Antisense inhibition of laminin-8 expression reduces invasion of human gliomas *in vitro*

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Abstract

Using gene array technology, we recently observed for the first time an up-regulation of laminin α4 chain in human gliomas. The data were validated by semiquantitative reverse transcription-PCR for RNA expression and immunohistochemistry for protein expression. Moreover, increase of the α4 chain-containing laminin-8 correlated with poor prognosis for patients with brain gliomas. Therefore, we hypothesized that inhibition of laminin-8 expression by a new generation of highly specific and stable antisense oligonucleotides (Morpholino) against chains of laminin-8 could slow or stop the spread of glioma and its recurrence and thus might be a promising approach for glioma therapy. We next sought to establish an *in vitro* model to test the feasibility of this approach and to optimize conditions for Morpholino treatment. To develop a model, we used human glioblastoma multiforme cell lines M059K and U-87MG cocultured with normal human brain microvascular endothelial cells (HBMVEC). Using Western blot analysis and immunohistochemistry, we confirmed that antisense treatment effectively blocked laminin-8 protein synthesis. Antisense oligonucleotides against both α4 and β1 chains of laminin-8 were able to block significantly the invasion of cocultures through Matrigel. On average, the invasion was blocked by 62% in cocultures of U-87MG with HBMVEC and by 53% in cocultures of M059K with HBMVEC. The results show that laminin-8 may contribute to glioma progression and recurrence not only as part of the neovascularization process but also by directly increasing the invasive potential of tumor cells. (Mol Cancer Ther. 2003;2:985–994)

Introduction

Glial tumors are the leading cause of cancer death in children.[1] Overall, they account for 1.4% of all cancers and 2.4% of all cancer deaths. Average survival time for low-grade astrocytoma or oligodendroglioma patients is 6–8 years. It decreases to 3 years for patients with anaplastic astrocytoma and drops to 12–18 months for glioblastoma multiforme (GBM). Currently, these tumors are treated by surgical removal, radiation therapy, chemotherapy, or their combinations. Most GBMs are highly invasive and rapidly develop recurrences at the primary site. Tumor prognoses and responses to therapy can vary greatly even with the same histological diagnosis (1). It is generally recognized that the improvement of prognosis, prediction of response to treatment, and development of novel effective therapeutic approaches for glial tumors may largely depend on the introduction into clinical practice of novel specific markers involved in the development of different gliomas and their subsequent recurrences.

Attempts have been made to establish and characterize a number of glioma markers, such as glial fibrillary acidic protein, vimentin, synaptophysin, and nestin. Determination of differential expression of these markers (immunophenotyping) in gliomas, however, has not altered existing therapeutic approaches, treatment success rates, or disease outcome prediction (1, 2). Researchers then sought to identify novel glioma markers using powerful gene array technology (3–6). Recently, our group described a new molecular marker of glial tumors, laminin-8, which was differentially expressed in malignant tumors compared with benign tumors and normal brain tissues (4).

All laminins consist of three covalently linked chains, α, β, and γ. To date, 15 members of this family (isoforms) that are present in different basement membranes (BMs) and that may serve different functions (7–9) have been described. Laminins interact with cells through various receptors. Most of these receptors belong to the family of integrin heterodimers, although other molecules including dystroglycan complex and Lutheran blood group glycoprotein were also shown to bind to laminins. In different

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[1] American Cancer Society, Brain and Spinal Cord Tumors in Adults (http://www.cancer.org/eprise/main/docroot/CRI/content/CRI_2_4_1X_What_are_the_key_statistics_for_brain_and_spinal_cord_tumors_3?sitearea=CRI).

cell types, integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, and $\alpha_7\beta_1$ have been reported to have the capability to bind to laminins. Specific laminin isoforms bind some but not all of these different integrins, and each integrin can bind to more than one laminin isoform (9, 10).

