BCL-2 promotes migration and invasiveness of human glioma cells

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Abstract Malignant progression in gliomas is correlated with increased migratory capacity which involves metalloproteolytic activity. Here, we report that ectopic expression of BCL-2 in two malignant glioma sublines markedly promoted glioma cell migration from spheroids and invasion into Matrigel-coated membranes. Invasion of fetal rat-brain aggregates was enhanced by BCL-2. Zymography revealed activation of matrix metalloproteinase-2 (MMP-2) in BCL-2-expressing cells. BCL-2 expressing cells showed an increase in MMP-2/-3/-12 (LN-18), and MMP-9/-12 and cell surface urokinase-type plasminogen activator (u-PA) (LN-229) mRNA and a reduction in tissue inhibitors of metalloproteinases (TIMP)-2 mRNA (LN-229). Taken together, we propose a novel function for BCL-2 in the malignant phenotype of glioma cells, that is, to enhance migration and invasion by altering the expression of a set of metalloproteinases and their inhibitors.

Key words: Glioma; BCL-2; Migration; Invasion; Matrix metalloproteinase; Tissue inhibitor of metalloproteinase; Zymography

1. Introduction

Malignant gliomas are characterized pathologically by an extensive infiltration of the surrounding normal brain tissue. Therefore, a better understanding of migratory and invasive mechanisms of glioma cells may result in new therapeutic approaches. Glioma invasion is thought to rely on the modification of cell adhesion and proteolysis of extracellular matrix components [1]. Activation of matrix metalloproteinases (MMP-2/-9) is observed in high grade gliomas but not in low grade gliomas. The ability of glioma cells to spread on myelin depends on metalloproteolytic activity [2]. Recently, the tumor suppressor gene *p16/CDKN2* has been shown to down-regulate MMP-2 expression in the human SNB19 glioma cell line, resulting in the suppression of glioma invasion in vitro [3]. Also, cells transfected with an antisense cell surface urokinase-type plasminogen activator (u-PA) receptor revealed decreased invasiveness [4]. Successful migration and invasion of glioma cells requires their resistance to the endogenous death program of apoptosis once the cell has detached from the primary tumor tissue. BCL-2, the prototype inhibitor of apoptosis, has been suggested to regulate cell-cell interactions [5], including integrin-dependent regulation of cell adhesion via r-ras [6]. Vice versa, BCL-2 expression is up-regulated by $\alpha_5\beta_1$ integrins, preventing apoptosis when cells are detached from matrix, or down-regulated in endothelial cells by an $\alpha_v\beta_3$ integrin antibody, with subsequent induction of apoptosis [7]. Further, BCL-2 promotes the metastatic potential of the human breast cancer cell line MCF7 in vivo and migratory and invasive properties in vitro [8]. These observations prompted us to examine a possible link between BCL-2 and cell motility in human glioma cells. In the present study, we show that ectopic expression of BCL-2 changes the expression patterns of metalloproteinases and their inhibitors towards a more invasive phenotype. Further, BCL-2-expressing cells show enhanced migration and invasion in Matrigel invasion as well as in fetal rat brain confrontation assays.

2. Materials and methods

2.1. Cell lines and cell culture

LN-18 and LN-229 cells were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, and 0.5% Geneticin (Boehringer, Mannheim, Germany). The generation of glioma cell sublines expressing the murine BCL-2 protein has been described [9]. Immunodetection of BCL-2 was done using the mouse anti-Bcl-2 polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) [10].

2.2. Assessment of viability and clonogenicity

Cell growth, generation times and survival were assessed by crystal violet staining. Clonogenicity was assayed by suspending the cells in 2 ml medium/well of a six-well plate and counting the number of macroscopically visible clones (>0.15 mm in diameter) after 10–19 days. The number of cells invaded through Matrigel was determined by the microculture tetrazolium (MTT) assay.

2.3. Matrigel invasion assay

Invasion of glioma cells in vitro was measured by the invasion of cells through Matrigel-coated transwell inserts (Becton Dickinson, Heidelberg, Germany) [11]. Briefly, transwell inserts with 8 μm pore size were coated with a final concentration of 0.78 mg/ml of Matrigel. Cells were trypsinized and 200 μl of cell suspension (3×10^5 cells/ml) per condition were added in triplicate wells. 500 μl 3T3 conditioned medium was added to the lower well. After 48 h incubation, the cells that passed through the filter into the lower wells were quantitated by MTT assay, and expressed as a percentage of the sum of the cells in the upper and lower wells [12]. Cells on the lower side of the membrane were fixed, stained with haemalaun and sealed on slides. Quantification of invasion through the coated membranes was done by counting stained cells using a microgrid. Cells were counted twice by two independent investigators. Inter-observer variation was below 5%.

