

Cathepsin L Affects Apoptosis of Glioblastoma Cells: a Potential Implication in the Design of Cancer Therapeutics

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Abstract. *Background: Previous studies indicate that Cathepsin L (CatL) is involved in brain tumour progression. Here, CatL in tumour cell invasion and apoptosis has been studied. Materials and Methods: Human glioblastoma cell line U87 was transfected with CatL cDNA in sense and antisense orientations. The in vitro invasiveness was tested in modified Boyden chambers. Apoptosis was determined by fluorescent staining, caspase activity, and by Bax and Bcl-2 mRNA levels. Results: Surprisingly, the invasiveness of U87 cells was not impaired by genetic down-regulation of CatL expression. In the CatL antisense clones, the apoptotic rate induced by either intrinsic or extrinsic stimuli was increased, whereas CatL sense transfection seemed to protect the cells from apoptosis. Conclusion: Increased chemoresistance of tumour cells may be associated with increased levels of CatL and may have potential application in gene therapy, which would augment the apoptosis of glioblastoma cells induced by chemotherapy.*

One of the most prominent features of malignant brain tumours is their local invasive growth, where tumour cells invade the neighbouring healthy brain tissue. The diffuse growth of glioblastoma multiforme, the most progressive type of glioma, prevents successful surgical removal of these tumours and therefore shortens patient survival. Eventual proteolytic modification of brain extracellular matrix (ECM) components, such as laminin, fibronectin, collagen and others, would facilitate the invasive spread of tumour cells (1). Proteolytic enzymes, including lysosomal cathepsins (Cats), mediate this invasion process (2). On the other hand, Cats may also affect other processes, such as apoptosis (3).

Cathepsins are lysosomal proteases of various classes, by far the largest being the family of cysteine endopeptidases,

which share close structural and functional characteristics with the plant cysteine endopeptidase papain, which represents the model of clan C1A peptidases (see Merops data base classification at www.merops.sanger.ac.uk). Cats participate in many normal processes, but reportedly also play a role in various pathological processes, including cancer progression (4-7). In many types of cancer, including brain tumours, increased expression of cysteine Cats (8-12) and their altered subcellular trafficking (13) were reported. In general, the increased cysteine Cats activity can be modulated by their natural inhibitors (4), synthetic inhibitors (10), antibodies (8, 14-15), and by genetic manipulation (16-17), all affecting tumour cell phenotypes and presenting potential candidates for adjuvant therapy. However, the therapeutic goal in cancer is not only to target invasion, but also to selectively trigger tumour cell death, apoptosis. Malfunctioning of apoptosis is a crucial step in carcinogenesis and also affects increased resistance to therapy (18). Various apoptotic pathways, which depend on the cell type and triggering stimuli, generally converge into caspase activation and result in DNA degradation and cell death. Under physiological conditions, lysosomes and their constituents do not seem to participate in this cytosolic process, but under oxidative stress, resulting in disintegration of the lysosomal membranes, the intrinsic apoptotic pathway is triggered, possibly upstream of caspases (19). Cathepsin-mediated apoptosis possibly involves cleavage of Bid (20-21), which would affect the delicate balance between more than twenty of the Bcl-2X family proteins, such as the pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins. Over-expression of the anti-apoptotic Bcl-2 protein was implicated in tumour resistance to treatment and may co-exist with a high concentration of execution caspases (22). As glioblastoma cells express cathepsins B and L (CatB and CatL, respectively) at much higher levels than normal glial cells, apoptosis triggering could be highly efficient if lysosomal contents were released into the cytosol, thus sensitizing tumour cells to cytotoxic agents; however, this is not observed (23). In contrast, it was demonstrated that triggering the intrinsic (mitochondria-mediated) pathway of apoptosis by staurosporine (STS) in

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a primary culture of anaplastic astrocytoma (IPTP) cells was impaired in the presence of higher levels of CatL activity, whereas genetic ablation of CatL resulted in an increased apoptotic response to STS (16). In the present study, further investigation of the role of CatL, not only in the intrinsic, but also in the extrinsic apoptotic pathways, induced by STS and tumour necrosis factor α (TNF- α), respectively, in the malignant glioblastoma cell line U87, confirmed the anti-apoptotic role of CatL.

Materials and Methods

Cell line and transfection. Human permanent glioblastoma cell line U87 was obtained from the American Type Culture Collection and cultured in MEM medium supplemented with 10% foetal bovine serum, 2.5 mM L-glutamine, and 1% penicillin/streptomycin (all Euroclone, Milano, Italy) at 37°C and 5% CO₂. The cells were grown to 80% confluence before harvesting. Full length PreProCatL cDNA, in both sense and antisense orientations (16), was cloned into mammalian vector pcDNA3.1(-), (Invitrogen, Paisley, UK). U87 cells (10⁶) were transfected with 5 μ g of these constructs using the calcium phosphate precipitation method (24). Stably transfected clones were selected for 3-5 weeks in the presence of geneticine (Gibco, Paisley, UK; 400 μ g/ml). Several CatL sense transfected clones, U87/Sn, CatL antisense transfected clones, U87/Asn, and empty-vector transfected clones, U87v, resistant to geneticine, were isolated.

