EXTENDED REPORT

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

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Background: Histone acetylation/deacetylation has a critical role in the regulation of transcription by altering the chromatin structure.
Objective: To analyse the effect of trichostatin A (TSA), a streptomyces metabolite which specifically inhibits mammalian histone deacetylases, on TRAIL-induced apoptosis of rheumatoid arthritis synovial fibroblasts (RASF).
Methods: Apoptotic cells were detected after co-treatment of RASF with TRAIL (200 ng/ml) and TSA (0.5, 1, and 2 μmol/l) 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 $p21^{Waf/Cip1}$ by TSA was analysed by western blot. Expression of the TRAIL receptor-2 (DR5) on the cell surface of RASF was analysed by flow cytometry. Levels of soluble TRAIL were measured in synovial fluid of patients with RA and osteoarthritis (OA) by ELISA.
Results: Co-treatment of the cells with TSA and TRAIL induced cell death in a synergistic and dose dependent manner, whereas TRAIL and TSA alone had no effect or only a modest effect. RASF express DR5 (TRAIL receptor 2), but treatment of the cells with TSA for 24 hours did not change the expression level of DR5, as it is shown for cancer cells. TSA induced cell cycle arrest in RASF through up regulation of $p21^{Waf1/Cip1}$. Levels of soluble TRAIL were significantly higher in RA than in OA synovial fluids.
Conclusion: Because TSA sensitises RASF for TRAIL-induced apoptosis, it is concluded that TSA discloses sensitive sites in the cascade of TRAIL signalling and may represent a new principle for the treatment of RA.

Rheumatoid arthritis (RA) is a chronic inflammatory disease that results in the progressive destruction of joints.[1] Apart from B cells, T cells, and macrophages, activated RA synovial fibroblasts (RASF) are the major players in the process of joint destruction. Current treatments for RA aim at inhibiting inflammation and, specifically, at suppressing 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) has a critical role in gene regulation by altering the chromatin structure.[2] Inhibitors of HDAC act effectively as 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 HDAC inhibitors TSA and FK228 induce expression of the cell cycle regulators p21 and p16 and inhibit the expression of tumour necrosis factor α in affected tissues in arthritic animals.[5 6] In this study, we ask whether TSA can sensitise RASF to tumour necrosis factor related apoptosis-inducing ligand (TRAIL)-induced apoptosis.

MATERIAL AND METHODS

Synovial tissue specimens were obtained during synovectomy and arthroplastic surgery from seven patients with RA (two male, mean (SD) aged 64 (7) years; five female, aged 69 (10) years, mean (SD) disease duration 27 (13) years) and three patients with osteoarthritis (OA; one female, aged 79 years, two male, aged 66 (9) years). All patients with RA were receiving treatment (5×prednisone, 2×methotrexate, 1×indometacin (Indocid) and/or 2×sulfasalazine (Salazopyrin). RASF were isolated from synovial tissues, digested by collagenase, and used from passages 4–8 as described.[7] All patients with RA fulfilled the American College of Rheumatology criteria.[8] Synovial fluids from patients with RA and OA were obtained during joint aspiration from 16 patients with RA and 16 patients with OA. Samples were centrifuged at 1300 *g* for 15 minutes and stored at −70°C until analysed. All experiments were performed with permission from the local ethics committees and informed consent was obtained from all patients to participate in the present study.

After treatment of RASF with TSA (0.5, 1, and 2 μmol/l) in combination with TRAIL (200 ng/ml), apoptotic cells were detected by flow cytometry using propidium iodide/annexin-V-FITC staining (Roche, Basel, Switzerland). The effect of TRAIL (200 ng/ml; Alexis, Lausen, Switzerland), TSA (2 μmol/l; Sigma, Buchs, Switzerland), and the co-treatment on cell proliferation was assessed using the MTS CellTiter96®AQ_{ueous} Cell Proliferation Assay (Promega, Wallisellen, Switzerland). Induction of the cell cycle inhibitor $p21^{Waf1/Cip1}$ by TSA was analysed by western blot. RASF (2×10^6/well) were incubated in the absence or presence of TSA (2 μmol/l) and lysed in Laemmli buffer on days 1, 4, and 14. Protein extracts from RASF were analysed by 10% sodium