2.4. Glioma spheroids

Multicellular glioma spheroids were cultured in 25-cm^2 culture flasks base-coated with 0.75% Noble Agar (Difco Laboratories, Detroit, MI, USA) prepared in DMEM [13]. Briefly, 3×10^6 cells were suspended in 10 ml medium, seeded onto 0.75% agar plates, and

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Abbreviations: ECM, extracellular matrix molecules; MMP, matrix metalloproteinase; u-PA, cell surface urokinase-type plasminogen activator; TIMP, tissue inhibitors of metalloproteinases

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cultured until spheroids had formed. Spheroids of about 200 μm diameter were selected for the experiments.

2.5. Migration assay

The area covered by glioma cells migrating from a tumor spheroid explanted on a plastic surface was used as an index of cell migration. Spheroids were transferred individually to 96-well plates (Falcon), which were uncoated or coated with collagen type IV at 4°C overnight at 10 μg/ml PBS and blocked with 1% BSA containing 200 μl serum free medium. Every 24 h for 4 days, the radial distance of migration was determined after subtraction of the initial spheroid diameter at time zero from the diameter of the area covered with cells migrated from the spheroid.

2.6. Fetal rat-brain aggregates

Fetal rat-brain aggregates were obtained from 18-day-old fetuses of BD9 rats. The brains were aseptically removed and placed into a sterile tissue-culture plate containing PBS. The brain tissue was minced, washed in PBS, and dissociated by serial trypsinization. Single cell suspensions were obtained and plated into agar-coated 24-well plates at an average cell amount of one brain per well in 2 ml medium. After 48 h, aggregates were transferred to new plates and cultured for 19 days. Aggregates with about 200 μm diameter were used in further experiments [13].

2.7. Confrontation assays

Invasion of the glioma spheroids into fetal brain aggregates was analyzed by morphometry using the MCID digitalization system (IMAGING Research, Ontario, Canada). Briefly, tumor spheroids and rat brain aggregates were transferred in triplicate to individual wells of a 96-well plate, base-coated with agar. With the help of a sterile syringe and a microscope, tumor spheroids and fetal brain aggregates were placed in close contact to each other. Images were obtained at 24-h intervals.

2.8. Cell adhesion assay

Wells of 96-well plates were coated with the individual extracellular matrix (ECM) proteins at 4°C overnight at 1–10 μg/ml PBS and blocked with 1% BSA. A total of 4×10^4 cells were added per well in 100 μl of DMEM/1% BSA. Cells were allowed to attach for 45 min at 37°C in a CO_2 incubator. After incubation, plates were gently washed with Dulbecco's PBS containing Ca^{2+}/Mg^{2+} (DPBS). Adherent cells were fixed and stained with 0.2% crystal violet in 10% ethanol. Following three washes with PBS, the dye was extracted with 50 mM sodium phosphate, pH 4.5, in 50% ethanol and the absorbance measured at 540 nm.

2.9. Zymography of MMP-2

Analysis of MMP-2 was performed with sodium dodecyl sulfate-polyacrylamide gels impregnated with 0.1% gelatin (w/v) and 10% polyacrylamide (w/v). This assay is based on the digestion of gelatin and subsequent loss of Coomassie staining at the locus of migration of gelatinolytic enzymes. Cells were grown in 25-cm^2 culture flasks (Falcon) in DMEM containing 10% FCS until they reached 80% confluency. Cells were washed and maintained in serum-free medium, and the conditioned medium collected after 48 h. Four parts of medium containing equal amounts of protein (50 μg) from the conditioned medium were mixed with one part of Laemmli sample buffer (minus reductant) [14] prior to electrophoresis. Gels were run at a constant current and then washed twice for 30 min in 50 mM Tris-HCl, pH 7.5, plus 2.5% Triton X-100, and then incubated overnight at 37°C in 50 mM Tris-HCl, pH 7.6, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% NaN_3. Gels were stained with Coomassie Brilliant Blue R-250 and then destained.

