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How to cite

FISH, Richard, KRUITHOF, Egbert. Evidence for serpinB2-independent protection from TNF-alpha-induced apoptosis. In: Experimental cell research, 2006, vol. 312, n° 3, p. 350–361. doi: 10.1016/j.yexcr.2005.11.003

This publication URL: https://archive-ouverte.unige.ch/unige:36191

Publication DOI: 10.1016/j.yexcr.2005.11.003

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SCIENCE DIRECT

Experimental Cell Research 312 (2006) 350 – 361

Experimental Cell Research

www.elsevier.com/locate/yexcr

Research Article

Evidence for serpinB2-independent protection from TNF-α-induced apoptosis

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Received 12 August 2005, revised version received 28 October 2005, accepted 5 November 2005

Abstract

Clade B serine proteinase inhibitors (serpins) are intracellular proteins, whereas most of their identified targets are extracellular. A proposed intracellular role for these inhibitors is protection from apoptosis. We investigated the contribution of serpinB2 (plasminogen activator inhibitor-2, PAI-2) activity in TNF-α-induced apoptosis. PAI-2 is expressed in many normal and transformed cell types, particularly after stimulation with inflammatory cytokines. PAI-2 has been linked to protection from TNF-α-induced apoptosis, and a stabilizing interaction with the retinoblastoma protein (Rb1) has been proposed. We examined the activity of PAI-2 in TNF-α-induced apoptosis using HeLa, Isreco-1 and HT1080 cell lines. Stimulation with TNF-α protected each cell type from apoptosis induced by TNF-α and cycloheximide. Protection correlated with an increase in PAI-2 expression in IS-1 and HT1080 cells but not in HeLa cells where PAI-2 mRNA and protein were undetectable. PAI-2 was overexpressed in each cell type but gave no protection from TNF-α-induced apoptosis measured by cell viability, annexinV binding and caspase-3/7 activity. We detected wild-type Rb1, unchanged TNF receptor levels and induction of other apoptosis-protective factors in all cell types. In conclusion, elevated PAI-2 levels do not protect cells from TNF-α-induced apoptosis, and the protective effect of prior stimulation with TNF-α does not require PAI-2.
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Keywords: PAI-2; Apoptosis; SerpinB2; TNF

Introduction

Clade B serine proteinase inhibitors (serpins) are expressed largely intracellularly, whereas most of their identified target enzymes are found in the extracellular space. The activity of several human clade B serpins was reported to be associated with protection from pro-apoptotic stimuli [1]. This suggests the presence of unidentified intracellular targets that participate in apoptosis. SerpinB2, the plasminogen activator inhibitor type-2 (PAI-2), was initially characterized as an inhibitor of urokinase [2]. The intracellular localization of PAI-2, due to an inefficient signal peptide sequence, has led to several studies attempting to uncover further intracellular roles and targets for this inhibitor.

PAI-2 expression is rapidly induced by TNF-α in many cell types [2]. The first study linking PAI-2 to cell death demonstrated that incubation of HT1080 cells with TNF-α increased PAI-2 expression and gave protection from cell death induced by a second TNF-α stimulation in the presence of a protein synthesis inhibitor [3]. HT1080 cell clones overexpressing PAI-2 were also protected from TNF-α/cycloheximide-induced cell death, leading to the hypothesis that PAI-2 was a cellular protector from TNF-α-induced cell death. Indeed, cell clones expressing antisense PAI-2 were more sensitive to the apoptotic challenge than the parent cell line, and populations of cells with increased PAI-2 expression and apoptosis resistance were enriched by rounds of apoptotic stress [3].

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doi:10.1016/j.yexcr.2005.11.003

TNF-α-induced cell death was also investigated in HeLa cell clones overexpressing PAI-2 and antisense PAI-2 in a study linking PAI-2 to programmed cell death. Dickinson et al. showed that PAI-2 overexpressing clones were protected from apoptosis [4]. Furthermore, they observed that HeLa clones expressing a reactive center mutant of PAI-2 (R380A) or a mutant lacking the CD interhelical loop were not protected [4,5]. Recently, PAI-2 was shown to interact with the transcriptional regulator protein Rb1 [6] and to stabilize this protein in HeLa cells. A direct link between this interaction and a protective effect on apoptosis has not been demonstrated.

Other studies have also addressed a possible protective effect of PAI-2 on cell death. Gan et al. demonstrated that addition of PAI-2 protein to culture medium inhibited apoptosis in murine macrophages incubated with Mycobacterium avium and indomethacin, which suggests an anti-apoptotic role for PAI-2 in the cell environment [7]. Transgenic mice with keratinocyte-specific expression of PAI-2 permitted the progressive growth of induced cutaneous papillomas, compared to control animals where tumors regressed after the induction period. This was linked to reduced cell death in the tumors of the transgenic mice [8]. Compared to control mice, PAI-2-deficient mice showed no apparent differences in organ histology, cell morphology or cell culture growth characteristics of cell types known to express high levels of PAI-2 [9].

We investigated the activity of PAI-2 in TNF-α-induced apoptosis using three human tumor cell lines: HeLa (cervical), IS-1 (colorectal) and HT1080 (fibrosarcoma). PAI-2 expression levels were increased in IS-1 and HT1080 cells upon stimulation with TNF-α, whereas we found no evidence for PAI-2 expression in HeLa cells, even after stimulation with TNF-α. Despite this difference, incubation of each cell type with TNF-α protected them from cell death in a subsequent stimulation in apoptotic conditions. Using lentiviral vectors, each cell line was transduced for functional overexpression of PAI-2. None of the transduced cell populations showed protection from TNF-α-induced apoptosis, compared to the parent cell lines, using cell viability, annexinV/propidium iodide binding, and caspase-3/7 activities as read-outs. In contrast to results described using clonally selected cells, our results show that PAI-2 does not protect populations of tumor cells from TNF-α-induced apoptosis, and that protection mediated by pre-incubation with TNF-α is not PAI-2-dependent.

Materials and methods

Cell lines

Isreco-1 colon carcinoma (IS-1) and HT1080 fibrosarcoma cells were a gift from Dr. B. Sordat (ISREC, Epalinges, Switzerland). 293T cells were a gift from Dr. D. Trono (Geneva University Medical Centre, Switzerland). HeLa cervical carcinoma cells were purchased from the European collection of cell cultures, ECACC number: 93021013. All cells were maintained in DMEM supplemented with 10% fetal bovine serum and 10 mM HEPES, pH 7.4 (all purchased from Invitrogen). All cell lines were routinely tested for the presence of mycoplasma, using the Mycoalert (Cambrex) reagent and the manufacturers protocol, and were negative.

PAI-2 cDNA constructions and site-directed mutagenesis

The human type 2 PAI-2 (SERPINB2) open reading frame was amplified by PCR from the pJ7 clone [10] introducing 5′ NheI and 3′ NotI restriction sites. This fragment was subcloned into the NheI/NotI sites of pTRE2hyg (Clontech) and the entire sequence verified by DNA sequencing. The insert was further subcloned between the BamHI/SalI sites of ploxCW, a gift from Dr. P. Salmon, Geneva University Medical Centre. ploxCW PAI-2 then served as the gene transfer plasmid when producing lentiviral vectors, with PAI-2 expression under the control of the CMV immediate early promoter. PAI-2 R380A (Arg 380 to Ala mutation) was also generated by PCR using overlap extension of two PCR products [11], designed to introduce the mutation and the same flanking restriction sites as the wild-type sequence. PAI-2 R380A was subcloned to pTRE2hyg, as for the wild-type open reading frame, sequenced and transferred to ploxCW. Oligonucleotide sequences are available upon request.