Abbreviations: HDAC, histone acetylation/deacetylation; OA, osteoarthritis; RA, rheumatoid arthritis; RASF, rheumatoid arthritis synovial fibroblasts; TBST, Tris buffered saline containing 0.1% Tween 20; TRAIL, tumour necrosis factor related apoptosis-inducing ligand; TSA, trichostatin A

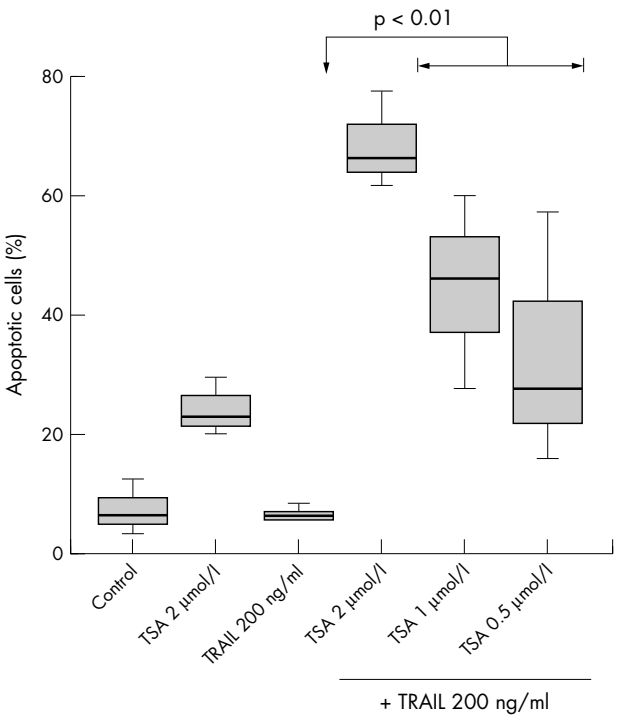

Figure 1 Box plot of the dose dependent induction of apoptosis by co-treatment with TSA (0.5, 1, and 2 µmol/l) and TRAIL (200 ng/ml) in RASF (n = 3). Apoptotic cells were analysed after 4 days by annexin-V-FITC/propidium iodide staining with flow cytometry ($p \leqslant 0.01$, Mann-Whitney U test).

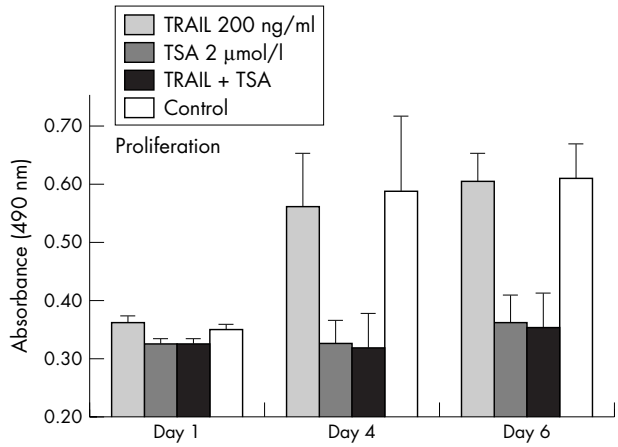

Figure 2 Effect of TSA and TRAIL on cell proliferation. Four RASF 2×10^3 cells/200 µl/well were incubated in a 96 well plate in the presence of 2 µmol/l TSA or 200 ng/ml TRAIL, or both. Cell proliferation was assessed 2 hours after the addition of the Reagent Solution of the MTS Cell Proliferation Assay on days 1, 4, and 6. Results show optical density at 490 nm, each bar represents the mean (SD) of four patients (each six replicates).

dodecyl sulphate-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. The membrane was blocked for 2 hours with Tris buffered saline (TBS) containing 0.1% Tween 20 (TBST) and 5% dehydrated skimmed 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 3×15 minutes with TBST, and incubated with horseradish peroxidase conjugated goat-antimouse IgG secondary antibodies at room temperature for 1 hour. After washing with TBST, the bound antibodies were detected by visualisation using enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK). Cells were detached with accutase (PAA Laboratories), then incubated with 2 µmol/l TSA for 24 hours. After incubation, expression of surface DR5 was analysed by flow cytometry using antihuman DR5 antibodies (R&D, clone MAB6311). Levels of soluble TRAIL were measured in hyaluronidase pretreated (10 mg/ml, 37°C, 1 hour; Sigma, Buchs, Switzerland) synovial fluid from patients with RA and OA by enzyme linked immunosorbent assay (ELISA; Diaclone Research, Besancon, France).