2.10. RT-PCR for metalloproteinases, u-PA and TIMPs

The expression of MMP-2, MMP-3, MMP-9, MMP-12, u-PA, TIMP-1, TIMP-2, and GAPDH was examined by RT-PCR as described [18]. For each gene product, amplification of cDNA was in the linear range under the cycling conditions.

3. Results and discussion

3.1. BCL-2 gene transfer enhances migration and invasiveness of LN-18 and LN-229 glioma cells

Polyclonal LN-18 and LN-229 cell lines expressing murine BCL-2 were generated as described [9]. Endogenous BCL-2 levels were very low in LN-18 cells, and strongly elevated by BCL-2 gene transfer in both cell lines (Fig. 1A). There was no significant difference of doubling times or colony formation between BCL-2 and neo transfected cell lines (Fig. 1B). The anti-apoptotic effects of ectopic BCL-2 expression in these human glioma cell lines have been characterized previously [9]. To examine whether BCL-2 is involved in glioma cell migration or invasion, we performed tumor spheroid migration assays, on plastic or collagen IV, a metalloproteinase-2/-9 proteolysis substrate. BCL-2-transfected LN-18 and LN-229 cells showed significantly more migratory activity than neo control cells at 24, 48, 72 and 96 h both in the absence of coating and in the presence of collagen IV (Fig. 1C) ($P<0.05$, *t*-test). There was significantly more migration in LN-229 BCL-2 cells on collagen IV compared with plastic at 24 and 48 h and in LN-18 BCL-2 cells at 72 and 96 h ($P<0.05$, *t*-test). In principle, these differences in migration could be due to expression of different adhesion molecules in the BCL-2-transfected cells. However, cell adhesion assays excluded that enhanced migration was due to modification of cell adhesion to collagen IV or integrin or non-integrin substrates. We assessed the adhesion of the cell lines to different substrates: collagen IV, poly-L-lysine (a non-integrin substrate), and the integrin substrates, vitronectin and fibronectin, in a standard cell adhesion assay [15]. There was no difference between the BCL-2-transfected and the neo cells in any of these assays (data not shown).

The differences in migration on collagen IV could be due to an up-regulation of collagenases in the BCL-2 cells, thus degrading collagen IV and thereby loosening cell-matrix interactions. This could also influence the invasive potential of the glioma cell lines. Therefore, we tested the invasiveness of the transfected cell lines in Matrigel Boyden chamber assays. Interestingly, there was a significant increase in invasiveness in the BCL-2 expressing sublines compared with the neo controls (Fig. 2A). At 48 h, invasion increased from 14 to 36% in LN-18 and from 33 to 68% in LN-229 cells, as assessed by the number of cells in the lower wells (Fig. 2B). A similar effect became apparent when comparing the number of cells adhering to the lower side of the membranes.