Clonal selection. Positive CatL clones were discriminated by specific PCR primers using a quantitative RT-PCR method (Perkin Elmer, Wellesley, USA). RNA was extracted from cells using the total mRNA Isolation Kit (Promega, Madison, USA). One μ g of RNA was reverse transcribed using a High-Capacity cDNA Archive Kit and the resulting cDNA was amplified using specific primers and probes with the TaqMan Universal PCR Master Mix (all Applied Biosystems, Foster City, USA). Amplification of 18S rRNA was performed as an internal control. Probe 5'-CTTGGGTCTCCCTATAGTG-3' was the same for all clones. The primer sequences were as follows: 5'-CACTGCTTACTGGCTTATCGAAATTA-3' (forward) and 5'-GCTCGAGTCTAGAGGGCCCGT-3' (reverse) for U87v, 5'-AGAACCCACTGCTTACTGGCTTAT-3' was forward primer for all CatL transfected cells, 5'-CGGATACACACTCGAGTCGAGTC-3' and 5'-CAGGGCTGGTGACACCTAC-3' were reverse primers for U87/Asn and U87/Sn, respectively.

RNA analysis by quantitative RT-PCR. Quantitative RT-PCR was also used to measure CatL, Bax and Bcl-2 mRNA expression. RNA was extracted, reverse transcribed and PCR amplified with specific primers and probes, as described above. Amplification of 18S rRNA was performed as an internal control. The sequences used were: for Bax, 5'-CGAGTGGCAGCTGACATGTTT-3' (forward primer), 5'-TCCCGGAGGAAGTCCAATG-3' (reverse primer) and 5'-AACTGGTGCAGGCCCTGTGCA-3' (probe); for Bcl-2, 5'-GCGCACGCTGGGAGAA-3' and 5'-TCCCGCATCCCACTCGTA-3' (forward and reverse primers, respectively), 5'-GAAGTACATCCATTATAAGCTGTCGAGAGGG-3' (probe); for CatL, 5'-AGCGTCTACCCGAACCTG-3' (forward primer), 5'-TTGTGCATCGCCTTCCACT-3' (reverse primer) and 5'-TTGTGCATCGCCTTCCACT-3' (probe).

Cathepsin protein and activity measurement

(i) Preparation of cell homogenates. Cells were homogenized by freezing in liquid nitrogen and thawing at 37°C (3 times) in 50 mM Tris buffer, pH 6.9, containing 0.05% (v/v) Brij 35, 0.5 mM dithiothreitol (DTT), 5 mM EDTA, 0.5 mM paramethylsulphonyl fluoride (PMSF) and 10 mM pepstatin A. The homogenates were centrifuged at 12,000 xg for 15 min and the supernatants were used for measurements.

(ii) Enzyme-linked immunosorbent assay (ELISA). ELISA assays for human CatL and CatB (Krka d.d. Novo mesto, Slovenia) were performed according to the manufacturer's instructions. These tests recognize the precursor and the active enzyme forms, as well as enzyme-inhibitor complexes. Cathepsin content was expressed in ng/mg of total protein. Total protein concentrations were determined using the Bradford assay (Bio-Rad protein assay reagent, Bio-Rad, USA) with bovine serum albumin as the standard.