Along with type IV collagens, nidogens, and perlecan, glycoproteins of the laminin family are the major constituents of brain microvessel BMs (7, 11, 12). These BMs have a complex structure and are produced by both endothelial and glial cells (12). Endothelial cells contribute laminins containing α4 and α5 chains to these BMs, whereas glial cells synthesize laminins containing α1 and α2 chains (12). In human brain capillary BMs, we have recently observed a weak expression of the α4 chain-containing laminin-9. Interestingly, during progression of human gliomas, the expression of capillary BM laminins containing α4 chain switches from the predominant laminin-9 (α4β2γ1) to laminin-8 (α4β1γ1; 4). Laminin-8 and its receptors, integrins $\alpha_3\beta_1$ and $\alpha_6\beta_1$, appear to be important to the functioning of endothelial cell BMs, which play a role in the maintenance of the blood-brain barrier (13, 14). Recently, the association of laminin α4 chain with angiogenesis has been demonstrated *in vivo* and *in vitro* (15). Some cultured glioma cell lines can also produce α4-containing laminins. Laminin-8 is thought to play a role in cell migration during development, wound healing, and angiogenesis (7, 9, 13).

Because laminin-8 appears to be associated with GBM recurrence *in vivo*, we hypothesized that it might play a role in tumor invasion. We explored this possibility *in vitro* using single cultures and cocultures of brain microvascular endothelial cells, normal fetal brain astrocytes, and several GBMs. We sought to analyze whether the patterns of laminin chain expression in cell culture would be similar to those seen in normal brain and in gliomas and whether inhibition of laminin-8 expression by an antisense approach would alter glioma invasiveness through a reconstituted BM (Matrigel).

Antisense oligonucleotides (oligos) that bind and inactivate specific RNA sequences may be the best tools for studying gene function, regulation of gene expression, and interactions between gene products. Highly specific antisense oligos that mimic the DNA template for RNA production are used to bind to the cRNA and to prevent protein translation (16, 17). Antisense oligos are the fastest, simplest, and most cost-effective tools for testing new therapeutic targets for drug development. The antisense approach was used in our present study to inhibit the expression of laminin-8 in cell culture.

Our results show that normal cultured astrocytes and endothelial cells mostly express laminin-9 as seen in normal brain tissue. Glioma cells predominantly express laminin-8, again similar to the *in vivo* situation. Most importantly, antisense blocking of laminin-8 chain expression resulted in the inhibition of glioma invasion through Matrigel. These data show that laminin-8 may be important for glioma invasion and could be a potential target for antitumor therapy.

Materials and Methods

Coculture of Gliomas, Astrocytes, and Brain Endothelial Cell Lines

Two types of human GBM cell lines (M059K and U-87MG; American Type Culture Collection, Rockville, MD), a normal human brain microvascular endothelial cell line (HBMVEC; obtained from Dr. Ken Samoto, Japan) and normal human fetal brain astrocytes HAST 040 (Clonexpress, Inc., Gaithersburg, MD), were used. M059K cell line was maintained in DMEM/F-12 medium, FCS, supplements, and antibiotics as below. U-87MG cells were cultured in Eagle's MEM with 10% FCS, L-glutamine, sodium bicarbonate, nonessential amino acids, antibiotics, and sodium pyruvate. The HAST 040 cell line was cultured in 50:50 DMEM/F-12 supplemented with 5% FCS and antibiotics (25 μg/ml of gentamicin and 2.5 μg/ml of fungizone) during regular maintenance of astrocytes. The medium was replaced with fresh medium every third day to maintain optimal growth. HBMVEC cells were cultured in RPMI 1640 with 10% FCS, 10% NU serum, sodium pyruvate, L-glutamine, nonessential amino acids, and antibiotics. Cell lines were maintained at 37°C in a humidified 5% CO_2 incubator and subcultured with trypsin-EDTA every 3–4 days. Cell lines were cocultured at a glioma/endothelium ratio of 5:1 in four-well chambers and examined at different time points (24 h, 3 days, and 5 days). Cocultures of normal human astrocytes HAST 040 and HBMVEC cells were cultured at the same ratio of 5:1 in four-well chambers and examined at different time points (24 h, 3 days, and 5 days).

