Trichostatin-A sensitizes rheumatoid arthritis synovial fibroblasts for TRAIL-induced apoptosis


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Abstract

Histone acetylation/deacetylation plays a critical role in the regulation of transcription by altering the chromatin structure. The effect of Trichostatin A (TSA), a streptomyces metabolite which specifically inhibits mammalian histone deacetylases, was analyzed for TRAIL-induced apoptosis of rheumatoid arthritis synovial fibroblasts (RASF).

Apoptotic cells were detected after co-treatment of RASF with TRAIL (200 ng/ml) and TSA (0.5; 1 and 2 µM) by flow cytometry using propidium iodide/ annexin-V-FITC staining. Cell proliferation was assessed using the MTS-proliferation test. Induction of the cell cycle inhibitor p21^{Waf1/Cip1} by TSA was analyzed by Western blot. Expression of the TRAIL receptor-2 (DR5) on the cell surface of RA-SF was analyzed by flow cytometry. Levels of soluble TRAIL were measured in synovial fluid of patients with RA and OA by ELISA.

Most interestingly, co-treatment of the cells with TSA and TRAIL induced cell death in a synergistic and dose-dependent manner, whereas TRAIL and TSA alone revealed no or only a modest effect. RASF express DR5 (TRAIL-receptor 2), but treatment of the cells with TSA for 24 h did not change the expression level of DR5, as it was shown for cancer cells. TSA induced cell cycle arrest in RASF via upregulation of p21^{Waf1/Cip1}. Levels of soluble TRAIL were significantly higher in RA compared to OA synovial fluids.

Based on the fact that TSA sensitizes RASF for TRAIL-induced apoptosis, it is concluded that TSA reveals sensitive sites in the cascade of TRAIL signaling and could represent a novel principle for the treatment of rheumatoid arthritis.
Introduction
Rheumatoid arthritis (RA) is a chronic inflammatory disease that results in the progressive destruction of joints [1]. Apart from B, T cells and macrophages, activated RA synovial fibroblasts (RASF) are the major players in the process of joint destruction. Current therapies for RA aim to inhibit inflammation and specifically to suppress the immune response of T and B cells, but the destructive process driven by synovial fibroblast-like cells remains mostly unaffected.

Histone acetylation / deacetylation (HDAC) plays a critical role in gene regulation by altering the chromatin structure [2]. Inhibitors of HDACs are effective anti-cancer agents by inducing cell cycle arrest, cell differentiation or apoptotic cell death of transformed cells [3]. Trichostatin A (TSA), originally developed as an antifungal agent from Streptomyces hygroscopicus, is a highly potent and reversible inhibitor of HDAC [4], which is already active at very low concentrations. TSA causes histone hyperacetylation thereby modulating multiple gene expression and inhibiting the cell cycle in normal fibroblasts [2]. In RA, no imbalance of histone acetylation has been described so far. In synovial cells, the HDACIs TSA and FK228 induce the expression of the cell cycle regulators p21 and p16 and inhibit the expression of tumor necrosis factor (TNF)-alpha in affected tissues in arthritic animals [5, 6]. In the present study, we ask whether TSA is capable to sensitize RASF to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis.

Material and Methods
Synovial tissue specimens were obtained during synovectomy and arthroplastic surgery from 7 patients with RA (2 male in the age of 64±7; 5 females in the age of 69±10 years, mean disease duration 27±13 years) and 3 patients with osteoarthritis (OA) (1 female in the age of 79 years, 2 male in the age of 66±9 years). All RA patients were under treatment (5x Prednisone, 2x Methotrexate, 1x Indocid and/or 2x Salazopyrin). RASF were isolated from synovial tissues, digested by collagenase, and used from passages 4 to 8 as described [7]. All RA patients fulfilled the American College of Rheumatology (ACR) criteria [8]. Synovial fluids from patients with RA and osteoarthritis (OA) were obtained during joint aspiration from 16 patients with RA and 16 patients with OA. Samples were centrifuged at 1300g for 15 minutes and stored at −70°C until analyzed. All experiments were performed with the permission from the local ethics committees and informed consent were obtained from all patients to participate in the present study.