(iii) CatL activity measurement. Supernatants of cell homogenates were diluted with water and mixed 1:2 with 0.34 M sodium acetate (pH 5.5) containing 4 mM EDTA 0.1% (v/v) Brij 35 and 2 mM fresh DTT (all Sigma, St. Louis, USA). Samples were incubated for 30 min at 37°C to allow for full activation of CatL. To measure specific CatL activity, 100 μ l of water was added to the sample and two negative controls were used, first with 100 μ l of the specific CatL inhibitor CLIK-148 (CLIK-148 was a gift of Dr. N. Katunuma, Tokushima Bunri University, Japan) in 0.5 μ M final concentration and second with 100 μ l of a cysteine protease inhibitor, L-trans-epoxysuccinyl-Leu-3-methyl-butylamide ethyl ester (E64c, Bachem, Bubendorf, Switzerland), added in 16 μ M final concentration, to inhibit total cysteine protease activity. 100 μ l of the reaction buffer (0.34 M sodium acetate, pH 6.0, containing 4 mM EDTA, 0.1% (v/v) Brij 35 and 4 mM fresh DTT) was then added and incubated for 5 min at 37°C to allow proteinase-inhibitor interaction. The reaction was started by the addition of the substrate benzyloxycarbonyl-phe-arg-7-amino-4-methylcoumarine (Z-FR-AMC, Bachem, Bubendorf, Switzerland) to a final concentration of 16 μ M. After 90 min at 37°C, the reaction was terminated by the addition of iodoacetic acid (0.5 mM final concentration, Sigma, St. Louis, USA). The fluorescence of the product, 7-AMC, was measured at 460/370 nm using a spectrofluorimeter (Tecan, Groedig, Austria). The values were compared with those for standard 7-AMC solutions. Specific CatL activity was calculated by subtracting the activity measured in the presence of CLIK-148, from the total activity of cysteine proteases, which was determined as the difference between the total and background activity of the non-cysteine peptidases in the assay using a E64c (broad spectrum inhibitor of cysteine proteases). Specific activity was expressed in enzyme units (EU) per mg of total protein, with one EU being the amount of the enzyme releasing 1 nM of 7-AMC per min.

(iv) CatB activity measurement. The activity of CatB was measured using the same protocol, as described above, where the selective CatL inhibitor was replaced by a selective CatB inhibitor, CA-074Me, at a final concentration of 10 μ M.

Invasion assay. This was performed in modified Boyden chambers. The chambers (polycarbonate filters, Sigma, St. Louis, USA), with 12 μ m pores, were coated on the lower surface with fibronectin (25 ng/mm², Sigma, St. Louis, USA) and on the upper surface with 250 μ l of Matrigel (0.2 mg/ml, Becton Dickinson, Franklin Lakes, USA), laminin (5 μ g/ml, Sigma, St. Louis, USA), fibronectin or

collagen IV (both 10 µg/ml, Sigma, St. Louis, USA), in serum free medium (SFM). The filters were dried overnight and reconstituted with 200 µl of SFM for 1 h at 37°C. The upper chamber of each well was seeded with 2x10⁵ cells in 0.5 ml SFM and 1.5 ml of 1:1 mixture of 24 h-conditioned SFM and fresh SFM was added to the lower chamber. The cells were incubated at 37°C for 21 h on Matrigel, fibronectin and collagen IV and for 45 h on laminin. 1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, USA) at 0.5 mg/ml final concentration was then added to both chambers. After 3 h at 37°C, the formazan crystals were collected separately from the upper and lower chambers, pelleted by centrifugation and dissolved in 1.0 ml of dimethyl sulfoxide. Absorbance was measured at 570 nm (reference filter 690 nm) on a spectrofluorimeter (Tecan, Groedig, Austria). The percentage of cells penetrating the substrate (invasive cells) was calculated as the ratio of the number of cells in the lower compartment to the sum of cells in both compartments. To test the inhibition of invasion, in the parallel assays, the inhibitors were added in the media when the cells were seeded on the substratum in both, the upper and the lower chamber at the final concentration of 0.5 µM CLIK-148, 50 µM of E64d (Bachem, Bubendorf, Switzerland), and 0.5 µM of methylated L-trans-epoxysuccinyl-Ile-Pro-OH propylamide (CA-074Me), which are below the toxic concentrations (as determined previously).

Assessment of apoptosis. Apoptosis of cells in culture was triggered by STS (Sigma, St. Louis, USA) and by TNF-α (gift of Dr. Viktor Menart, National Institute of Chemistry, Ljubljana, Slovenia) together with actinomycin D (ActD; Sigma, St. Louis USA; 2 µg/ml). STS was used at 1 µM final concentration, and TNF-α at 5 and 10 ng/ml. Apoptosis was assessed using differential fluorescence staining, measuring the enzymatic activity of caspases and by determining the ratio between gene expression of Bax and Bcl-2.

(i) *Fluorescence staining.* The stage of apoptosis was determined visually using a technique based on the differential uptake of the fluorescent DNA-binding dyes, acridine orange and ethidium bromide (25), depending on membrane integrity and nuclear morphology. A cell suspension was stained with 1:1 (v/v) acridine orange/ethidium bromide mix and examined under a Nikon Eclipse E800 fluorescence microscope equipped with a B2-A filter. 200 cells per slide were counted. For all cells, apoptosis was determined as the ratio of late apoptotic cells, counted after induction of apoptosis with STS, to the control cells (without STS).

(ii) *Caspase -3 and caspase-7 activity assays.* Effector caspase (-3 and -7) activities were determined on cells in culture using the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, Madison, USA), according to the manufacturer's instructions. Fluorescence was measured at 530 nm with excitation at 485 nm on a spectrofluorimeter (Tecan, Groedig, Austria). Activity of caspases -3 and -7 is presented as the ratio of the activity in cells after induction of apoptosis to that in the control cells (no apoptosis induction, background caspase activity), measured after 0 h, 4 h, 8 h, 12 h, 15 h and 24 h induction with STS.