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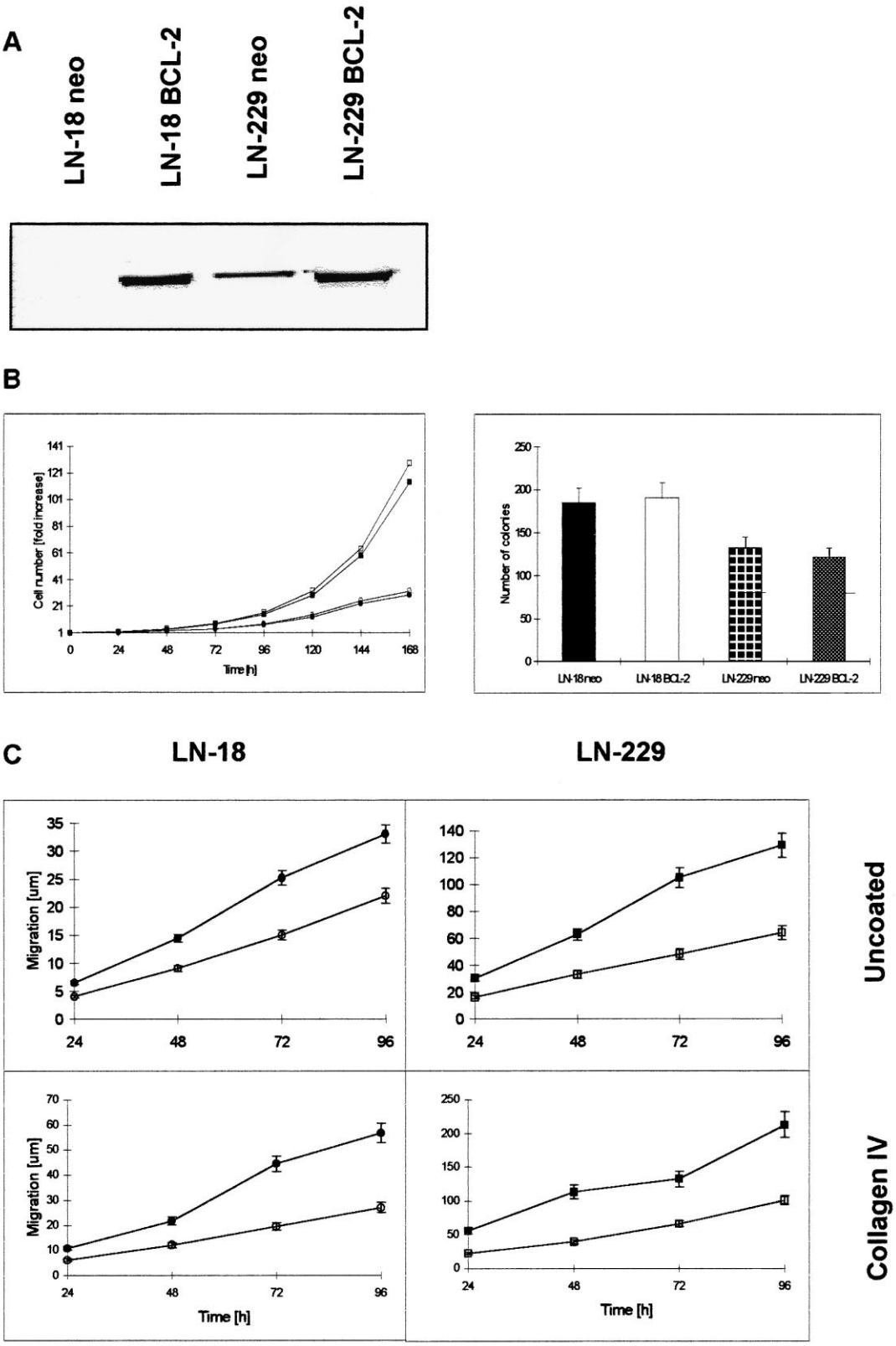
Fig. 1. BCL-2 gene transfer promotes migration of human malignant glioma cells. A: Immunoblot analysis for BCL-2 was performed as described in Section 2. B: 10^3 cells per well were seeded in 96-well plates and growth assessed at 24-h intervals by crystal violet staining (LN-18 neo (open circles), LN-18 BCL-2 (filled circles), LN-229 neo (open squares) and LN-229 BCL-2 (filled squares); $n=3$, S.E.M. $<10\%$). Data are expressed as ratios of optical density units at 570 nm (upper panel). The clonogenicity of LN-18 or LN-229 neo or BCL-2 cells was assessed on six-well plates in 2 ml medium as described in Section 2. Macroscopically visible colonies (0.15 mm) derived from 3×10^3 cells seeded per well were quantified after 17 days (mean S.E.M., $n=3$) (lower panel) ($P>0.05$, *t*-test). C: Tumor cell spheroids derived from LN-18 neo (open circles), LN-18 BCL-2 (filled circles) (LN-18 left side); LN-229 neo (open squares), or LN-229 BCL-2 (filled squares) (LN-229 right side) were prepared and seeded (uncoated well upper panel, collagen IV lower panel) as documented in Section 2. The median distance in μm from the center of the spheroid minus the diameter of the spheroid was measured for 50 exemplary, migrated cells (S.E.M. $<10\%$). Data are expressed as mean of three independent experiments performed in triplicate ($P<0.05$, *t*-test).

3.2. Acceleration of spheroid invasion into rat brain aggregates by BCL-2

To examine the invasiveness of BCL-2 transfected LN-18 and LN-229 glioma cells in a second paradigm that more closely resembles the in vivo situation, we co-cultured tumor spheroids and fetal rat brain aggregates (Fig. 3). Tumor spheroids from LN-18 and LN-229 BCL-2 cells invaded the rat brain aggregates significantly more extensively than neo control cells. The comparative time course of rat-brain aggregate invasion by neo and BCL-2 cells is depicted in Fig. 3C.