Data are expressed as the mean (SD). Mann-Whitney test was used for statistical analysis. Values of $p<0.05$ were considered significant.

RESULTS

In our study, RASF treated with TRAIL (200 ng/ml) showed no induction of apoptosis. TSA at a dose of 2 µmol/l 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 ($p \leqslant 0.01$; n = 3 each) (fig 1). Cell cycle arrest was induced by TSA (2 µmol/l) on day 1 and was still present on days 4 and 6 ($p \leqslant 0.001$) (fig 2) and accompanied by the induction of $p21^{Waf1/Cip1}$ in RASF (fig 3). In contrast, TRAIL (200 ng/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 (2 µmol/l) for 24 hours did not change the expression level of DR5 on RASF (41 (10)%). Of interest, detachment of the cells with accutase was essential because 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) than in OA synovial fluids (mean 62 (77) pg/ml, n = 16, $p \leqslant 0.001$) (data not shown).

DISCUSSION

In this study, we show that TSA, an inhibitor of histone deacetylases, has a dual effect on RASF; it sensitises RASF for TRAIL-induced apoptosis and, furthermore, effectively inhibits their proliferation through up regulation of $p21^{Waf1/Cip1}$. After this sensitisation, TRAIL is the main inductor of apoptosis. Because we demonstrate that patients with RA have increased levels of soluble TRAIL in synovial fluids in comparison with patients with OA, the application of non-toxic levels of TSA could become a new therapeutic principle in the treatment of RA.

Previous studies have shown that targeting TRAIL receptor-2 (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 treatment 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 trypsinisation of the cells strongly reduced the presence of DR5 on their surface. This fact may explain the discrepancies between the studies. Data on TRAIL mediated apoptosis in RASF are contradictory. In our study, TRAIL alone did 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 2–4 hours.[13 14] These discrepancies may be related to the different sources and concentrations of TRAIL used in the studies.

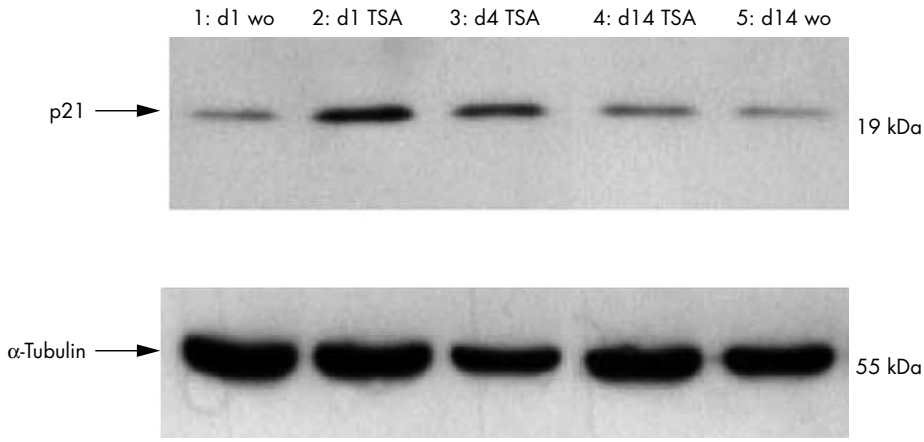

Figure 3 Western blot showing expression of $p21^{Waf1/Cip1}$ and α-tubulin by RASF at 19 kDa and 55 kDa. Whole cell extracts were analysed from cultured RASF (2×10^6/well) incubated in the absence or presence of TSA (2 μmol/l) on days 1, 4, and 14.

TSA can induce apoptosis and cell cycle arrest in cancer cells. The main targets of the HDAC inhibitors are histones in the nucleosomes, but other proteins can also be acetylated by HDAC. In this regard, it could be shown that HDAC inhibitors selectively modulate expression of about 2% of the genes in cultured tumour 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 affect 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 tumour cell growth at levels that have less or no toxicity for normal cells. In further experiments we showed 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 signalling complex (DISC) is formed and redirects the death signal to cell survival. As for the Fas receptor, FADD binds directly to the TRAIL receptors. Decreasing FLIP expression sensitises cells to death ligands.[21]

Because we know that TSA sensitises RASF for TRAIL-induced apoptosis, we conclude that TSA discloses sensitive sites in the cascade of TRAIL signalling and, thus might represent a new principle for the treatment of RA.

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