Induction of PAI-2 expression and apoptosis

2 to 3 × 10^5 cells per well were seeded into 6-well culture dishes the day prior to a 16-h stimulation with 10 ng/ml recombinant human TNF-α (R&D Systems). Cell lysates for Western blotting and RNA isolation were prepared as described previously [12]. For experiments measuring apoptosis after a prior incubation with TNF-α, 1 to 2.5 × 10^5 cells per well of each cell type were seeded in 6-well plates the day before a 16-h stimulation with 10 ng/ml TNF-α. Apoptosis was then induced for 6 h with 10 ng/ml TNF-α and 10 μg/ml cycloheximide (Sigma).

RNA isolation and quantitative RT-PCR

Total cellular RNA was isolated using kits from Qiagen. cDNA was generated using ImPromII (Promega) reverse transcriptase using the manufacturers instructions, typically with 1 μg total RNA per sample. Quantitative RT-PCR was performed as described previously [12] using an Applied Biosystems Prism 7000 instrument and SYBR green master mix reagent (Applied Biosystems) and data analyzed using the ΔC_T method with GAPDH as the control mRNA. 5′ to 3′ oligonucleotide sequences, were as follows: GAPDH forward GGTGAAGGTCGGAGTCAAC, GAPDH reverse CCATGGGTGGAATCATATTG, PAI-2 forward GGGTCAAGACTCAAACCAAAG, PAI-2 reverse

CCTTTGAAGTAGACAGCATTC cFLIP forward CCCTCACCTTGTTTCGGACTAT, cFLIP reverse AATGAGGACACATCAGATTTATCCAA, A20 forward CTCGGCTATGACAGCCATCA, A20 reverse TTAACAAGTGGAACAGCTCGGATT. Individual samples were subjected to duplicate or triplicate PCRs and ΔC_T values calculated. Error bars represent the standard deviation of triplicate experiments.

Lentiviral vector production and transduction of target cells

Lentiviral vector production, using a 3-plasmid transfection of HEK 293T producer cells, and target cell transduction, has been described previously [12,13]. Briefly, 2.5×10^6 293T cells were seeded into 100-mm round cell culture dishes the day prior to transfection using the calcium phosphate precipitation technique with a combination of pMDG VSV env (5 μg), pR8.91 (15 μg) and ploxCW (5 μg). All plasmids were gifts from Dr. P. Salmon, Geneva University Medical Centre. ploxCW bears either the wild-type PAI-2 open reading frame or the R380A PAI-2 mutant, for expression driven by the CMV immediate early promoter. The next day, the medium was replaced, and producer cells were left for a further 24 h. Conditioned medium was then harvested and filtered with 0.45-μm sterile filters. This viral stock was then used in a 1:1 ratio with fresh medium on cells plated 24 h previously in 6-well culture plates at 5×10^4 cells per well. Cells were centrifuged for 1 h at 350 *g* in the presence of vector and 8 μg/ml of polybrene (Sigma). The next day, medium was replaced, and cells were left to proliferate for 3 days. No apoptosis assays were performed on cells until the stability of transduction, and transduction efficacy, was assessed by flow cytometry, normally 2 weeks after transduction.

Western blotting and PAI-2 activity assay

Immunoblotting for detection of human PAI-2 in cell lysates, and in functional complexes with u-PA, has been described previously [12]. For quantification, we used recombinant human PAI-2 as a standard and compared band intensities of samples to standards.

Detection of cell surface TNFR1

TNF receptor 1 levels on each cell type used were assessed by flow cytometry. Approximately 1×10^6 cells in suspension were labeled with either 1 μg of monoclonal antibody MABTNFR1-B1 (BD Pharmingen) or 1 μg of IgG2a isotype-matched control antibodies (DAKO), followed by goat anti-mouse–FITC secondary antibodies (Kappel). All incubations and washes were done in PBS/1% bovine serum albumin (BSA). Cells were washed twice between primary and secondary antibodies and then three times after the secondary antibody. All incubations, centrifugations and washes were done at 4°C to avoid receptor trafficking from cell membranes. Labeled cells were analyzed by flow cytometry on a FACScan instrument (Becton Dickinson).

Apoptosis measured by annexinV/propidium iodide staining

Cells were incubated with medium alone, 10 ng/ml TNF-α, 10 μg/ml cycloheximide or TNF-α and cycloheximide for 6 h in 6-well plates seeded the previous day with 1 to 2×10^5 cells per well. All cells were recovered by pipetting detached cells and incubation at room temperature in 1 mM EDTA in PBS to detach adherent cells. Samples were centrifuged for 3 min at 200 *g*, rinsed once in PBS, centrifuged again and resuspended in FITC-labeled annexinV/propidium iodide (PI) according to the manufacturers instructions (Becton Dickinson, Apoptosis Detection kit). Labeled cells were analyzed by flow cytometry on a FACScan Instrument (Becton Dickinson).

Cell proliferation assay

10^3 cells per well in 100 μl medium were plated in 96-well plates on day 0. Biological activity in these wells, measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) was measured on days 1, 2, 3 and 4, at 24-h intervals using the same reagent incubation time on each day. Absorbance measurements were made at 490 nm, and values for wells containing medium alone were subtracted from all data. Absorbance values were shown to be directly proportional to cell numbers prior to this proliferation analysis.

Cell viability assay

1 to 2×10^3 cells per well were distributed in 96-well plates the day prior to incubation with 5-fold serial dilutions of TNF-α, in the presence of 10 μg/ml cycloheximide, for 16 h. TNF-α alone had no effect on cell viability at the concentrations and time periods shown. Incubation with cycloheximide alone reduced the viable cell values by no more the 25%, compared to untreated cells at the end of the incubation period, with no apparent cytotoxicity. After 16 h, cell viability in each well was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay, according to the manufacturers instructions. All data points were measured at $n = 6$. Blank well values on each plate were subtracted from experimental data. Absorbance values in experiments with HeLa cell data were analyzed using a sigmoidal dose–response curve using Prism software (GraphPad, CA). Data from experiments using IS-1 and HT1080 cells gave linear dose–responses, and all data were normalized to percent cell viability. Data for cells treated with 10 μg/ml cycloheximide alone was used as 100% viability.

Caspase-3/7 activity assay

Caspase-3/7 activity in each cell type was measured using the Caspase-Glo™ 3/7 Assay (Promega). The assay uses a DEVD peptide-based caspase substrate. Upon caspase-3 and/or -7 activation, the peptide is cleaved off to yield aminoluciferin. This acts as substrate for luciferase, provided in the assay, and yields light. Cells were seeded into 96-well plates the day prior to a 6-h incubation with or without 10 ng/ml TNF-α and 10 μg/ml cycloheximide. The assay was performed according to the suppliers instructions and luminescence measured using a Molecular Devices FlexStation instrument. Reagents alone control values were subtracted from all data.

Rb1 cDNA sequencing

Total cellular RNA was isolated from HT1080, HeLa and IS-1 cells using kits from Qiagen. cDNA was generated using ImPromII (Promega) reverse transcriptase using the manufacturers instructions, typically with 1 μg total RNA per sample. The C-pocket region of the Rb1 open reading frame was amplified by PCR using the following oligonucleotides: forward 5′ CATGCTGTTCAGGAGACATTC 3′ and reverse 5′ AATGAATCCAGAGGTGTACAC 3′. 637-bp PCR products amplified from each cell line cDNA were purified and sequenced by the DNA sequencing facility at the CMU, Geneva. Wild-type Rb1 sequences were found for each cell type, corresponding to the sequence accession number M15400 at the NCBI Entrez nucleotide Internet site.