After treatment of RASF with TSA (0.5, 1 and 2 µM) in combination with TRAIL (200ng/ml), apoptotic cells were detected by flow cytometry using propidium iodide/annexin-V-FITC staining (Roche; Basel, Switzerland). The effect of TRAIL (200ng/ml; Alexis; Lausen Switzerland), TSA (2 µM Sigma, Buchs, Switzerland) and the co-treatment on cell proliferation was assessed using the CellTiter96®AQeous Cell Proliferation Assay (Promega, Wallisellen, Switzerland). Induction of the cell cycle inhibitor p21 Waf1/Cip1 by TSA was analyzed by Western blot. RA-SF (2x10^6/well) were incubated in the absence or presence of TSA (2µM) and lysed in Lämml buffer on days 1, 4 and 14. Protein extracts from RASF were analyzed by electrophoresis on 10% SDS-PAGE gel, and transferred onto a nitrocellulose membrane. The membrane was blocked for 2 h with TBS containing 0.1% Tween 20 (TBST) and 5% dehydrated skim milk. Blots were then incubated overnight at 4°C in the presence of monoclonal antibody to p21 Waf1/Cip1 (Cell Signaling) or α-tubulin (Sigma). The blots were washed 3x15 min with TBST, and incubated with HRP-conjugated goat-anti mouse IgG secondary antibodies at room temperature for 1 h. After washing with TBST, the bound antibodies were detected by visualization using enhanced
chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK). Expression of surface DR5 was analyzed by flow cytometry after incubation of the cells with 2 µM TSA for 24 h after detachment of the cells with accutase using mouse anti-human DR5 antibodies (R&D, clone MAB6311). Levels of soluble TRAIL were measured in hyaluronidase pre-treated (10 mg/ml, 37°C, 1 h, Sigma; Buchs, Switzerland) synovial fluid from patients with RA and OA by ELISA (Diaclone Research, Besancon, France). Data are expressed as the mean ±SD. Mann Whitney test was used for statistical analysis. P values less than 0.05 were considered significant.

Results
In our study, RASF treated with TRAIL (200ng/ml) showed no induction of apoptosis. TSA at a dose of 2 µM induced apoptosis in 24.2±5% of the cells. Most interestingly, co-treatment with TSA and TRAIL induced cell death synergistically and dose-dependently in 69±8% of the cells after 4 days (p<0.01; n=3 each) (Figure 1). Cell cycle arrest was induced by TSA (2µM) on day 1 and was still present on days 4 and 6 (p≤0.001) shown in Figure 2 and accompanied by the induction of p21 Waf1/Cip1 in RASF (Figure 3). In contrast TRAIL (200ng/ml) did not influence cell proliferation significantly. Surface DR5 was detected in 41±2% of RASF and in 9±2% OASF (n=3 each). Treatment of the cells with TSA (2µm) for 24 h did not change the expression level of DR5 on RASF (41±10%). Of interest, detachment of the cells with accutase was essential, since trypsin strongly reduced the cell surface expression profile of DR5 (8±2%). Levels of soluble TRAIL were significantly higher in RA synovial fluids (mean 1116±1215 pg/ml, n=16) compared to OA synovial fluids (mean 62±77 pg/ml, n=16, p<0.001) (data not shown).

Discussion
In the present study, we show that TSA, an inhibitor of histone deacetylases, has a dual effect on RASF; it sensitizes RASF for TRAIL-induced apoptosis and furthermore, effectively inhibits their proliferation via up-regulation of p21 Waf1/Cip1. After this sensitization, TRAIL is the main inductor of apoptosis. Since we demonstrate that patients with RA have increased levels of soluble TRAIL in synovial fluids when compared to patients with OA, the application of non-toxic levels of TSA could become a new therapeutic principle in the treatment of RA.

