T and NKT leukocyte subsets of OA patients, as measured by flow cytometry. The second approach to test this hypothesis could be the analyzes of granulysin-mediated NK cell cytotoxicity in different time points using major histocompatibility complex (MHC) devoid, NK sensitive K562 cells of human erythroleukaemia cell-line, as targets in classical *in vitro* model for the assessment of NK cell activity [38]. ^{51}Cr release assay measures necrosis/lysis [39] very precisely [40], but the measurement of NK cell mediated target cell apoptosis could be more suitable for evaluating granulysin activity. Late