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Manipulation of apoptosis as a treatment modality in rheumatoid arthritis

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Rheumatoid arthritis (RA) is an inflammatory and destructive joint disease. Present treatment modalities although beneficial in approximately 40% of RA patients, are insufficient to prevent severe disability in a high number of nonresponders. Thus, new treatment approaches for RA are required. Apoptosis is programmed cell death. Insufficient apoptosis in the inflamed RA synovia leads to intra-articular accumulation of highly differentiated B and T lymphocytes and invasive growth of macrophages and fibroblasts. It might also prevent response to antirheumatic treatment. The intracellular events supporting long-term cell survival in RA include low sensitivity to the ligation of professional death receptors (Fas and tumor necrosis factor receptor) and the increase in caspase and mitochondrial activity inhibitors. The authors summarize the attempts to increase apoptosis in RA by stimulation through death receptors, targeting proteins of the inhibitor of apoptosis protein family and by using the inhibitors of transcriptional pathways and of the cell cycle. Increasing susceptibility of RA synovia to apoptosis may be an attractive therapy in addition to the existing treatment modalities.

Keywords: apoptosis, cell cycle, Fas, inhibitor of apoptosis protein, proteasome inhibitors, rheumatoid arthritis, therapy, transcriptional regulation

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Rheumatoid arthritis (RA) is the most common inflammatory joint disease with a prevalence approximately 1% in the general population. RA is an important social issue affecting middle-aged, professionally-active women and leading to severe disability within 5 years in nearly half of all patients. Despite using an optimal cytokine-targeting treatment regimen, only a small proportion of RA patients achieve remission and 28–56% of patients do not show any signs of clinical response [1]. Morphologically, RA is characterized by the influx of inflammatory leukocytes into the synovial tissues, the uncontrolled proliferation and hyperplasia of synovial tissue resulting in formation of pannus on the surface of joint cartilage and the invasive growth of hyperplastic synovial tissue into the cartilage leading to cartilage and bone destruction. Three main cell types have been shown to be important for the pathogenesis of RA, T and B lymphocytes, macrophage-like synoviocytes and fibroblast-like synoviocytes [2]. The analyses of synovial fibroblasts from patients with RA reveal features of transformed, long-living cells, such as the presence of somatic mutations, expression of oncogenes and resistance to apoptosis [3–5]. Resistance to apoptosis is suggested as the main characteristic contributing to synovial hyperplasia and joint destruction [6,7]. Mechanisms protecting RA synovial tissue from apoptosis possibly lead to a poor response to immunomodulating drugs. Thus, increasing susceptibility of RA synovia to apoptosis may be a valuable addition to the existing treatment modalities. In this review, the authors analyze the information regarding apoptosis dysfunction in RA synovium and suggest possible methods of apoptosis regulation in the clinical setting.

Apoptosis pathways

Apoptosis is a tightly regulated process of elimination of aging cells without disrupting cellular integrity [8,9]. Two major mechanisms are known to initiate apoptosis: extracellular, by the activation of receptors and structures on the cell membrane; and intracellular, by the release of mitochondrial content into cytoplasm. Both pathways induce expression of apoptosis genes and activation of the caspase cascade, resulting in DNA fragmentation.

The extracellular/receptor-driven apoptosis pathway is activated by the ligation of death receptors (DR3, DR4 and DR5), belonging structurally to the tumor necrosis factor (TNF) receptor family, or by the activation of Fas (CD95) by its interaction with the Fas ligand (FasL). The FasL–Fas interaction forms the basis for T-cell-mediated cytotoxicity, while TNF-receptor-mediated apoptosis occurs in macrophages, fibroblasts and dendritic cells. Besides TNF, DRs 4 and 5 are activated by TNF-related apoptosis-inducing ligands (TRAILs). Following linking and oligomerization of Fas or DRs, the cytoplasmatic part of the receptor recruits adaptor molecules (Fas associated death domain [FADD] or TNF-receptor-associated death domain [TRADD]) activating caspase 8 and the remaining downstream

caspases. Besides the activation of effector caspases, caspase 8 cleaves a proapoptotic protein, Bid, triggering a mitochondria-related pathway.