Detection of Rb1

Rb1 protein was detected in the nuclear fraction of whole cell lysates. Cells were lysed in 100 μl buffer A: 10 mM HEPES–NaOH, pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 200 mM sucrose, 0.5% Nonidet-P40, 1 mM PMSF and 1× Complete Mini protease inhibitor cocktail (Roche), for 10 min on ice. Nuclei were pelleted by centrifugation at 3500 *g* for 5 min in a refrigerated centrifuge. Cytosolic fractions were collected by pipetting and remaining nuclei washed once in 100 μl buffer A. Nuclei were lysed in 50 μl of buffer B: 20 mM HEPES, pH 7.8, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 × Complete Mini protease inhibitor cocktail (Roche) for 30 min on ice. Debris was pelleted by centrifugation at 10,000 *g* for 10 min at 4°C. Nuclear extracts were collected by pipetting and mixed with an equal volume of buffer C: 20 mM HEPES, pH 7.8,100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 1 mM PMSF and 1 × Complete Mini protease inhibitor cocktail (Roche). Nuclear extract protein content was determined using the BIO-RAD Protein Assay (Biorad). Extracts were separated by electrophoresis and transferred to nitrocellulose, as for other immunoblots. Mouse anti-human Rb1 monoclonal antibodies were used to detect full-length Rb1 (clone G3-245 Becton Dickinson, Pharmingen). Goat anti-mouse-HRP antibodies (Biorad) were used as secondary antibodies and ECL reagents (Amersham) for revelation. Equal gel lane loading was confirmed by ponceauS staining (not shown).

Results

TNF-α stimulation protects cells from apoptosis induced by TNF-α in the presence of cycloheximide

The first report on the role of PAI-2 in TNF-α-induced cell death demonstrated that pre-incubation with TNF-α gave protection to HT1080 cells in a subsequent stimulation with TNF-α in the presence of cycloheximide [3]. This correlated with PAI-2 induction, and overexpression of PAI-2 in clonal HT1080 cell lines appeared to mimic the protective effect. The same clonal selection approach was used in a subsequent study, using HeLa cells [4]. We began our study by investigating whether TNF-α incubation protected wild-type HeLa, IS-1 and HT1080 cells from apoptosis induced by TNF-α in the presence of cycloheximide. 16-h incubation with or without 10 ng/ml TNF-α was followed by a 6-h incubation with 10 ng/ml TNF-α in the presence of 10 μg/ml cycloheximide. Similar incubations were used in previous studies of PAI-2 in apoptosis. Using cell surface annexinV binding and propidium iodide chromatin staining, we were able to measure apoptosis induction by flow cytometry. In preliminary experiments, cell lines were shown to undergo measurable apoptosis using a 6-h incubation with 10 ng/ml TNF-α and 10 μg/ml cycloheximide (apoptotic conditions), without TNF-α pre-incubation (data not shown). Pre-incubation with TNF-α gave each cell type measurable protection from apoptosis, compared to untreated cells. Fig. 1A shows this protective effect as the percent of cells which are both annexinV and propidium iodide negative, after the incubation in apoptotic conditions. This criterion was chosen as the 6-h incubation with TNF-α and cycloheximide represents a snap-shot of the apoptotic process, with some cells in the challenged population showing no staining, some showing early apoptosis (annexin V^+), others in late apoptosis (annexin V^+/PI^+) and finally some necrotic cells (annexin V^-/PI^+). Untreated cells or cells treated with TNF-α alone showed no significant changes in annexin V^-/PI^- cells over the incubation period (data not shown).

To determine whether the protective effect of TNF-α pre-stimulation correlated with PAI-2 induction, each cell type was incubated for the same 16 h with 10 ng/ml TNF-α used for the experiments in Fig. 1A. By quantitative RT-PCR, we measured clear induction of PAI-2 mRNA in IS-1 and HT1080 cells (Fig. 1B) and