Previous studies showed that targeting TRAIL-R2 (DR5) alters the apoptosis of synovial cells. RASF with high levels of surface DR5 were highly susceptible to anti-DR5 mediated apoptosis, therefore such strategies were propagated as a potential therapy for RA [9]. Miranda-Carus et al. reported high levels of DR5 on RA synovial fluid cells, whereas both OASF and normal skin fibroblasts were negative for this receptor and consequently resistant to anti-DR5 mediated apoptosis [10]. In contrast, Perlman et al. found no expression of DR5 on synovial fibroblasts [11]. Our results showed stronger expression of DR5 on RASF than on OASF. We also observed that trypsinization of the cells strongly reduced the presence of the DR5 on their surface. This fact may explain the discrepancies between the studies.

TRAIL-mediated apoptosis in RASF is discussed controversially. In our study, TRAIL alone does not induce apoptosis in RASF. This is in agreement with studies from Park et al. [12], whereas other groups reported TRAIL-induced apoptosis in RASF within 2 to 4 hours [13, 14]. These discrepancies may be related to different sources and concentrations of TRAIL used in the studies.

TSA can induce apoptosis and cell cycle arrest in cancer cells. The main targets of the HDAC inhibitors are the histones in the nucleosomes, but also other proteins can
be acetylated by HDACs. In this regard, it could be shown that HDAC inhibitors selectively modulate expression of about 2% of the genes in cultured tumor cells [15]. HDAC inhibitors up regulate the expression of DR5 in several cancer cells and enhance TRAIL-mediated apoptosis [16, 17]. In the present study, however, TSA did not effect the surface expression of DR5 on RASF.

Gene transfer or application of recombinant TRAIL effectively ameliorated disease progression in several animal models for RA [18-20]. Furthermore, numerous studies have demonstrated that HDAC inhibitors selectively inhibit tumor cell growth at levels that have less or no toxicity for normal cells. In further experiments we could show that TSA significantly reduces the expression of mRNA of the antiapoptotic molecule FLIP. FLIP binds to the Fas-associated death domain (FADD) when the death-inducing signaling complex (DISC) is formed and redirects the death signal to cell survival. Similar to the Fas receptor, FADD binds directly to the TRAIL receptors. Decreasing FLIP expression sensitizes cells to death ligands [21].

Based on the fact that TSA sensitizes RASF for TRAIL induced apoptosis, we conclude that TSA reveals sensitive sites in the cascade of TRAIL signaling and, thus could represent a novel principle for the treatment of rheumatoid arthritis.
Figure legends

Figure 1
Box blot of the dose-dependent induction of apoptosis by co-treatment with TSA (0.5, 1 and 2 µM) and TRAIL (200 ng/ml) in RASF (n=3). Apoptotic cells were analyzed after 4 days by annexin-V-FITC / propidium iodide (PI) staining with flow cytometry (p≤0.01, Mann-Whitney U-test).

Figure 2
Effect on cell proliferation. Four RA-SF 2x10³ cells / 200ul / well were incubated in a 96 well plate in the presence of 2 µM TSA or 200 ng/ml TRAIL or both. Cell proliferation was assessed 2 hours after the addition of the Reagent Solution of the MTS Cell Proliferation Assay on days 1, 4 and 6. Results shows optical density at 490 nm, each bar represents the mean±SD of 4 RA-SF of 6 replicates.

Figure 3
Western blot showing expression of p21^Waf1/Cip1 and α-tubulin by RASF at 19 kda and 55 kda. Whole cell extracts were analyzed from cultured RA-SF (2 x 10⁶/well) incubated in the absence or presence of TSA (2 µM) on day 1, 4 and 14.
References


Figure 1
Figure 2

Proliferation

Absorbance (490 nm)

- Trail 200ng/ml
- TSA 2µM
- TRAIL+TSA
- Control

day 1  day 4  day 6
Figure 3

- p21: 19 kDa
- α-tubulin: 55 kDa
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