(ii) *Determination of Bax: Bcl-2 ratio.* mRNA expression of the pro-apoptotic Bax and anti-apoptotic Bcl-2 genes was measured using quantitative RT-PCR (see above) and the ratio of the two mRNA levels was calculated.

Statistical analysis. Data are presented as mean values of at least three independent measurements and standard errors of the mean

(SEM). Statistical significance of the comparisons between samples was determined using the Student's *t*-test and differences at $p < 0.05$ were considered statistically significant.

Results

Cathepsin L expression. The U87 glioblastoma cell line was transfected with PreProCatL cDNA of CatL in both sense and antisense orientations. Transfected cells were analysed for CatL protein and activity levels. Two of 19 CatL sense clones resistant to geneticine, showing the highest CatL expression (U87/Sn-8 and U87/Sn-9) and two of 15 antisense clones with the lowest CatL expression (U87Asn-6 and U87Asn-7), were chosen for further analysis. Figure 1 shows that CatL down-regulation by antisense transfection of U87 cells resulted in significantly lower expression of CatL protein and activity (reduced between 30% and 65%) than that in the control cell line U87v, which was the U87 line, transfected with empty vector. In the U87/Sn transfected clones, CatL protein was 2.4 to 3.1 times higher than in the control, whereas CatL activity was raised by about 50%. PCR analysis showed an increase in CatL mRNA of 50% and 30% in Sn-9 and Sn-8, respectively, and a less than 10% (non-significant) reduction in the U87/Asn clones, compared to U87v. CatL transfection did not affect protein and activity levels of CatB (data not shown).

Effect of CatL gene expression and of cysteine protease inhibitors on U87 cell invasion in vitro. The invasive potential of U87 cells and its CatL transfectants into different components of ECM was tested. All the cells, U87/Sn, U87/Asn, and U87v, penetrated Matrigel and fibronectin better than laminin or collagen IV (Figure 2). The percentage of invading control cells (U87v) was 15-20% for the Matrigel and fibronectin substrates and approximately 10% for laminin and collagen IV. Independent of the substrate, the two U87/Sn clones were significantly more invasive than the U87/Asn clones and the control, whereas the invasiveness of the U87/Asn clones was not significantly different from the U87v control.

The ability of cell-penetrating inhibitors, such as the general cysteine protease inhibitor E64d, the CatB selective inhibitor CA-074Me, and the CatL selective inhibitor CLIK-148, to reduce the invasion through Matrigel and fibronectin, was tested. Figure 3A shows that invasion of the U87v control through Matrigel and fibronectin, was inhibited by E64d by 45% and 57%, respectively. The invasion of both U87/Sn clones (Figure 3B) and both U87/Asn clones (Figure 3C) through fibronectin was also significantly inhibited ($p < 0.05$) by E64d, as was their invasion through Matrigel (not shown). CA-074Me and CLIK-148 significantly inhibited the invasion through fibronectin of U87v, but only up to 15% (Figure 3A), as well

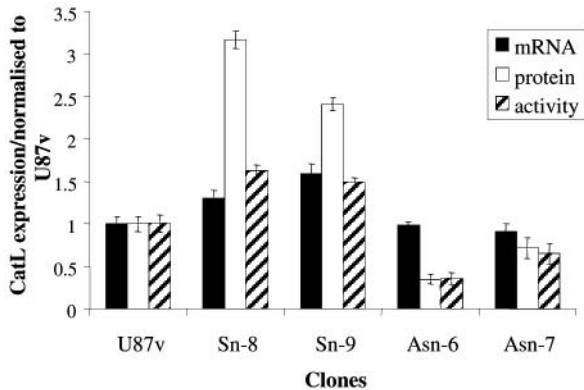


Figure 1. *CatL* mRNA, protein and activity in U87 clones. Two U87/Sn (Sn-8 and Sn-9) and two U87/Asn (Asn-6, Asn-7) clones are compared to the control clone, U87v. *CatL* expressions were determined as described in Materials and Methods and normalised to the U87v (=1). *CatL* protein and activity, but not mRNA, of both U87/Asn clones were significantly ($p < 0.05$) lower than in the control U87v. U87/Sn transfectants express significantly ($p < 0.05$) more *CatL* at all levels of expression. Error bars indicate SEM of three independent measurements.