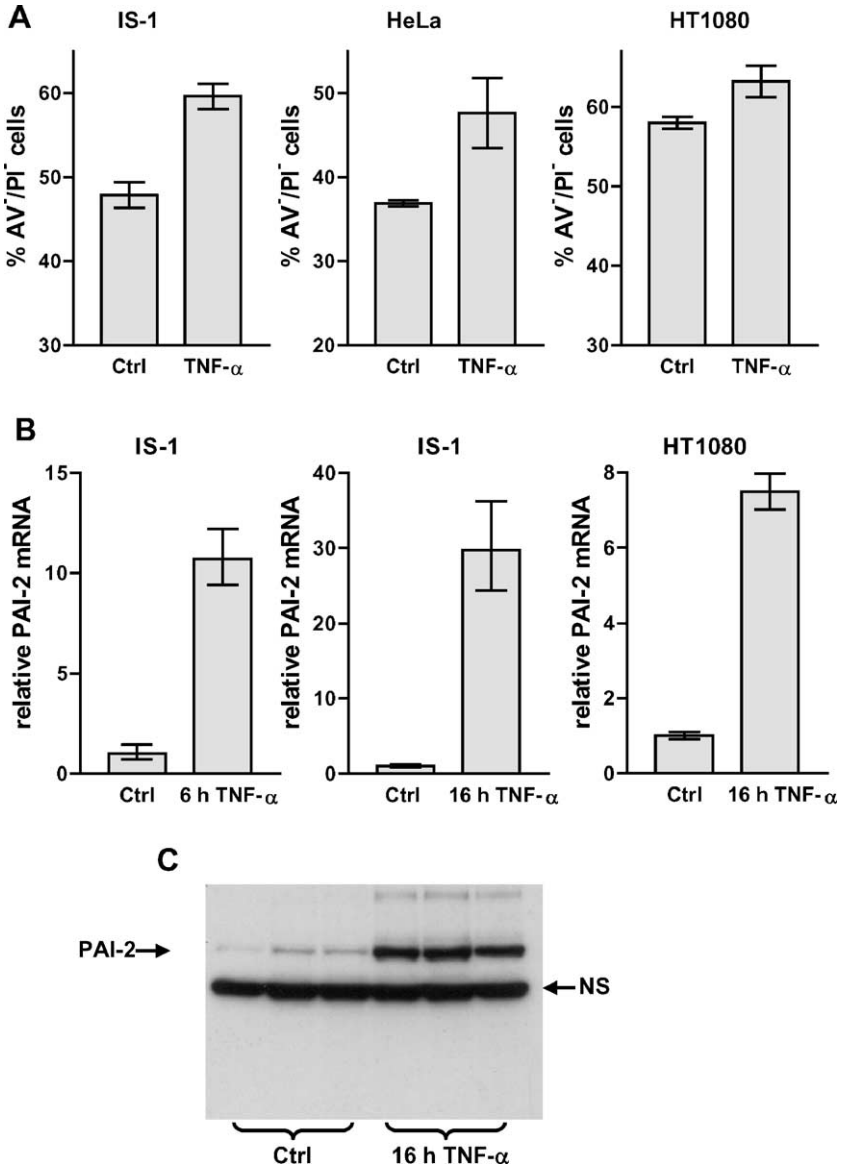


Fig. 1. TNF-α protects IS-1, HeLa and HT1080 cells from apoptosis and is accompanied by increased PAI-2 expression in IS-1 and HT1080 cells. In panel A, IS-1, HeLa and HT1080 cells were incubated for 16 h with (TNF-α) and without (Ctrl) 10 ng/ml TNF-α and then subjected to apoptotic conditions (10 ng/ml TNF-α with 10 μg/ml cycloheximide) for a further 6 h. Percentage annexin V–FITC/propidium iodide negative cells was determined by flow cytometry (%AV^-/PI^-). Error bars represent SEM, $n = 3$. In panel B, PAI-2 mRNA levels were measured by quantitative RT-PCR of total RNA isolated from IS-1 cells with and without a 6- and 16-h stimulation with 10 ng/ml TNF-α and HT1080 cells with a 16-h stimulation with the same TNF-α. Data are expressed relative to a value of 1 for non-stimulated cells. Error bars represent SD, $n = 3$. In panel C, cell lysates from IS-1 cells incubated for 16 h with and without 10 ng/ml TNF-α were subjected to immunoblotting using anti-human PAI-2 monoclonal antibodies and HRP-labeled secondary antibodies. A single non-specific band (NS), detected in IS-1 cell lysates, was used as a loading control, as was Ponceau S staining (not shown). The bands corresponding to PAI-2 are indicated. Analyses were performed in triplicate, hence, the three lanes for each condition.

increased PAI-2 antigen by immunoblotting in IS-1 cells (Fig. 1C). PAI-2 mRNA levels were higher after 16 h than after 6 h in IS-1 cells (Fig. 1B), suggesting a continued accumulation of mRNA with prolonged, apoptosis-protective, TNF-α stimulation. No PAI-2 mRNA or protein was measured in HeLa cells with or without TNF-α stimulation (data not shown). Endogenous PAI-2 activity could not, therefore, have contributed to the apoptosis-protective effect of TNF-α pre-stimulation in HeLa cells shown in Fig. 1A.

Generation of cell lines overexpressing functional and mutated PAI-2

To investigate further the role of PAI-2 in protection from apoptosis, we generated cell lines, which overexpress functional PAI-2. To avoid phenotypes associated with clonal selection, we used lentiviral vectors for delivery of expression cassettes leading to stable overexpression of PAI-2, as described previously [12]. Retroviral vectors were used for similar reasons in a recent investigation of TNF receptor-1-

induced apoptosis mechanisms [14]. IS-1 cells were transduced with the PAI-2 vector and the PAI-2 antigen activity measured. The generation and characterization of PAI-2 overexpressing IS-1 cells were described previously [12]. HT1080 and HeLa cells were also transduced with the same vector as well as a vector leading to expression of PAI-2 R380A, an inactive mutant [4]. Fig. 2A shows flow cytometry analysis of HeLa and HT1080 cells transduced

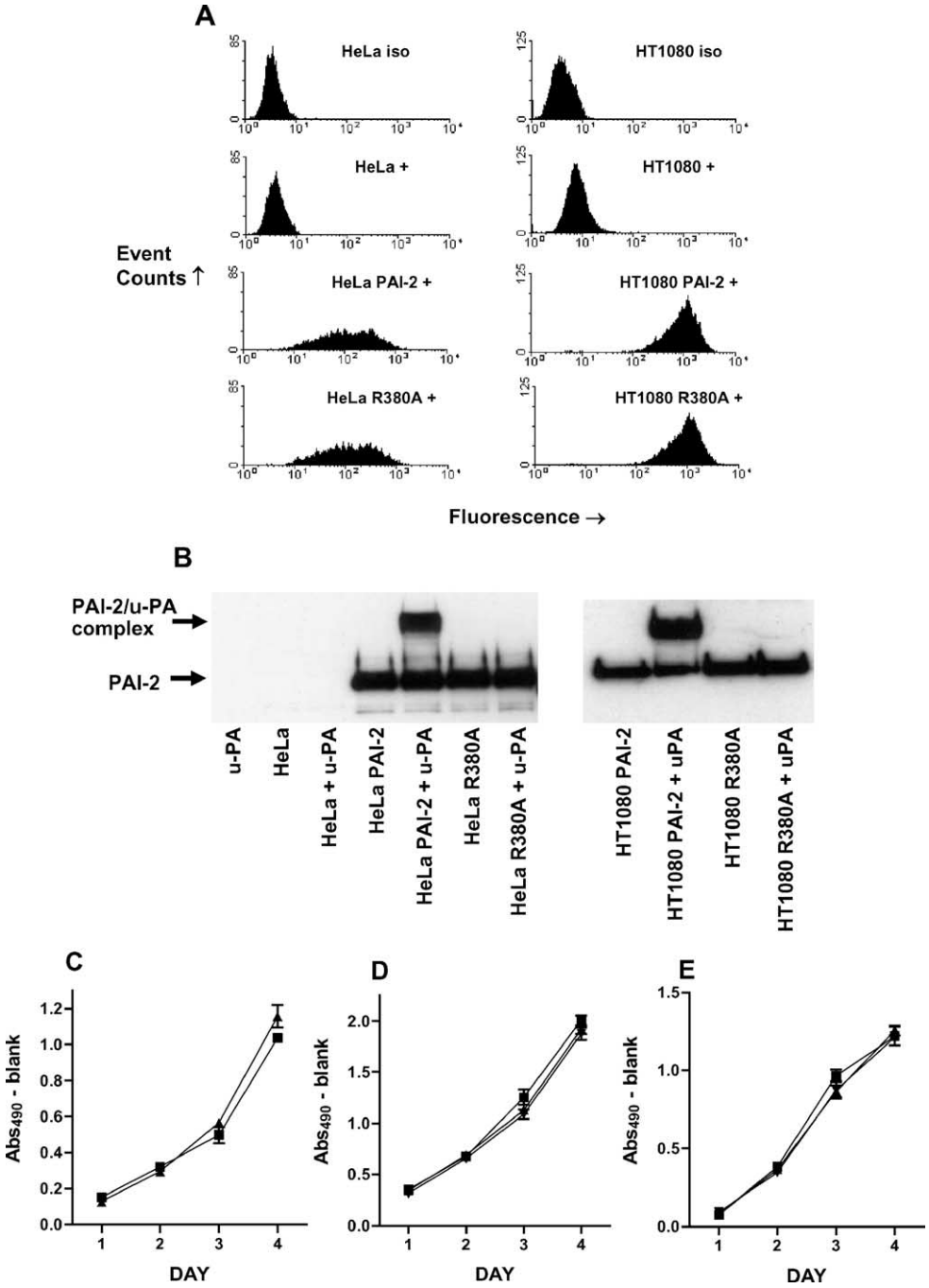
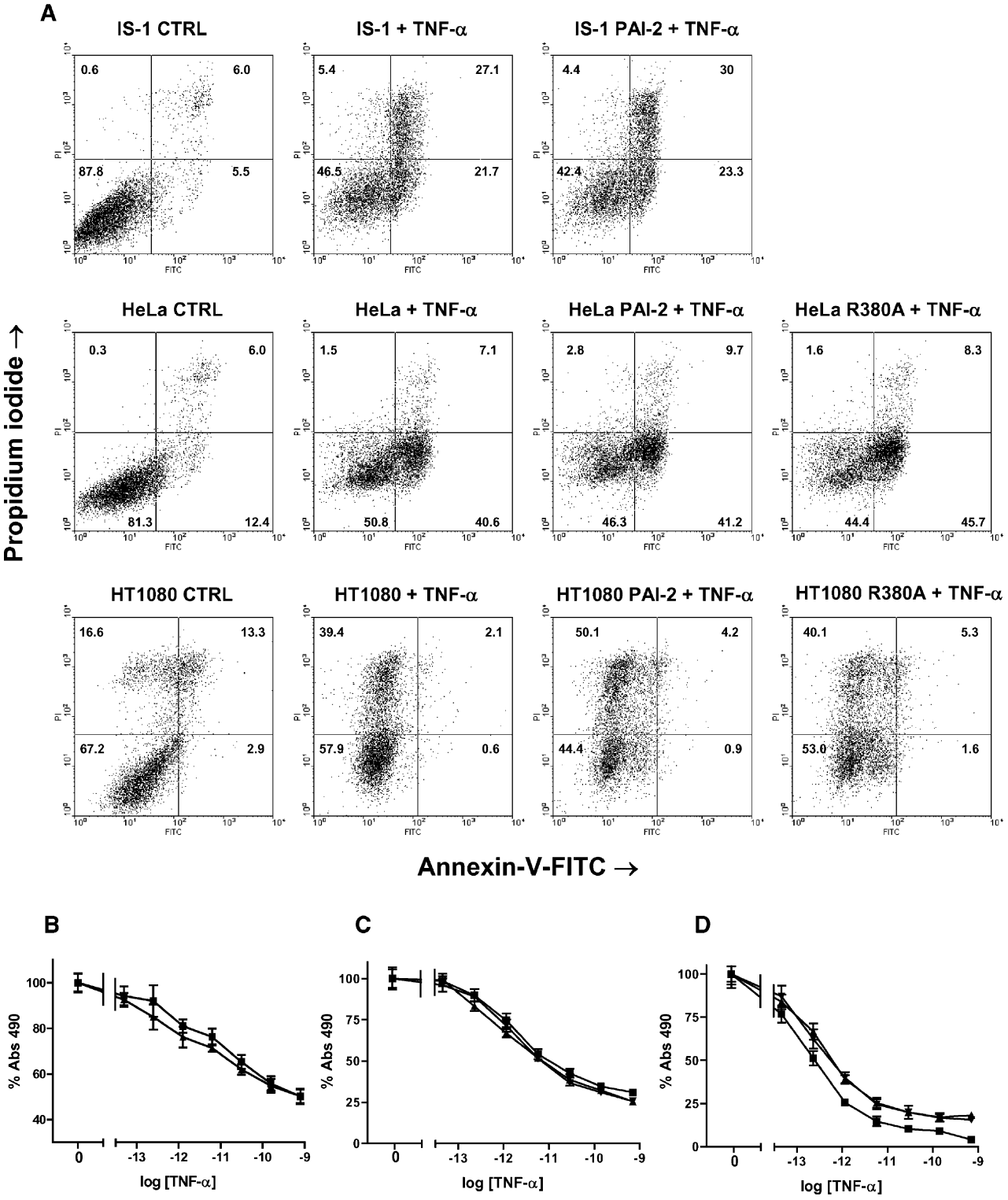