The initiation of intracellular/mitochondrial mechanism triggered apoptosis occurs following the release of cytochrome C and/or second mitochondria-derived activators of caspase (Smac/DIABLO) from mitochondria into cytoplasm. In the cytosole, cytochrome C binds to apoptotic peptidase-activating factor (Apaf)-1 and triggers its complex formation with caspase 9. Smac/DIABLO acts as a proapoptotic protein by directly binding the proteins of the inhibitor of apoptosis proteins (IAPs) family, preventing them from interaction with caspases. The activation of effector caspase 3, by Apaf-1/caspase 9 or caspase 8, initiates a serial cleavage and the activation of downstream caspases, resulting in cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins, inhibitor subunits of endonuclease and destruction of housekeeping cellular functions. The described changes lead ultimately to morphological manifestations of apoptosis, such as DNA condensation and fragmentation and membrane blebbing.

Mechanisms regulating apoptosis

FasL–Fas signaling is inhibited by Fas-associated phosphatase (FAP)-1, FADD-like interleukin (IL)-1β-converting enzyme inhibitory protein (FLIP) and soluble decoy receptors. These decoy receptors antagonize stimulation of Fas by competing with the FasL. Cytochrome C release is regulated by the group of proteins named the Bcl-2 family. The Bcl-2 family includes proapoptotic members, such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk, and anti-apoptotic members such as Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1, blocking the release of cytochrome C. The balance between Bcl-2 and Bax determines the fate of the cell. Bcl-2 is named for its role in B-cell lymphoma, as it was the first proto-oncogene identified that promotes neoplastic expansion not by driving cell division, but rather by preventing cell death [10]. The caspases downstream of caspase 3 are controlled by the family of IAPs. The IAP family consists of XIAP, cIAP-1 and -2, NIAP, Bruce, survivin and livin. The IAPs stop apoptosis by binding directly to and degrading active caspases.

Recent studies indicate that molecules controlling cell-cycle check points such as p53 are tightly connected with the regulation of apoptosis. p53 is a primary component of cell-cycle regulation, controlling the G_1 and G_2 check points [11]. The p53 protein functions as a transcription factor regulating downstream genes important in cell-cycle arrest, DNA repair and apoptosis. After DNA damage, p53 holds the cell at a G_1 checkpoint preventing the progression of the cell cycle until DNA damage can be repaired. If the damage is irreversible, apoptosis will ensue. p53 mediates apoptosis through the induction of proapoptotic proteins Bax, noxa and Perp, and simultaneously inactivating the gene of anti-apoptotic survivin [12]. The loss or malfunctioning of p53 leads to inhibition of apoptosis and accumulation of individual mutations typical for a long-living cell.

Nuclear factor (NF) κB is a nuclear transcription factor that regulates expression of a large number of genes involved in inflammation and apoptosis. NFκB has been shown to have both anti- and proapoptotic functions that are determined by the nature of the death stimuli. Under physiological conditions, the activation of NFκB induces resistance to apoptosis through the activation of TNF-receptor-associated factor, IAP and XIAP. However, in certain circumstances, activation of NFκB leads to the activation of proapoptotic proteins, such as interferon-regulated factor 1, c-myc, p53, and caspase 1. PI3 kinase is a multifunctional kinase playing a central role in the cell survival, proliferation, motility and tissue neovascularization. PI3-kinase activates the kinase Akt and exerts dual effects on apoptosis: both phosphorylating IκB and liberating active NFκB, combined with activating Mdm2, which blocks p53, phosphorylates antiapoptotic Bad and inactivates caspase 9. Akt kinase is regulated by the phosphate and tensin homolog (*PTEN*) tumor suppressor which functions as a phosphatase, possessing both lipid and protein phosphatase activity *in vitro*. *PTEN* is the second most frequently mutated human tumor-suppressor gene after *p53* [13].