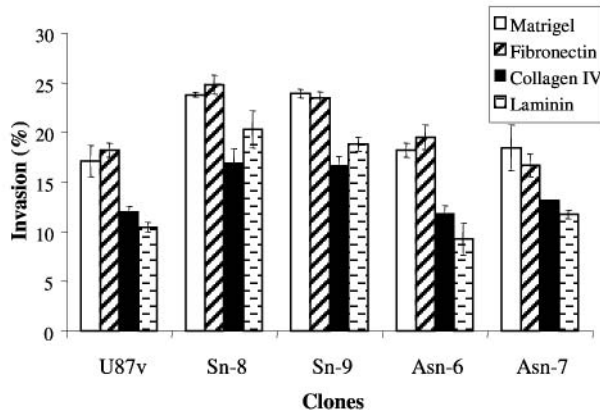


Figure 2. Invasive potential of U87 clones into Matrigel, fibronectin, laminin and collagen IV. The percentage of cells penetrating through different ECM components was calculated as described in Materials and Methods. The invasion of U87 clones through Matrigel and fibronectin was higher than through laminin and collagen type IV. Both U87/Sn showed significant ($p < 0.05$) increase of invasion through all substrates. The differences between U87/Asn clones and the control, U87v, were not significant. Error bars indicate SEM of three independent experiments.

as of the U87/Sn clones (Figure 3B), but did not impact the invasion through fibronectin of U87/Asn (Figure 3C). CA-074Me and CLIK-148 did not affect the invasion rates of U87/Sn and U87/Asn clones through Matrigel.

Effect of CatL gene expression on apoptosis of U87 clones. The intrinsic pathway of apoptosis was triggered with STS and the extrinsic pathway with TNF- α . The rate of apoptosis

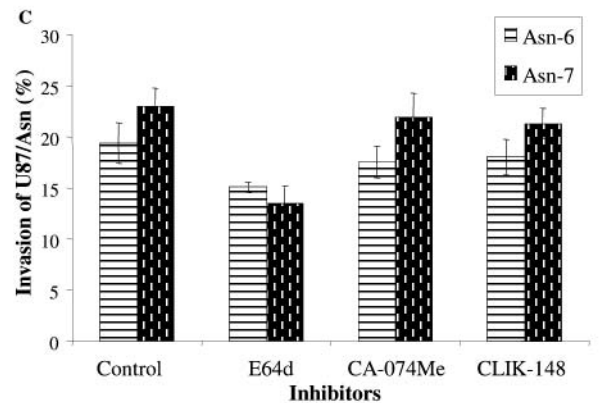
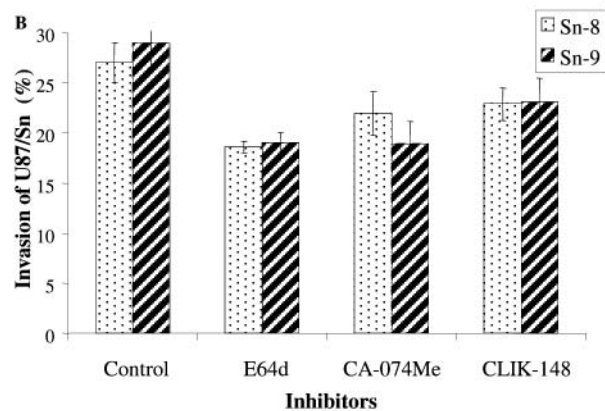
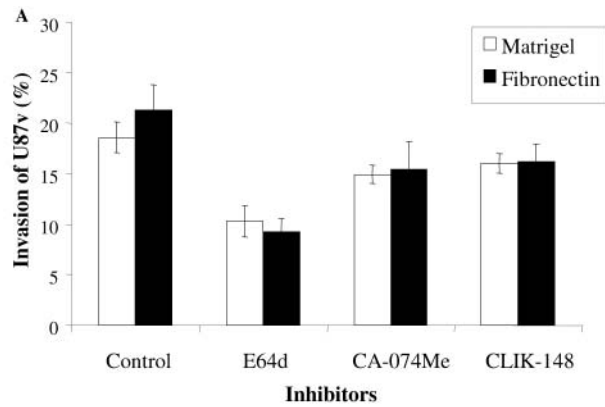


Figure 3. Effect of cysteine protease inhibitors on U87 clones invasion. A) Invasion of the control cells, U87v. Invasion of the U87v through Matrigel and fibronectin was significantly inhibited by E64d ($p = 0.003$ and $p = 0.0004$, respectively). CA-074Me significantly inhibited U87v invasion through Matrigel ($p = 0.043$) and fibronectin ($p = 0.019$), whereas CLIK-148 significantly reduced invasion of U87v through fibronectin ($p = 0.042$). B) Invasion of U87/Sn clones through fibronectin. Invasion of U87/Sn clones was significantly inhibited by E64d ($p = 0.002$ and 0.009 , for Sn-8 and Sn-9 clone, respectively), and somewhat less, but still significantly ($p < 0.05$) by CA-074Me and CLIK-148. C) Invasion of U87/Asn clones through fibronectin. Invasion of U87/Asn clones was significantly inhibited by E64d ($p = 0.017$ and 0.022 , for Asn-6 and Asn-7, respectively). CA-074Me and CLIK-148 did not affect the invasion of U87/Asn clones. In all experiments, E64d was used at $50 \mu\text{M}$, whereas CA-074Me and CLIK-148 were used at $0.5 \mu\text{M}$ concentrations. The percentage of invasive cells was calculated as described in Materials and Methods. Error bars indicate SEM of three independent experiments.