Fig. 2. Characterization of IS-1, HeLa and HT1080 cells transduced with lentiviral vectors for overexpression of PAI-2 and PAI-2 R380A. HeLa and HT1080 cells were transduced with lentiviral vectors leading to expression of wild type and R380A PAI-2. In panel A, flow cytometry was used to assess expression levels and transduction efficiencies. Cells were permeabilized and labeled with anti-human PAI-2 monoclonal antibodies (+), or an isotype control (iso), and secondary PE-labeled anti-mouse antibodies. Transduced IS-1 cells were described previously [12]. In panel B, the u-PA binding activity of the PAI-2 in each transduced HeLa and HT1080 cell type was assessed by immunoblotting cell lysates with anti-PAI-2 antibodies with and without prior incubation with u-PA. Wild-type PAI-2 shows u-PA complex formation in both cell types, whereas the R380A mutant fails to bind. In panels C, D and E, cell proliferation rates were compared for each transduced cell type, compared to non-transduced cells. Each cell type was grown in 96-well plates for 5 days (0 to 4) and cell viability assessed using a colorimetric assay, determined at 24-h intervals on days 1, 2, 3 and 4. Error bars represent SD, $n = 6$ to 8. In C, IS-1 (■) and IS-1 PAI-2 (▲) are compared. In panel D, Hela (■), HeLa PAI-2 (▲) and HeLa R380A (▼) are compared. In panel E, HT1080 (■), HT1080 PAI-2 (▲) and HT1080 R380A (▼) are compared.

with PAI-2 vectors, permeabilized and stained with anti-PAI-2 antibodies and phycoerythrin-labeled secondary antibodies. This clearly shows the efficacy of population-wide lentiviral vector-mediated transduction, as all HeLa and HT1080 cells, as well as IS-1 cells [12] overexpressed PAI-2. Fig. 2B shows immunoblots of HeLa, HeLa PAI-2, HeLa R380A, HT1080 PAI-2 and HT1080 R380A cell lysates with and without incubation with low molecular weight u-PA. Band shifts upon u-PA incubation demonstrate the functional activity of the wild-type overexpressed protein but not the R380A mutant. Similar results were observed for the overexpressed proteins in IS-1 cells (Reference [12] and data not shown). Using semi-quantitative immunoblotting, we quantified PAI-2 in cell lysates from each of the



wild-type PAI-2-transduced cell lines. IS-1 PAI-2 cells express 1 μg PAI-2/mg of total cell lysate, for HeLa PAI-2 cells, we measured 2 μg PAI-2/mg of total cell lysate and for HT1080 PAI-2 cells 40 μg PAI-2/mg of total cell lysate. These values are higher than those described previously in studies on the role of PAI-2 in TNF-α-induced apoptosis, for example, 132 ng/mg measured by ELISA in Reference [4]. We also quantified secreted glycosylated PAI-2 from HT1080 PAI-2 cells and observed that approximately 6% of total PAI-2 was in conditioned medium after a 16-h incubation. All secreted PAI-2 was glycosylated, whereas all cell-associated PAI-2 in each overexpressing cell line was not.

We have also measured PAI-2 mRNA levels in each overexpressing cell line. In IS-1 and HT1080 cells, which express endogenous PAI-2, mRNA levels in the overexpressing cells were 130-fold and over 1000-fold higher than in the non-transduced cell lines, respectively. Such increases are much higher than those obtained after stimulation with TNF-α (as in Fig. 1B).

To control for possible effects on the growth of each transduced cell line, cell proliferation assays were performed over a 4-day period. A 96-well-based assay was used which measures directly the biological activity in the wells, with a colorimetric read-out. Figs. 2C, D and E show that the transduced IS-1, HeLa and HT1080 cells have the same growth kinetics as the parent cell lines, respectively.