Intracellular degradation of proteins is achieved by the proteasome complex. The binding of ubiquitin molecules to lysine residues in proteins is one way to destine the protein for degradation by the S26 proteasome, the multicatalytic protease. By contrast, the binding of sentrin to a protein will protect the protein from degradation. The ubiquitination determines the activity and concentration of the apoptosis regulating Fas- and TNF-receptor adaptive proteins, proteins of the Bcl family

and IAPs, as well as the fate of transcription factors p53, stress kinases and inhibitory proteins of the IκB family.

Pro- & anti-apoptosis activities in RA

Multiple disturbances in the apoptotic machinery have been observed in synovia of RA patients. Inflammation with accumulation of cytokines and other products from activated macrophages and lymphocytes results in the upregulation of NFκB and PI3 kinase activity. Activation of NFκB and PI3 kinase signaling makes RA synovial cells insensitive to TNF-mediated apoptosis. By contrast, TNF induces synovial lymphocytes to proliferate. Neither inhibition of NFκB nor PI3 kinase alone induces apoptosis in RA synovial fibroblasts. However, the neutralization of NFkB and PI3 kinase makes the RA cells susceptible to TNF-mediated apoptosis. RA synovial T cells and fibroblasts express Fas, but not FasL, and do not undergo spontaneous apoptosis *in vivo*. However, 20–90% of cells are sensitive to Fas-mediated apoptosis in experimental conditions. Increased levels of soluble Fas, neutralizing FasL stimulation, in RA synovial fluid have been suggested as a possible explanation for the low apoptotic rate in the Fas-positive cells in the RA synovium [14]. Fas-deficient mice spontaneously developed chronic synovitis, which further supports the importance of the disruption of Fas–FasL signaling in the pathogenesis of RA [15]. Moreover, erosive arthritis will develop following exposure of these mice to bacterial infection [16].

Resistance to Fas-induced apoptosis in RA synovium correlates with a marked increase in expression of sentrin-1[17]. Binding of sentrin-1/small ubiquitin-related modifier (SUMO) to a given protein results in the prevention of ubiquitin-related processing and degradation of the protein. Sentrin-mediated protection has been shown for a number of proteins, including p53 and IκBα. Overexpression of synoviolin, described recently as ubiquitin ligase, is also associated with apoptosis recovery [18].

Upregulation of antiapoptotic molecules belonging to the Bcl family and the caspase 8 inhibitor FLIP has been reported repeatedly in RA [19] and has been shown to contribute to the pathogenesis of experimental arthritis [20,21]. Among the antiapoptotic genes, *Bcl-xL*, *survivin* and *Bcl-2* were reported to be overexpressed in RA fibroblast-like cells and T cells, suggesting deficient control of synovial hyperplastic growth. It has been shown recently that the expression of *Bcl-2* and *Bcl-xL* in synovial tissue is enhanced following stimulation with IL-15. In addition, increasing apoptosis of RA synovial fibroblasts occurs when IL-15 stimulation is stopped [20]. The presence of extracellular survivin in blood and synovial fluid of RA patients was associated with destructive joint disease [22]. Less is known regarding the role of mitochondria-induced apoptosis in the pathogenesis of joint inflammation. Recent studies on synovial fluid showed the low cytochrome C pool in inflamed RA joints [23].

Overexpression of *p53* in the synovial tissue of RA patients has been reported in several studies [24,25]. Further studies on the cell-cycle regulators indicated that *p53* in RA synovia is active [26]. It has been suggested that *p53* has a protective role preventing synovial hyperthrophy and invasive growth in RA by abrogation of the cell cycle and inducing apoptosis [25,27]. Somatic mutations in the *p53* gene have been reported in RA synoviocytes and mononuclear cells, but not in RA skin or control osteoarthritis synovium [24,28]. Strikingly, overexpression of *p53* was present in the synovial tissue of RA patients, independently of proliferation and even during the remission of articular inflammation [26,29]. Finally, the direct pathogenetic role of *p53* mutation in RA was proved by demonstration of an increased cellularity and severe cartilage destructions in the *p53* knockout mice [30].