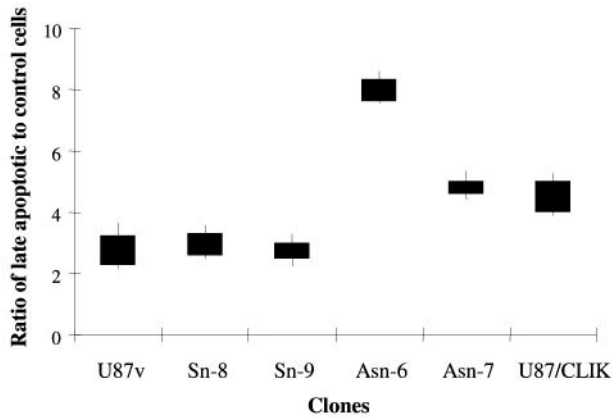


Figure 4. The proportion of late apoptotic cells in U87 clones. Apoptosis was induced with 1 μ M STS for 4 h and the number of late apoptotic cells determined by fluorescent staining as described in Materials and Methods. In one experiment, U87v cells were pre-incubated with CLIK-148 (0.5 μ M, 24 h) before induction of apoptosis. The proportion of late apoptotic cells was significantly higher ($p < 0.05$) in U87/Asn clones and in cells pre-treated with CLIK-148, than in U87/Sn clones and the control, U87v.

was evaluated using three methods and the optimal apoptosis induction time for each method was determined experimentally. Figure 4 shows the ratio of late apoptotic cells, after induction of apoptosis with STS for 4 h, to that of the control cells (cells without STS treatment) in U87v, U87/Sn and U87/Asn transfected clones, and in U87 cells pre-treated with the CatL-selective inhibitor, CLIK-148, as observed using differential fluorescence staining. The number of late apoptotic cells was significantly higher in U87 cells with reduced CatL expression – in both U87/Asn clones and in U87 cells pre-treated with CatL inhibitor. This number in U87/Sn cells did not differ significantly from the control.

Effector caspase-3 and -7 activities were determined after the induction of apoptosis with STS for various times and with TNF- α /ActD for 12 h and 24 h (Figure 5). Up to 8 h of STS treatment, the activity of caspases-3 and -7 was very similar to the baseline activity (without STS) in all clones tested. After 12 h of STS treatment, caspase-3 and -7 activity reached its peak in the U87/Asn clones, and was 2-3 times higher than in the U87v clone. Caspase-3 and -7 activity of U87/Sn clones and U87v was highest after 15 h of STS treatment. Similarly, when apoptosis was triggered with TNF- α /ActD, after 24 h significantly higher caspase-3 and -7 activity was observed in both U87/Asn clones whereas the activity in U87/Sn clones was lower than in the U87v clone. Treatment with 5 ng/ml of TNF- α /ActD for 24 h and with 10 ng/ml of TNF- α /ActD for 12 h, gave very similar results (Figure 5).

mRNA expression of the pro-apoptotic Bax and anti-apoptotic Bcl-2 genes in U87 clones also varied with