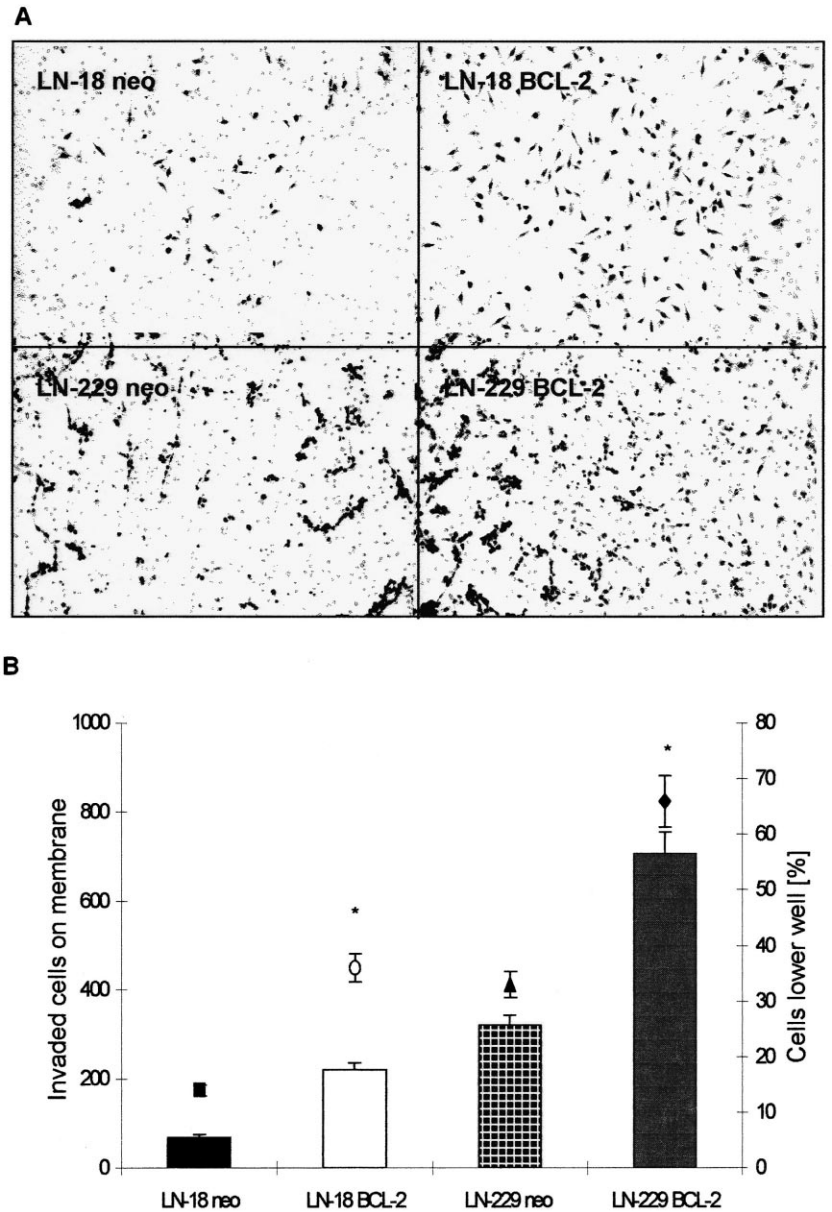


Fig. 2. BCL-2 gene transfer promotes invasion into Matrigel in LN-18 and LN-229 glioma cells. A: Glioma cells invading through Matrigel-coated 8-μm pore size transwell inserts were stained as described in Section 2. B: The number of invading cells was quantified by counting stained cells in random fields of the membrane (bars, left axis) or quantifying cells within the lower well by MTT assay (symbols, right axis). All experiments were performed three times in triplicate and counting was done independently by two investigators. Significance in both assays was calculated using the unpaired *t*-test (*$P < 0.05$, *t*-test).