Influence of contemporary antirheumatic treatment on apoptosis

Modern treatment of RA is based on the use of a dihydrofolate reductase inhibitor, methotrexate (MTX). Although apoptosis has been suggested as a methotrexate-induced effect on RA lymphocytes and synovial monocytes [31], other mechanisms may also play a role in the anti-inflammatory effects of methotrexate [32]. Analogously, it has been reported that other frequently used disease-modifying agents in patients with RA, for example sulfasalazine, cyclosporin A and gold compounds, might function through the induction of apoptosis. The benefit of RA treatment using biological substances is evident. The induction of apoptosis is suggested as the main mechanism through which TNF-α inhibitors alleviate inflammation. Polymorphisms in the *FasL* and *caspase-9* genes are predictive of beneficial effect following treatment with TNF-α inhibitors [33]. There are contradictory reports regarding the sensitivity of different cell populations to apoptosis achieved by TNF-α antagonists. Infliximab and adalimumab induce apoptosis by ligating membrane-bound

TNF α and induce apoptosis in T lymphocytes and monocytes, but not in synoviocytes of RA patients [34–36]. Being a TNF-α receptor analog, etanercept neutralizes soluble TNF α with no connection to the cell surface. Treatment with etanercept increases apoptosis of synovial fluid monocytes [37]. The lack of specificity of conventionally used antirheumatic drugs and simultaneous promotion of both pro- and anti-apoptotic signals, indicates a need for more selective therapeutic agents interfering with apoptosis during RA.

Development of genetic therapy for RA

Prevention of intracellular proapoptotic signals in RA synovium may be achieved by:

- Direct inhibition of IAPs or proteasome enzymatic activity using synthetic inhibitors
- Short inhibitory RNA or oligonucleotide sequences preventing transcription of apoptosis inhibitors
- Supporting naturally occurring inhibitors (recombinant proteins or genetic transfectans with continuous production of the protein of interest)

The use of biological antirheumatic drugs has proved that delivery of the macromolecule may provide a beneficial effect. However, due to the short half-life of cytokines this mode of treatment appears to be expensive and often insufficient to stop the complex pathological process of joint inflammation. Efficient methods of intra-articular gene delivery systems have been established, including plasmid DNA and replication-deficient viral vectors [38]. These new methods allow the precise intracellular targeting of pathological processes and permit the transcriptional regulation of gene expression in response to pharmacological agents. Regulated gene expression is an important issue for chronic conditions, such as RA, being characterized by phases of relapse and remission. Therapeutic genes may be delivered directly into the patient (*in vivo*) or indirectly into isolated cells (*ex vivo*). Both cultures of mobile (T cells, B cells, or dendritic cells) and transplanted islets of synovial fibroblasts have been used for genetic manipulations.

Future therapeutic approaches to enhance apoptosis during RA

Fas ligation

Administration of antagonistic anti-Fas antibodies or FasLs has been shown to be effective in abrogation of collagen-induced arthritis [39,40]. Treatment of Fas-deficient mice with FasL-expressing cells reduced synovial hyperplasia and lymphocyte infiltration in affected joints. Local injection of anti-Fas antibodies targeted FasL-expressing cells, leading to their apoptosis and a significant reduction in cell number within the synovial tissue. However, clinical use of direct Fas ligation is unacceptable due to adverse effects on the liver. Suppression of FLIP, one of the intracellular inhibitors of the Fas-dependent pathway, by antisense transfection [41] or CD40 ligation [42] significantly increased sensitivity to Fas-mediated death stimuli in cell cultures. Therapeutic approaches using *ex vivo* manipulated dendritic cells to express the proapoptotic ligands FasL and TRAIL have been successful for the elimination of autoreactive T cells and neutrophils in established arthritis [43–46]. The favorable effect of TRAIL on the course of collagen-induced arthritis has been proved, both by exacerbation of arthritis following the administration of soluble DR5 blocking TRAIL and in contrast, reduction of arthritis was achieved by treatment with TRAIL-expressing dendritic cells [45].