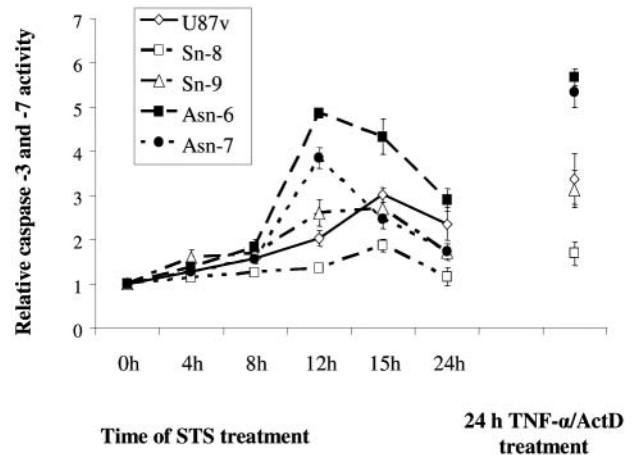


Figure 5. Caspase-3 and -7 activity in U87 clones. Apoptosis was triggered by STS and by TNF- α /ActD. Caspase-3 and -7 activity was measured as described in Material and Methods at the indicated time intervals of STS treatment and 24 h after TNF- α /ActD treatment. Error bars indicate SEM. The baseline caspase activity (in non-treated cells) was taken as 1. Caspase-3 and -7 activity of both U87/Asn clones peaked at 12 h and was significantly higher ($p < 0.05$) than in the control, U87v. In U87/Sn and the U87v, caspase-3 and -7 activity reached its highest value after 15 h. The Sn-8 clone exhibited significantly lower ($p = 0.002$) caspase-3 and -7 activity than U87v, whereas Sn-9 did not. At 24 h of STS treatment, caspase-3 and -7 activity in all clones decreased. When apoptosis was induced with TNF- α /ActD, significantly higher ($p < 0.05$) caspase-3 and -7 activity was observed in both U87/Asn clones and significantly lower caspase-3 and -7 activity in Sn-8 ($p = 0.018$), than in the control U87v after 24 h.

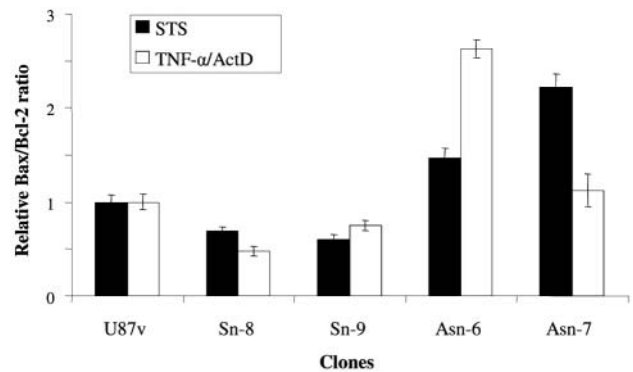


Figure 6. Bax/Bcl-2 ratio in U87 clones. Apoptosis was triggered by STS for 4 h (full bars) and by TNF- α /ActD for 24 h (open bars). mRNA levels of Bax and Bcl-2 were determined by quantitative RT-PCR as described in Materials and Methods. The ratio of the two genes was normalised to that of U87v (=1). Error bars indicate SEM. STS treatment resulted in a significantly higher Bax/Bcl-2 ratio in Asn-6 ($p = 0.024$) and Asn-7 ($p = 0.009$) clones and a significantly lower ratio in Sn-8 ($p = 0.028$) than in the control, whereas in the Sn-9 clone, it was not significantly different from the control. With TNF- α , both U87/Sn clones had a significantly lower Bax/Bcl-2 ratio ($p < 0.05$), and Asn-6 a significantly higher ($p = 0.002$) ratio than in U87v. The Asn-7 clone was not significantly different from the control U87v.

duration of STS treatment. The time course of Bax mRNA expression and of the Bax/Bcl-2 ratio in U87/Asn differed from that in U87/Sn clones and the U87v control. The differences were greatest at 4 h and diminished gradually after 8 h of STS treatment (data not shown). For this reason, comparisons of Bax/Bcl-2 mRNA ratio were made at 4 h of STS treatment (Figure 6). The ratio was lower in the U87/Sn clones, indicating reduced apoptotic response to STS, whilst being significantly higher in the U87/Asn clones than in the control U87v, indicating elevated apoptotic responses to STS. Similar results were obtained when apoptosis was induced by TNF- α /ActD.

Discussion

CatL is over-expressed in various types of cancer and has been shown to be associated with invasiveness and/or tumorigenicity of various tumour cells, including breast (4, 26), myeloma (17), melanoma (27) and glial tumour cells (9, 28, 12). Its ability to hydrolyse components of the ECM, such as collagen IV, fibronectin, laminin and Matrigel (29), may classify this enzyme as relevant for tumour cell invasion into normal surrounding tissue and into blood vessels. To better define the function of CatL in glioma invasion, we applied an antisense strategy, by stably transfecting the glioblastoma cell line U87 with a mammalian expression vector carrying the complete cDNA of CatL in sense and antisense orientations, as we reported previously for the primary anaplastic astrocytoma IPTP cell line (16). The CatL transfection produced clones with both higher and lower expression of CatL protein and activity, but did not affect the expression of CatB or stefins. In contrast to IPTP cells (16) and osteosarcoma cells (30), where transfection with CatL antisense oligonucleotides reduced invasiveness, U87/Asn clones were not less invasive than the control in various ECM proteins, such as complex Matrigel and single protein substrates, fibronectin, collagen and laminin. In addition, the CatL siRNA method was not effective in reducing the invasion of U87 through Matrigel (Kenig and Lah, unpublished observation). This may be explained by the different protease expression profiles of the more invasive U87 cells compared with less malignant IPTP ones. For example, more malignant U87 and other glioblastoma cells lines also express higher levels of CatB (8) and other proteases (9), which may compensate for low CatL activity to facilitate the *in vitro* invasion. Indeed, CatB inhibition by the antisense strategy was also effective in diminishing the invasive potential of U87 cells (31). Furthermore, even for cancer cells of the same origin, invasiveness may depend on different sets of proteases (32). Alternatively, highly malignant glioblastoma cells may easily alter their cell morphology during invasion, towards that of amoeboid-like movement, which is less dependent on proteolysis of