Functional PAI-2 overexpression does not protect cells from TNF-α-induced apoptosis

We monitored apoptosis induced in the cell lines described in Fig. 2. First, cells were subjected to apoptotic conditions for 6 h, and annexin V/propidium iodide staining was measured by flow cytometry. Functional PAI-2 overexpression gave no protection to IS-1, HeLa or HT1080 cells as very similar annexin V/propidium iodide flow cytometry profiles were obtained for each cell type, with or without PAI-2 overexpression (Fig. 3A). Second, cell viability was measured using a colorimetric assay, after a 16-h incubation in the presence of cycloheximide and a range of TNF-α concentrations. This prolonged assay was used to assess the relative number of surviving cells after a longer lasting apoptotic stress. Dose–response curves of absorbance at different TNF-α concentrations were generated for each cell type (Figs. 3B–D). PAI-2 overexpressing IS-1 and HeLa cells gave super-imposable curves with their respective parent cell lines, as did PAI-2 mutant overexpressing HeLa cells. HT1080 cells were slightly more sensitive to the treatment than were HT1080 PAI-2 and HT1080 R380A cells. As all HT1080 cell types reached the lowest cell viability between 10^{-10} and 10^{-11}M TNF-α, but a lower cell viability compared to control cells was measured in HT1080 cells at this concentration, a small TNF-α-resistant population may be present in the transduced HT1080 PAI-2 and HT1080 R380A cells. As both PAI-2 and inactive mutant PAI-2 expressing cells show this small resistance, it is not due to PAI-2-mediated protection.

The activity of caspase-3 or -7 was measured in cell lysates from all cell types, with and without 6-h incubation with 10 ng/ml TNF-α and 10 μg/ml cycloheximide. The assay uses a DEVD peptide-based caspase substrate. Upon caspase-3 and/or -7 activation, the peptide is cleaved off to yield aminoluciferin. This acts as substrate for luciferase and yields light. Luminescence is therefore proportional to caspase-3 and/or -7 activities in apoptotic cells. Fig. 4 demonstrates that PAI-2 overexpression does not affect caspase-3/7 activity in IS-1(A), HeLa (B) and HT1080 (C) cells. Transduced HT1080 cells showed slightly lower caspase-3/7 activity than HT1080 cells, but as both wild-type and inactive mutant (R380A) PAI-2 overexpression showed this difference, we conclude that the lower caspase-3/7 activity is not due to overexpression of active PAI-2.

PAI-2 overexpression does not affect TNFR1 expression

To investigate possible changes in TNF receptor 1 expression levels in the transduced cells used in this study, which could alter the responses of cells to TNF-α in the apoptosis assays we have used, we measured TNFR1 levels in all cell types by flow cytometry after staining with TNFR1-specific or isotype control antibodies. TNFR1 was detected in all cell types, and no differences in expression levels were seen between transduced or non-transduced cells (Fig. 5).

Detection of full-length Rb1

It has been suggested previously that an apoptosis protective function of PAI-2 might be mediated via an interaction with the C-pocket of the Rb1 protein [6]. We investigated the possibility that the cells we have used fail

Fig. 3. Overexpression of PAI-2 gives no protection to IS-1, HeLa and HT1080 cells from TNF-α-induced apoptosis. In panel A, all cell types described were incubated for 6 h in apoptotic conditions (10 ng/ml TNF-α with 10 μg/ml cycloheximide, +TNF-α in the figure), or with medium alone (CTRL), and labeled with annexin V–FITC and propidium iodide for analysis by flow cytometry. Annexin V–FITC labeling is shown on the *x*-axis and PI on the *y*-axis. Control data are shown for non-transduced cells of each cell line. Similar profiles were obtained for non-treated transduced cells of each cell line (data not shown). Incubation of each cell type with either TNF-α or cycloheximide alone gave similar annexinV-PI labeling to non-treated cells (data not shown). A quadrant is placed on each dot plot to distinguish healthy cells (lower left, annexin V–FITC$^-$/PI$^-$), early apoptotic cells (lower right, annexin V–FITC$^+$/PI$^-$), late apoptotic cells (upper right, annexin V–FITC$^+$/PI$^+$) and necrotic cells (upper left, annexin V–FITC$^-$/PI$^+$). Percentage cells in each quadrant are shown. In panels B–D, cell viability was measured by absorbance of a colorimetric substrate after a 16-h incubation in 10 μg/ml cycloheximide and 5-fold serial dilutions of TNF-α, starting at 12.5 ng/ml ($10^{-9.15}$ M), in each IS-1 (B), HeLa (C) and HT1080 (D) cell line, respectively. Data are adjusted to percentage absorbance values for each cell line after subtraction of blank absorbance values in the absence of cells. 100% absorbance was measured in the absence of TNF-α. Symbols are ■ for IS-1 cells and ▲ for IS-1 PAI-2 cells in panel (B), ■ for HeLa, ▲ for HeLa PAI-2 and ▼ for HeLa R380A in panel (C and ■ for HT1080, ▲ for HT1080 PAI-2 and ▼ for HT1080 R380A in panel D. Error bars represent SD, $n = 6$.

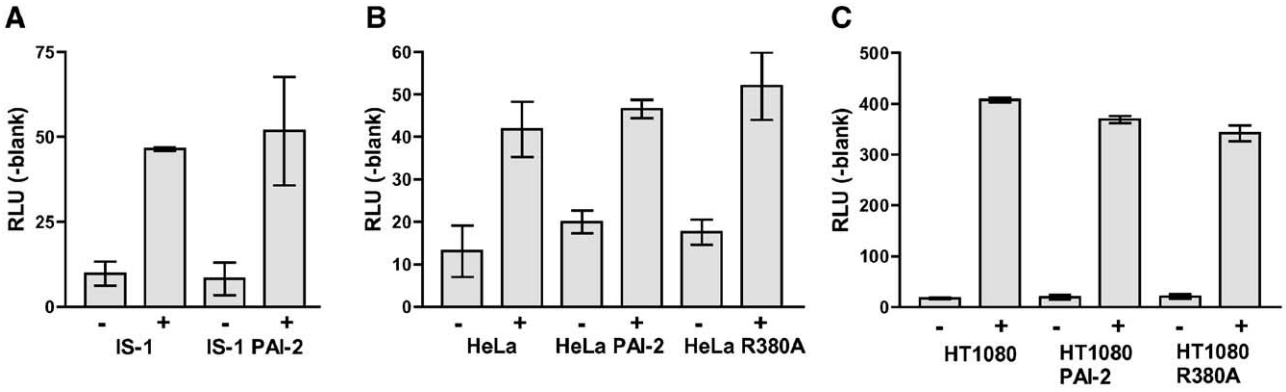


Fig. 4. PAI-2 overexpression has no effect on caspase-3/7 activity during apoptosis in IS-1, HeLa and HT1080 cells. The activity of caspase-3 and/or -7 in cell lysates was measured using an aminoluciferin-coupled caspase substrate. Cells were treated for 6 h with 10 ng/ml TNF-α and 10 μg/ml cycloheximide (+) or with medium alone (−). Cells were then lysed in a buffer containing the caspase-3/7 substrate. Relative Light Units (RLU) are proportional to caspase activity. Error bars represent SD, $n = 3$. Panel A shows data for IS-1 and IS-1 PAI-2 cells, panel B for HeLa, HeLa PAI-2 and HeLa R380A cells and panel C for HT1080, HT1080 PAI-2 and HT1080 R380A cells.

to express the Rb1 antigen and therefore fail to respond to overexpression of PAI-2. By immunoblotting, we detected the full-length Rb1 protein in all of the cell lines used in this study, which excludes this possibility (Fig. 6). In two of the three cell lines, IS-1 and HT1080, the levels of Rb1 antigen were comparable in transduced and non-transduced cells. In HeLa cells, we observed a slightly higher level of Rb1 antigen in transduced, compared to non-transduced cells. There was, however, no difference between wild-type PAI-2 or inactive PAI-2 R380A transduced cells (Fig. 6). In addition, we isolated cDNA from IS-1, HeLa and HT1080 cells and amplified the C-pocket region of the Rb1 cDNA by PCR. The sequence of the PCR products was identical for all three cell types and corresponded to the wild-type C-pocket sequence (data not shown).