Alternative ways of activating apoptosis have been explored, including use of nonprofessional death receptors. It is known that tissue inhibitor of metalloproteinases (TIMP)-3 mediates apoptosis induction by ligation and trimerisation of the Fas activating caspase-8-dependent cascade [47,48]. Transfection of synovial fibroblasts with adenoviral vectors encoding TIMP-1 and -3 protects efficiently from the invasive behaviour of RA synovial fibroblasts *ex vivo* and from cartilage degradation *in vivo* [49]. The expression of mammalian lectin, galectin-1, by autologous fibroblasts was effective for the treatment of established collagen-induced arthritis by inducing apoptosis of pathogenic T cells and promoting the shift to a therapeutic T helper (Th) 2 immune responses [50]. A synergy of proapoptotic effects was observed between recombinant TRAIL and synthetic retinoid receptor agonists potentiating mitochondrial and caspase-3-dependent apoptosis as well as downregulating *Bcl-2* and increasing expression of *Bad* [51].

Targeting IAPs

Adenoviral transfection of RA synovial fibroblasts with XIAP-antisense facilitated apoptosis in synovial tissue [52]. High *survivin* expression is associated with a decrease in *p53* expression [53] and a relative PTEN deficiency leading to activation of PI3-kinase pathway. Small interfering RNA (SiRNA) against survivin sensitized p53 mutated

cells to apoptosis [54]. Smac/DIABLO, a protein released from mitochondria together with cytochrome C, acts as a proapoptotic protein by physically preventing IAPs from caspase inhibition [55]. Therefore, peptide inhibitors of IAPs have been designed using the Smac protein as a template. Such a peptide sensitized resistant malignant cells *ex vivo* to TNF-induced apoptosis [56]. Interestingly, these Smac peptides have little or no activity without an additional apoptotic signal. Thus, the utilization of these peptides may be a valuable agent for combination therapy of arthritis. The inhibitors of 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase (statins) exert proapoptotic properties on lymphocytes by increasing the cytosolic Smac pool [57]. This may be one of the mechanisms explaining efficient, anti-inflammatory properties of statins in the adjuvant arthritis model in rats [58].

Proteasome inhibitors have a unique ability to activate caspases through a mitochondrial pathway that does not need a functional apoptosome [59]. In certain cases, the effect of proteasome inhibitors is mediated by Bcl-2 family members [60]. Migita and colleagues demonstrated an accumulation of p53 in RA synoviocytes following *in vitro* treatment with the protesome inhibitor, MG-132 [61]. In the animal model of streptococcal wall-induced arthritis, treatment with proteasome inhibitor, PS-341, resulted in the attenuation of clinical progression of arthritis with respect to inflammatory cell infiltration and cartilage destruction [62].

Cell-cycle inhibitors

Despite the fact that *p53* is an important component in the pathogenesis of arthritis, its direct inhibition may lead to significant damage of normal cells. In contrast, overexpression of *p53* inhibited IL-1β-mediated arthritis [63]. It has been suggested that a selective G_2 checkpoint abrogation, not involving *p53*, could be a preferable target [11]. Several synthetic inhibitors and peptides affecting check point kinase (CHK) 1 and/or CHK2 are presently undergoing clinical safety trails for cancer indications. The authors have shown recently that etoposide, a topoisomerase II inhibitor, has remarkable antierosive properties in collagen-induced arthritis [64].

Peroxisomal proliferator-activated receptor-γ agonists

Peroxisomal proliferator-activated receptor (PPAR)-γ agonists have recently shown efficiency in promoting apoptosis *in vitro* and alleviating adjuvant arthritis [65,66] and collagen-induced arthritis [67] by reducing cellularity in the synovia and cartilage destruction. A clinical study of pioglitasone in psoriatic arthritis demonstrated a significant reduction in disease activity during treatment [68]. Activation of PTEN in response to PPARγ agonists has been suggested as the major antiarthritic mechanism associated with an increase caspase-3-dependent apoptosis and increased expression of *p53* [69].