3.3. Up-regulation of metalloproteinases and u-PA and down-regulation of TIMP-2 in BCL-2 expressing cells

Since the cells expressing BCL-2 were more invasive in Matrigel and co-culture assays and since MMP-2 (72-kDa gelatinase/type IV collagenase = gelatinase A), MMP-3 (stromelysin-1), MMP-9 (92-kDa gelatinase/type IV collagenase = gelatinase B), tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and -2) and the cell surface urokinase-type plasminogen activator (u-PA) may play a vital role in glioma invasion [16], we determined their expression in neo versus BCL-2 transfected cells. RT-PCR was performed for a representative set of proteolytic enzymes: MMP-2, MMP-3, MMP-9, MMP-12, u-PA, TIMP-1, TIMP-2, and for GAPDH as a reference for equal amounts of cDNA template. There were prominent differences between LN-18 and LN-229 cells (Fig. 4A). While LN-18 neo showed strong expression of MMP-2, MMP-9, u-PA, TIMP-1, and TIMP-2, and a weak signal for MMP-3, LN-229 neo exhibited no signal for u-PA and MMP-3. The BCL-2 gene transfer into LN-18 cells resulted in a substantial up-regulation of MMP-3 and induction of MMP-12 mRNA expresssion, and a minor up-regulation of MMP-2 mRNA expression. TIMP-1, TIMP-2 and u-PA mRNA expression were unchanged. LN-229 BCL-2 glioma cells showed up-regulation of MMP-9 mRNA expression, whereas MMP-12 and u-PA were detected only in the transfected cells. The mRNA levels of MMP-2 were unchanged, as were TIMP-1 levels, but TIMP-2 mRNA levels were markedly down-regulated in BCL-2 transfected LN-229 cells. In essence, this might result in increased MMP-2 activity in the LN-229 BCL-2 cells as