Antisense Treatment of Glioma–Endothelial Cocultures

Morpholino (phosphorodiamidate morpholino oligomer) oligos custom made by Gene Tools, Inc. (St. Louis, MO) for laminin α4 and β1 chains were as follows:

α4 antisense 5′AGCTCAAAGCCATTTCTCCGCTGAC 3′,
α4 sense 5′GTCAGCGGAGAAATGGCTTTGAGCT 3′,
β1 antisense 5′CTAGCAACTGGAGAAGCCCCATGCC 3′,
and β1 sense 5′GGCATGGGGCTTCTCCAGTTGCTAG 3′.

Gene Tools protocol was used according to the company's recommendations. The new special delivery formulation consisted of a prepaired duplex of Morpholino oligo and partially complimentary DNA oligo together with a weakly basic delivery reagent, ethoxylated polyethylenimine. Morpholino oligos are stable and totally nuclease resistant so there is no need for redelivery. Cocultures of glioma cells with normal brain endothelium were treated with antisense oligos to laminin-8 α4 and β1 chains for selected time intervals (3 and 6 days), alone or in combination. To make the delivery mixture, 0.5-mM antisense laminin α4 or β1 chain or 0.5-mM sense oligos (negative control) and Morpholino/DNA stock solution (Gene Tools) were added to H_2O and mixed. Two-hundred-micromolar ethoxylated polyethylenimine special delivery solution was added, vortexed, and incubated at room temperature for 20 min to generate the complete

delivery solution. Medium was removed from a 24-h coculture and the solution with a specific oligo was added to cells, and placed into a CO_2 incubator. After 3 h, the delivery solution was aspirated and replaced with fresh serum-containing medium. Medium was changed every 2 days. Each oligo was assessed at four incubation time points: 2, 4, 6, and 8 days (coculture time being 3, 5, 7, and 9 days, respectively). Another set of controls included endothelial or glioma cells alone.

Immunohistochemistry

Cells were incubated in culture with or without Morpholino and at selected times were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and immunostained for laminin chains and endothelial cell markers. These markers included von Willebrand factor (Sigma Chemical Co., St. Louis, MO), CD31 (clone HC1/6, Cymbus Biotechnology/Chemicon International, Temecula, CA, and clone JC70A, DAKO Corp., Carpinteria, CA), CD34 (clone QBEnd 10, DAKO), and CD105 (clone P3D1, Chemicon). Uptake of Alexa Fluor 488-labeled acetylated low-density lipoprotein (Ac-LDL; Molecular Probes, Eugene, OR) was also used to identify endothelial cells. Briefly, cells were incubated for 24 h in medium with 5-μg/ml labeled Ac-LDL, washed, fixed, and permeabilized. Cells were then counterstained with 10-ng/ml 4′, 6-diamidino-2-phenylindole (DAPI; Sigma) to visualize nuclei and additionally immunostained for selected laminin chains. Primary monoclonal antibodies (mAb) and polyclonal antibody were used to the laminin α4 chain [mAb FC10 (18) and pAb 377 (4)], laminin β1 chain (mAb LT3; Upstate Biotechnology, Inc., Lake Placid, NY), and laminin β2 chain (mAb C4 obtained from the Developmental Studies Hybridoma Bank, Department of Biology, University of Iowa, Iowa City, IA).

Western Blot Analysis

Serum-free conditioned medium was obtained from the same number of cells in the same volume of medium from the cocultures that were cultured for the same periods. Conditioned media from cocultures were concentrated 10-fold by filtering through Centriplus filtration devices (Millipore, Bedford, MA) and proteins were separated using 3–8% gradient Tris-acetate SDS-PAGE (Invitrogen, Carlsbad, CA) under reducing conditions. Lysates of human glioma T98G, known to express laminin-8 (14), were used as a positive control. The gels were blotted onto nitrocellulose membrane (Invitrogen). The membranes were probed with mAbs followed by chemiluminescent detection using the enhanced chemiluminescence kit with alkaline phosphatase-conjugated secondary antibodies (Amersham, Piscataway, NJ). Antibodies were used to the laminin α4 chain [mAb 8B12 (14)] and β1 chain (mAb LT3). Antibody to fibronectin eighth type 3 repeat [mAb 568 (19)] was used to control for equal loading of gel lanes.