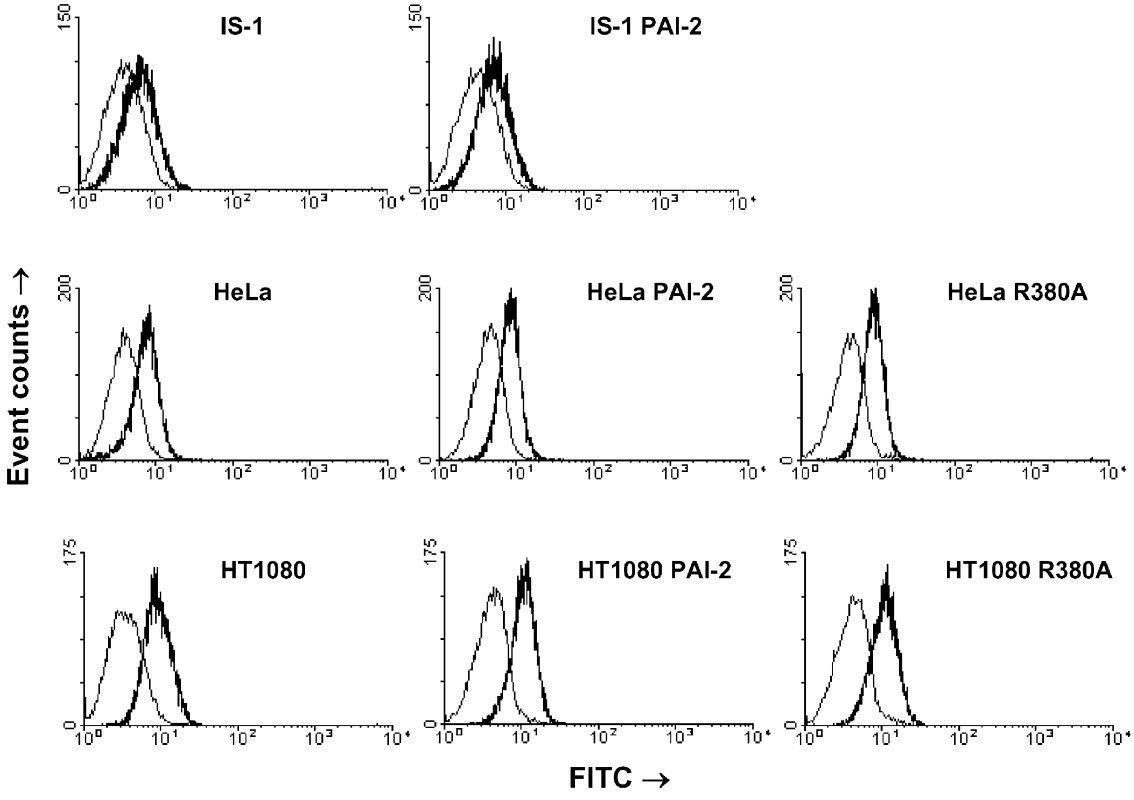


Fig. 5. Cell surface TNFR1 expression. Expression of TNFR1 was detected on the surface of all cell types used in this study. Cells were incubated in the presence of anti-TNFR1 monoclonal antibodies or an equivalent amount of isotype control antibodies. FITC-labeled secondary antibodies were used to detect the presence of each antibody type. Cells were analyzed by flow cytometry, data for isotype antibody-incubated cells are represented with the thin line in each histogram and TNFR1 antibody-labeled cells with the thick line.

Fig. 6. Detection of Rb1. Full-length Rb1 antigen was detected in the nuclear fraction of all cell types used in this study. Nuclear fractions were subjected to immunoblotting using anti-Rb1 monoclonal antibodies and HRP-conjugated secondary reagents. Expected bands for the Rb1 antigen were confirmed with relative molecular weight markers, and equal protein loading was used for each cell type and confirmed by Ponceau S staining (not shown).

Increased expression of A20 and cFLIP in TNF-α-stimulated IS-1, HeLa and HT1080 cells

We measured a protective effect of prior incubation with TNF-α on apoptosis induced by TNF-α and cycloheximide (Fig. 1A) and the absence of a protective effect of PAI-2 overexpression on apoptosis (Figs. 3 and 4). These data led us to investigate whether, in the cell lines used, incubation with TNF-α could increase the expression of other mRNAs for proteins with known apoptosis-protective effects. IS-1, HeLa and HT1080 cells were incubated with TNF-α for 16 h, an incubation which gives protection to cells from a subsequent apoptotic stimulus (Fig. 1A), and levels of A20 and cFLIP mRNAs were measured by quantitative RT-PCR. A20 and cFLIP are well-characterized inhibitors of TNF-α-induced apoptosis [15–20]. After 16 h, both mRNAs were upregulated in all cell types (see Fig. 7), suggesting that the protective effect we have measured (Fig. 1) could be accounted for by the presence of elevated levels of A20 and cFLIP activities.

Discussion

TNF-α is a potent cytokine which, on one hand, induces expression of inflammatory response proteins and, on the other, may induce apoptosis. In TNF receptor-expressing cells, TNF-α does not normally induce apoptosis because TNF-induced expression of anti-apoptotic proteins is sufficient to confer resistance to cell death pathways [14,21–23]. As a consequence, inhibitors of transcription or translation are often required to demonstrate TNF-α-induced apoptosis experimentally [24,25].