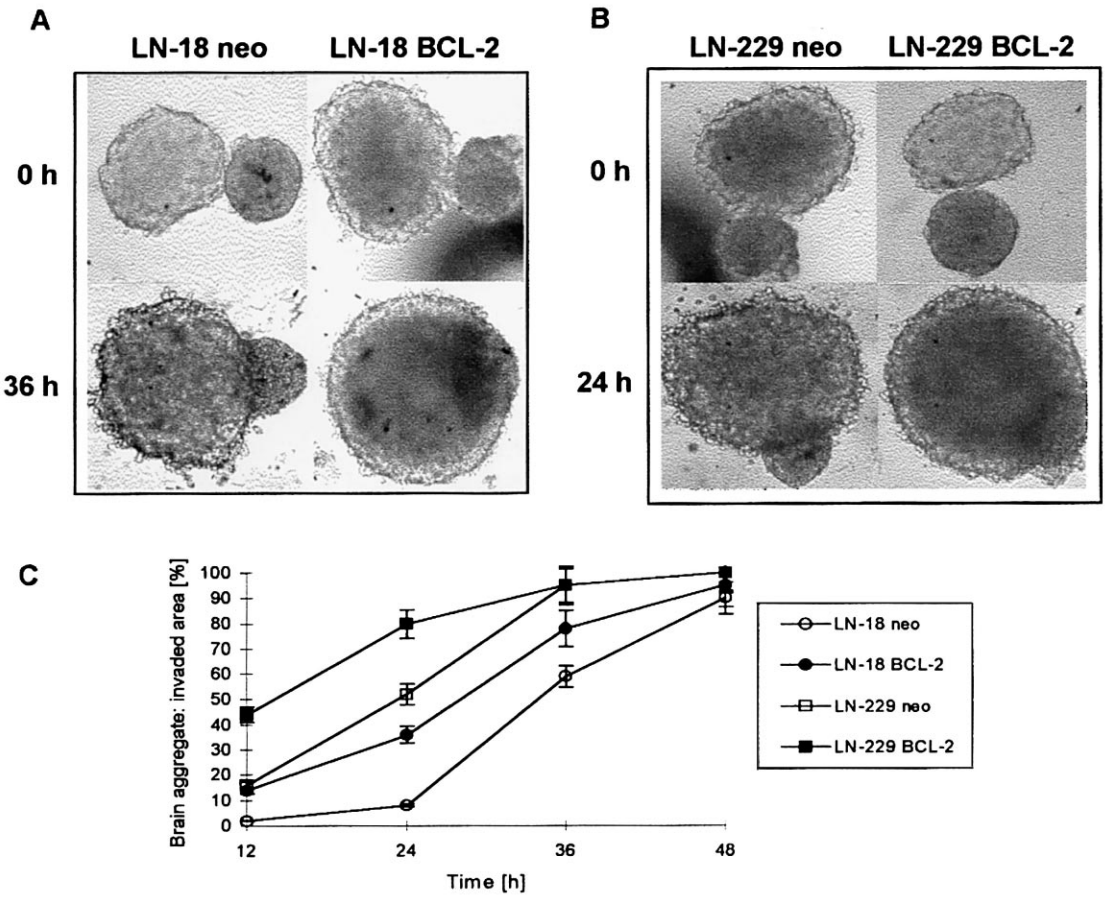


Fig. 3. BCL-2 enhances invasiveness of LN-18 and LN-229 glioma cells into fetal rat-brain aggregates. A, B: The experiments were done as documented in Section 2. The images (A: LN-18 at 0 h and 36 h; B: LN-229 at 0 h and 24 h) show the brain aggregate smaller and the tumor spheroid relatively larger. C: Invasion was determined using a light microscope and an image analysis system. Invasion was measured as percent of the area of the original rat brain aggregate invaded by tumor cells. Experiments were performed three times in triplicate.

described by Deryugina et al. [19]. These results are consistent with enhanced invasiveness of both the LN-18 and LN-229 BCL-2 transfected cells. For LN-18 BCL-2 cells, up-regulation of MMP-3 and MMP-12 and a moderate increase of MMP-2 mRNA expression without other significant changes seems to represent one profile which promotes migration and invasiveness. The results for the LN-229 glioma cells are similar but instead of an increase of MMP-2, MMP-9 is up-regulated, and TIMP-2 down-regulated. Additionally, these cells express no MMP-3, but there is an induction of u-PA expression, the ligand of the u-PA receptor, which is known to enhance invasion in glioblastoma and shedding of metastatic cells in prostate cancer [4], in the BCL-2 cells. The association of de novo MMP-12 expression with a more motile and invasive phenotype is a novel finding.

3.4. *BCL-2-mediated promotion of invasiveness correlates with increase in MMP-2 activity*

Next, we assessed whether BCL-2 promoted changes in metalloproteinase activity as determined by zymography assays. This assay shows the gelatinolytic activity of MMP-2 assessed by degradation of gelatin and maturation of MMP-2 by detection of cleaved forms. The gelatinolytic profile (Fig. 4B) shows that neo transfected LN-18 and LN-229 cells express the 68-kDa proform of MMP-2 in conditioned medium. Both glioma cell lines transfected with BCL-2 express a 62–64-kDa intermediate product, and the LN-18 BCL-2 cell line also shows a weak signal of the mature 59-kDa MMP-2 form, suggesting activation of MMP-2 in the BCL-2-transfected cells. In this regard, Deryugina et al. [17], studying interactions of TIMP-2, membrane type metalloproteinase-1 (MT1-MMP) and $\alpha_v\beta_3$ integrin in the activation of MMP-2, postulated that binding of soluble TIMP-2 to MT1-MMP results in the formation of a catalytic complex to activate pro-MMP-2. For interaction with the ECM, mature MMP-2 probably must bind to $\alpha_v\beta_3$ integrin. The authors concluded that MMP-2 of U251.3 glioma cells is not activated in vitro without exogenous expression of MT1-MMP [17].

In contrast, we observe MMP-2 activation in BCL-2-transfected cells (Fig. 4B), confirming and extending our finding of changes in the expression of MMP-3, MMP-9, MMP-12 and u-PA (Fig. 4A). To confirm the immediate importance of metalloproteinase activity for migration and invasion, we tested glioma cell invasion in the Boyden chamber assay or migration on matrices in the presence of different proteinase inhibitors. The inhibitors were directly added to the medium. Neither aprotinin, a blocker of serine proteinases, nor leupeptin, a blocker of cysteine proteinases, nor a combination of both, had an effect on migration (data not shown) or invasion (Fig. 4C,D). In contrast, 100 μM *O*-phenanthroline, a broad spectrum metalloproteinase inhibitor, strongly blocked migration (data not shown) and invasion of the LN-18 and LN-229