Cell Viability Assay

Cell numbers were measured with the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI). It was designed for the determination of the number of viable cells using MTS dye [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt]. According to the manufacturer's instructions, a small amount of the CellTiter 96 AQueous One Solution Reagent was added directly to culture wells, and after 3 h of incubation, the absorbance at 490 nm was recorded using an ELISA reader (Spectra Max Plus 384, Molecular Devices, Sunnyvale, CA). The quantity of formazan product as measured by the amount of 490-nm absorbance is directly proportional to the number of living cells in culture. All cell lines were treated exactly as described above in ''Antisense Treatment of Glioma-Endothelial Cocultures.'' For viability assay, cells were incubated after treatment with Morpholino sense and antisense oligos and/or delivery factor for 3 days, the average time point that was used in our experiments. Each experiment was performed in triplicate and was repeated twice.

In Vitro Invasion Assay

Invasion studies were conducted using the Matrigel BM matrix assay developed for quantitative measurement of tumor cell invasiveness. Most tested cells characterized as invasive and metastatic *in vivo* are able to invade Matrigel *in vitro* (20–22). We used BioCoat Matrigel invasion chambers (12-well cell culture inserts containing an 8.0-μm PET membrane with a uniform layer of Matrigel [Becton Dickinson, Bedford, MA]). The coated filters were rehydrated with warm serum-free DMEM (2 ml/chamber). The upper chamber was filled with 2.5×10^4 cells in serum-free medium. The lower chamber was filled with DMEM containing 5% FCS as a chemoattractant toward which the cells migrate. The chambers were incubated for 22 h at 37°C in a 5% CO_2 atmosphere. Cells from the upper surface of the filters were removed by scrubbing with a cotton swab and those migrating to the lower surface of the filters were fixed and stained with H&E. The number of cells that penetrated the filter was counted in 10 microscopic fields of each filter under 200× magnification in both experimental and special control membranes using a Zeiss Axiophot microscope connected to an image processing and measuring system (Hamamatsu, Japan). Percent invasion is expressed as mean cell number from invasion chamber to mean cell number from control chamber according to the manufacturer's recommendation. Assays were carried out in triplicates. Four independent experiments were performed for each type of coculture with each treatment.

Statistical Analysis

The data from the cell viability assay and invasion experiments were statistically evaluated by ANOVA test using GraphPad Prism 3 software program (GraphPad Software, San Diego, CA). $P < 0.05$ was considered significant.

Results

Immunohistochemistry of Endothelial Markers and Laminin Chain Expression in Untreated Cultures

Several endothelial markers were tested to select the best that might be used to reliably differentiate endothelial cells from normal and malignant astrocytes in cocultures. In

preliminary experiments, fluorescent Ac-LDL consistently labeled endothelial cells (23) much more uniformly than did antibodies against von Willebrand factor, CD31, CD34, or CD105.

Uptake of fluorescent Ac-LDL was, therefore, used to identify endothelial cells in subsequent experiments with cocultures. In pure endothelial cultures, most if not all cells displayed predominantly punctate fluorescence with a perinuclear distribution (Fig. 1). Cultures of normal astrocytes and glioma cell lines were largely negative (Fig. 1), although some cells showed low background fluorescence. Ac-LDL uptake allowed identifying positive endothelial cells in cocultures as well (Fig. 1).

Cultures were then immunostained for chains of laminin-8 and laminin-9. In accordance with the *in vivo* situation, cultured normal endothelial cells stained positive for α4 and β2 chains, compatible with the presence of laminin-9 (Fig. 2A). At the same time, staining for laminin-8 β1 chain was mostly negative (Fig. 2A). Normal fetal astrocytes did not appreciably stain for any tested laminin chain (Fig. 2A). In contrast, glioma U-87MG (data not shown) and M059K cells were positive for laminin-8 α4 and β1 chains but largely negative for laminin-9 β2 chain (Fig. 2A). These results were fully confirmed by Western blot analysis of conditioned media from cultures with equal protein loading (Fig. 2B).

In cocultures of normal astrocytes and HBMVEC, mostly α4 and β2 chains could be seen, with very little β1 chain expression (Fig. 3). However, in cocultures of glioma cells with HBMVEC, α4 and β1 chains were predominantly expressed (Fig. 3). An important finding was that HBMVEC, when cocultured with malignant astrocytes, started expressing laminin β1 chain in contrast with its absence in endothelial cells alone or in coculture with normal astrocytes (Fig. 3).