extracellular matrix (33). In contrast to the antisense U87/Asn clones, the invasiveness of the sense U87/Sn clones increased from 10% to 25%, indicating that CatL may further facilitate invasion, as has also been reported for osteosarcoma cells (30). Partial inhibition of invasiveness was observed for both the native U87 cells, as well as the CatL sense transfectants, in Matrigel and fibronectin when exogenous cysteine protease inhibitors, including the CatL selective inhibitor, CLIK-148, were introduced into cell culture media. This is in line with our previous data (10) as well as with Sivarapathi *et al.* (8), who partially inhibited the invasion of several glioblastoma and meningioma cells by CatL antibodies. However, the inhibition in the control and sense clones resulted in an invasion similar to that of the untreated CatL antisense clones. Similar results have been reported for two prostate cancer cell lines, where E64 completely inhibited their CatB and CatL activities, but only partially reduced their invasiveness (32), in line with previous observations (34). Therefore, we conclude that other endopeptidases also participate in cell invasion and may compensate for the lower CatL expression during the cell invasion process, as shown in the genetically manipulated clones of U87.

Lysosomal enzymes are actively involved in the autophagic pathways and in necrosis, and accumulating data suggest that caspase-independent apoptosis, induced by certain cytokines, is carried out *via* a "lysosomal pathway" (35-36). Oncogene-driven transformation of immortalized cells has been associated with significant increase of cathepsin expression and increased sensitization to TNF- α induced apoptosis (35). It has also been shown that lysosomal destabilization and consequent translocation of cathepsins from lysosomal lumen to the cytosol, resulting from various apoptotic stimuli (TNF- α , etoposide, H₂O₂), might be responsible for activating executioner caspase-3 (37-39). On this basis, it has even been suggested that high CatB and CatL levels in tumour cells may be exploited to selectively target the tumours, using apoptotic and lysosomotropic agents, thereby enhancing cell death. This proposal was opposed by Gewies and Grimm (40), who found that transient transfection of CatB and CatL into HeLa cells did not sensitize them to TNF- α -mediated apoptosis. However, in this study, we have shown that manipulation of CatL expression sensitized U87 cells to STS and TNF- α induced apoptosis. The number of late apoptotic cells induced with STS was higher in cells with reduced CatL expression. Our data suggest that these changes occur upstream of the Bax/Bcl-2 expression and of the effector caspase activation. Interestingly, triggering the extrinsic pathway of apoptosis by TNF- α affected the cells in a very similar way, indicating that anti-apoptotic signalling of CatL is an early event in the apoptosis. This study therefore does not support a pro-apoptotic activity for

CatL, but is in line with several other reports demonstrating a protective role of high CatL levels against apoptosis under conditions where lysosomes may remain intact (41-42). Anti-apoptotic activity of CatB and CatL has previously been suggested by the rapid induction of apoptosis in several cancer cells using the cysteine cathepsin inhibitor Z-Phe-Gly-NHO-Bz (43). The anti-apoptotic role of CatL over-expression is also supported by the findings that prolonged inhibition of the lysosomal proteolytic pathway is incompatible with cell survival, leading to apoptosis of neuroblastoma cells (44), and that apoptosis rates in keratinocytes and melanocytes were higher in CatL (-/-) than in CatL (+/+) hair follicles (45). More recently, enhanced susceptibility of CatL-deficient A549 lung cells to spontaneous and anti-Fas-induced apoptosis was reported (46), with a possible mechanism involving altered CatD processing by CatL. Although we may speculate that increased chemoresistance of tumour cells may be associated, or even due to, increased levels of CatL, the precise mechanisms of CatL in programmed cell death is not known yet. The evidence presented here supports the hypothesis that the cysteine protease, CatL, is relevant for apoptosis of glioblastoma cells as it contributes to their ability to resist various apoptotic stimuli. Targeting the elevated levels of CatL in human brain cancer by administration of CatL inhibitors or by genetic manipulation of its expression, would render tumour cells more vulnerable to chemotherapy and might prove beneficial in designing adjuvant therapies for treating glioma patients.

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