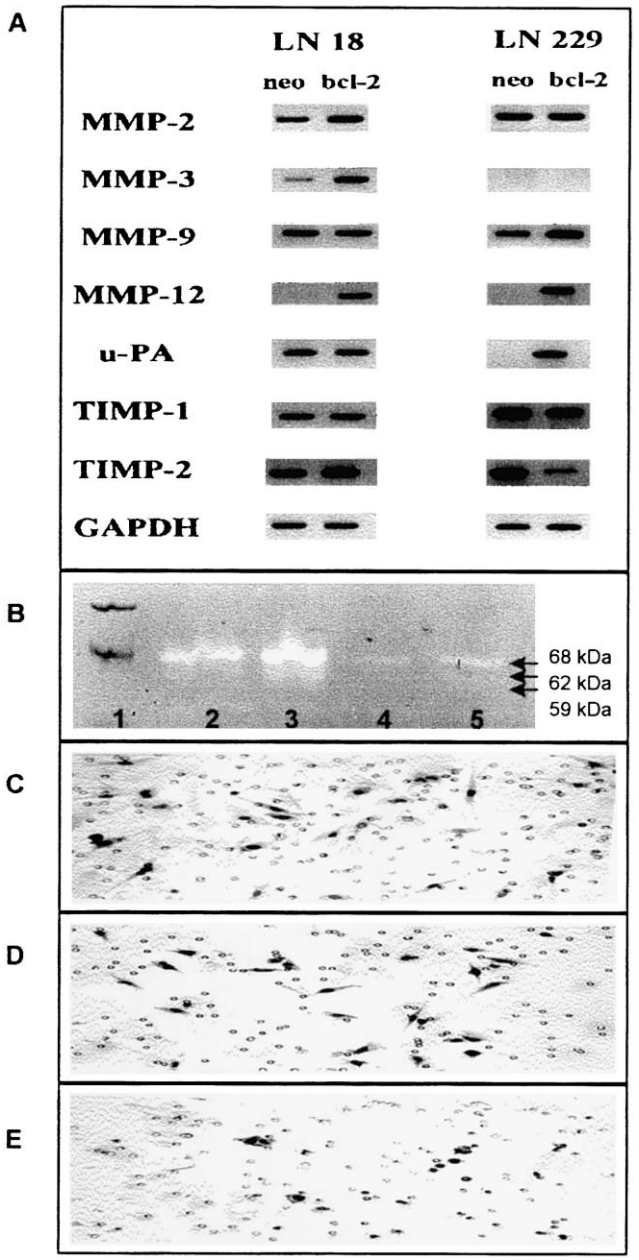


Fig. 4. Invasive profile of BCL-2 transfected cells correlated with metalloproteinase activation. A: Semi-quantitative PCR was performed as described in Section 2 [17]. Photographs of the agarose electrophoreses (LN-18 left panel, LN-229 right panel) are displayed. Analysis was performed semi-quantitatively by assessment of differences of intensity of ethidium bromide-stained bands. B: Gelatin zymography of MMP-2 in conditioned medium of neo and BCL-2 transfected cells was performed as described in Section 2. A 68-kDa band is displayed in LN-229 neo (lane 2), LN-229 BCL-2 (lane 3), LN-18 neo (lane 4), and LN-18 BCL-2 (lane 5) reflecting enzyme activity (lane 1 represents a molecular weight marker). C, D, E: LN-18 BCL-2 cells were examined as in Fig. 2C. The cells were untreated (C) or exposed to inhibitors of serine (3 μM aprotinin) and cysteine (10 μM leupeptin) proteinases (D) or 100 μM *O*-phenanthroline (E), a specific metalloproteinase inhibitor.

BCL-2 glioma cells by more than 75% (Fig. 4E). In parallel experiments, we could inhibit invasion of the neo cell lines in a similar fashion.

The present study provides evidence for a novel role for BCL-2 in maintaining a neoplastic phenotype in human glioma cells. Importantly, it remains unclear whether the promotion of migration and invasiveness is completely independent of the anti-apoptotic properties of BCL-2. The anti-apoptotic properties of BCL-2 in the same cell lines have previously been documented by protection from CD95-mediated, drug- and irradiation-induced apoptosis [9]. The specific changes in molecules involved in invasion (Fig. 4) lead us to propose that BCL-2 has specific effects on the molecules relevant for migration and invasion that warrant further investigation in an in vivo paradigm and might represent a novel target for cancer therapy.

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