Transduction pathway inhibitors

The transcription of genes encoding for apoptosis proteins is regulated by NFκB and PI3-kinase signaling pathways. The intra-articular use of a combination of antisense-targeting NFκB has been suggested as a promising approach in the treatment of arthritis [70]. However, the benefit of direct NFκB inhibition varied depending on the model of arthritis used [52,71]. The insufficient effect could be related to the short half-life of injected antisense sequences. Indeed, *ex vivo* transfection of synovial fibroblasts for permanent NFκB inhibition might be a promising option. The insertion of I κB kinase (IKK)-β overexpressing plasmid [72] or inactivation-resistant IKKβ mutant [73] into synovial fibroblasts ameliorated the severity of adjuvant-induced arthritis. A synthetic IKK inhibitor, BMS-345541, has also been shown to be efficient in improving the clinical course of collagen-induced arthritis, decreasing both synovial inflammation and joint destruction [74]. The intra-articular instillation of a peptide blocking the NFκB essential modulator (NEMO)-binding domain of IKK attenuated swelling, cell influx and cartilage destruction of the inflamed joints [75]. Inhibition of PI3-kinase facilitates TNF-induced apoptosis in synovial fibroblasts [76].

Future perspective

Restitution of apoptosis in the inflamed synovial tissue is an essential step in combating arthritis. Several approaches using pharmacological compounds and intracellular engineering have been tested and proved efficient in overcoming apoptosis resistance in RA synovia *in vitro* and in animal models of arthritis. Clinical studies of proapoptotic substances in patients with RA are awaited in the near future. Recent benefits in the treatment of RA using a cytokine-targeting approach encouraged enthusiasm in both patients and rheumatologists. A better understanding of the mechanisms initiating and perpetuating inflammation in RA will help identify the appropriate therapeutic

target for various stages of the joint disease. The combination of interventions inducing synergistic effects in remodeling intracellular processes and supporting a healthy cell cycle and apoptosis would be advantageous in the treatment of RA. Therapeutic modalities directed at the induction of apoptosis in the inflamed synovia will find their place in the treatment of RA. They will be a valuable addition to the existing disease-modifying and biological antirheumatic drugs.

Executive summary

Introduction

- Rheumatoid arthritis (RA) is an inflammatory and destructive joint disease.
- Present treatment modalities, although beneficial in approximately 40% of RA patients, are insufficient to stop severe disability in a high number of nonresponders.
- Thus, new treatment approaches for RA are required.

Apoptosis pathways

- Apoptosis is a programmed cell death initiated extracellularly by the activation of Fas and tumor necrosis factor (TNF) receptors on the cell surface and intracellularly by the liberation of cytochrome C from mitochondria into cytoplasm.

Mechanisms regulating apoptosis

- The regulation of apoptosis is controlled by specific inhibitory proteins (of the Bcl and inhibitor of apoptosis protein [IAP] families), the ubiquitin–sentrin proteasome system of protein elimination and, finally, by transcription factors governing apoptosis genes.

Pro- and anti-apoptosis activities in RA

- Insufficient apoptosis in the inflamed RA synovia leads to intra-articular accumulation of highly differentiated B and T lymphocytes and invasive growth of macrophages and fibroblasts destroying the cartilage.
- Resistance to apoptosis might also prevent response to antirheumatic treatment.
- The intracellular events supporting long-term cell survival in RA include, low sensitivity to the ligation of professional death receptors (Fas and TNF receptors) and the increase of caspase and mitochondrial activity inhibitors.

Influence of contemporary antirheumatic treatment on apoptosis

- The ability to potentiate apoptosis is reported for many of the antirheumatic drugs. The exact mechanism(s) of this effect remains unknown preventing regulation and direction of apoptosis by means of these drugs in clinical settings.

Development of genetic therapy for RA

- Gene therapy is a new approach at targeted interfering of processes at the single-cell level. It opens wide perspectives for the development of selective strategies in the treatment of RA.

Future therapeutic approaches to enhance apoptosis during RA

- Modulating apoptosis is obtained in animal models and human cell cultures. Experimental arthritis could be alleviated successfully by stimulation of death receptors, targeting proteins of the IAP family, using inhibitors of transcriptional pathways and inhibitors of the cell cycle.
- Similar approaches efficiently changed invasive, pro-inflammatory phenotype in rheumatoid synoviocytes.
- Increasing susceptibility of synovia to apoptosis in patients with RA may be an attractive therapy in addition to the existing treatment modalities.

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