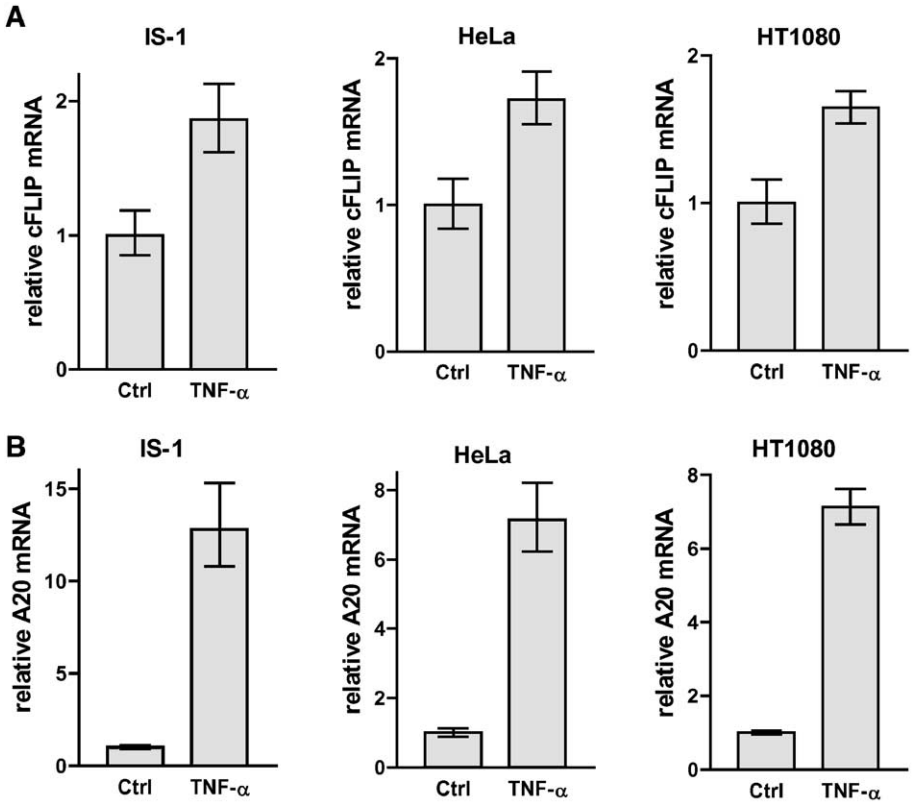


Fig. 7. TNF-α induces cFLIP and A20 expression in IS-1, HeLa and HT1080 cells. Expression of cFLIP (A) and A20 (B) mRNAs were measured in IS-1, HeLa and HT1080 cells by quantitative RT-PCR with (TNF-α) and without (Ctrl) a 16-h stimulation with 10 ng/ml TNF-α. Data were analyzed using the ΔC_T method, using GAPDH as the housekeeping gene. Data are normalized to a value of 1 for each gene in each non-stimulated cell type. Experiments were performed in triplicate and PCRs for each cDNA in duplicate, error bars represent SD (n = 3).

Several clade B serpins have been implicated as intracellular protectors from apoptosis, particularly in response to TNF-α [4,26–28] (reviewed in [1]). The targets for clade B serpin-mediated apoptosis resistance are unclear, and their identification would point to novel proteinase-dependent cell death pathways. SerpinB2 (PAI-2) expression is induced by TNF-α in many cell types and has been implicated as a protective factor in TNF-α-induced apoptosis [3,4]. Our original aim was to use different cell lines to study the role of PAI-2 as an anti-apoptotic molecule and to identify a putative intracellular target for this inhibitor.

For our study, we used three cell lines, of which HeLa and HT1080 have previously been used to generate PAI-2 overexpressing clonal cell lines that were resistant to TNF-α-induced cell death [3,4]. We studied the effect of TNF-α incubation on subsequent sensitivity of each cell line to an apoptosis-inducing combination of TNF-α and cycloheximide. Each of the three cell types had some protection from apoptosis after TNF-α pre-incubation. Endogenous PAI-2 was detected in only two of these cell lines where its expression was increased by TNF-α stimulation. HeLa cells had no detectable PAI-2 protein or mRNA, even after TNF-α stimulation. The absence of PAI-2 in HeLa cells excludes it as an endogenous apoptosis-protective factor in these cells. In addition, TNF-α pre-stimulation was accompanied by induction of other proteins known to provide defense from death receptor-induced apoptosis, A20 and cFLIP, in each of the cell types we used.

As tumor cell lines are notoriously heterogeneous, we used lentiviral vector-mediated PAI-2 gene delivery to stably overexpress PAI-2 in each cell type. With this approach, we observed that we could obtain stable overexpression of PAI-2 in a population of each cell line, with near 100% efficiency, thereby maintaining the heterogeneity of the parent cell lines and avoiding potential clonal biases. We verified that the overexpressed PAI-2 was functional by testing its ability to covalently bind to u-PA. As a control for the requirement of protease inhibiting PAI-2, we used the inactive R380A PAI-2 variant. In previous studies, clonal cell lines expressing this mutant were not protected from apoptosis induced by TNF-α and cycloheximide [4]. We observed no cytotoxicity of vector-transduced cells, compared to parent cell lines, and measured no changes in growth kinetics of stably transduced cell populations. PAI-2 mRNA and protein levels were markedly higher in transduced cells than in TNF-α stimulated cells.

Populations of IS-1, HeLa and HT1080 cells, transduced for overexpression of functional PAI-2, showed no protection from TNF-α-induced apoptosis, compared to the parent cell lines or HeLa and HT1080 cells transduced for expression of inactive PAI-2 R380A. This conclusion is based upon results from three different cell death assays: measurement of annexinV binding to apoptotic cell membranes, caspase activity in cells undergoing apoptosis and measurements of cell viability after prolonged apoptotic stress.

Apoptosis induced by TNF-α is thought to be mainly induced via TNFR1, which in contrast to TNFR2, bears a death domain in its cytoplasmic tail [22]. To verify whether overexpression of PAI-2, or lentiviral transduction, had modified the expression of TNFR1 in a direction that counteracts the potential protective effect of PAI-2, we measured cell surface TNFR1 levels in all cell lines used in our study. TNFR1 was detected in all cell lines, and no differences in cell surface expression levels were noted between non-transduced and transduced cells.

Darnell et al. reported that overexpression of PAI-2 protects the Rb1 protein from degradation [6]. An interaction between the carboxy-terminal C-pocket of Rb1 and PAI-2 was demonstrated. Rb1, identified as the prototype tumor suppressor, is a global regulator of transcription and influences cell cycle progression, apoptosis and differentiation [29]. The degradation of Rb1 is observed in death receptor-mediated apoptosis, and a non-degradable mutant Rb1 renders cells resistant to TNFR1-induced cell death [30].

If PAI-2-mediated protection from apoptosis requires wild-type Rb1 then the absence of protection by overexpressed PAI-2 could theoretically be due to an absence of Rb1 or a mutant of Rb1 in the cell lines used. By immunoblotting, we detected Rb1 at the expected relative molecular mass, and by DNA sequencing, we detected the wild-type C-pocket sequence of Rb1, in all the cell lines used. This excludes an absence of, or mutation of, Rb1 as an explanation of our conclusion that PAI-2 does not protect cells from TNF-α- and cycloheximide-induced apoptosis.

We detected increased levels of Rb1 antigen in transduced HeLa cells, compared to the parent cell line. However, as there was no difference in apoptosis sensitivity in these cells, the higher level of Rb1 appears to be insufficient to influence TNF-α-induced cell death. Furthermore, as the same increase in Rb1 was measured in wild-type PAI-2 and inactive PAI-2 overexpressing cells, we can exclude PAI-2 activity as a factor contributing to this increase. IS-1 and HT1080 cells showed no differences in Rb1 levels in transduced compared to non-transduced cells.

The efficient transduction of the cell lines used in our study gave high levels of PAI-2 expression. We demonstrated that the overexpressed protein formed stable complexes with u-PA. It seems prudent to consider that very high levels of overexpression may be counter-productive to the “anti-apoptotic” activity of PAI-2 if, for example, PAI-2 were part of a multimolecular complex where excessive PAI-2 levels sequestered binding partners from interacting with each other. However, the mechanism previously proposed for the protective effect of PAI-2 on TNF-α-induced apoptosis required a wild-type reactive center loop sequence, which infers a proteinase inhibitory activity. It is unclear how a proteinase involved in apoptosis would be less efficiently inhibited by high concentrations of a suicide substrate inhibitor, such as a serpin.

Taken together, our data are incompatible with a protective role for PAI-2 in TNF-α-induced apoptosis. This conclusion is based upon our finding that populations of cells with increased expression of functional PAI-2 are as sensitive to induction of cell death as cells which express low endogenous levels of PAI-2 or no PAI-2 at all. The potential intracellular target(s) for PAI-2 activity remain poorly defined and are the continued focus of future studies.

Acknowledgments

The authors wish to thank the Swiss Federation Against Cancer (KFS 1059-09-2000), the Swiss National Science Foundation (no. 32.061510.00) and the Geneva League Against Cancer for financial support. We also thank Christelle Viglino for technical assistance and Sylvie Dunoyer-Geindre and Nathalie Satta for the useful discussions.

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