These data show that cocultures of normal astrocytes and endothelial cells mostly expressed laminin-9 in accordance with our previous *in vivo* results (4). Furthermore, similar to the *in vivo* situation, glioma cells alone and in cocultures with endothelial cells mostly expressed laminin-8. Therefore, the established coculture system resembled the situation *in vivo* in both a normal and a tumor brain environment. The laminin expression data thus strongly suggest that glioma-endothelial cocultures may be a valid model to study further the inhibition of expression of laminin-8, a new glioma marker associated with tumor progression and recurrence development.

Cell Viability Assay

To test the potential toxicity of sense and antisense Morpholino oligos and the delivery factor ethoxylated polyethylenimine, cell viability was measured using MTS-based CellTiter 96 assay. The relative numbers of viable cells of three cell lines U-87MG, M059K, and HBMVEC, which had been treated with oligos and/or delivery factor, were compared with cell numbers of replicate cultures of corresponding cell lines without any treatment (taken as 100%). Cell viability for each cell line after oligo treatment in two separate experiments was higher than 90% (Fig. 4). This did not differ significantly from untreated controls ($P > 0.05$). Based on these data, we concluded that Morpholino oligos and/or delivery factor did not exert any significant toxic effect on any of the used cell lines.

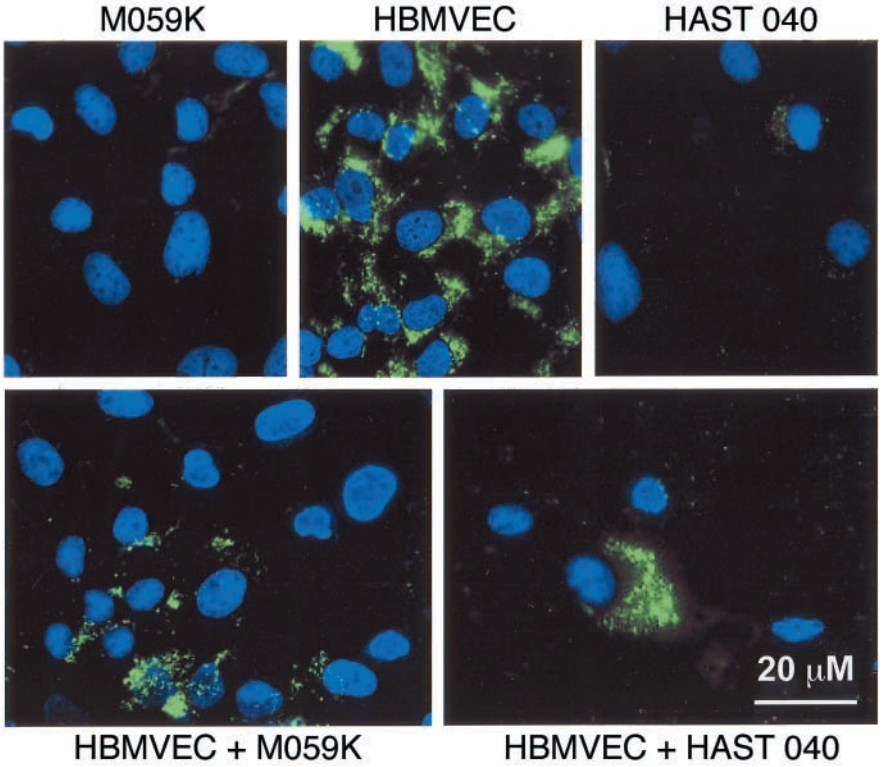

Figure 1. Uptake of Ac-LDL by various cultures and cocultures. Endothelial cells (*HBMVEC*) are positive (*green fluorescence*) but glioma cells (*M059K*) and normal astrocytes (*HAST 040*) are negative. In cocultures HBMVEC + M059K and HBMVEC + HAST 040, endothelial cells are positive, whereas other cells are negative. DAPI was used to counterstain cell nuclei (*blue fluorescence*).

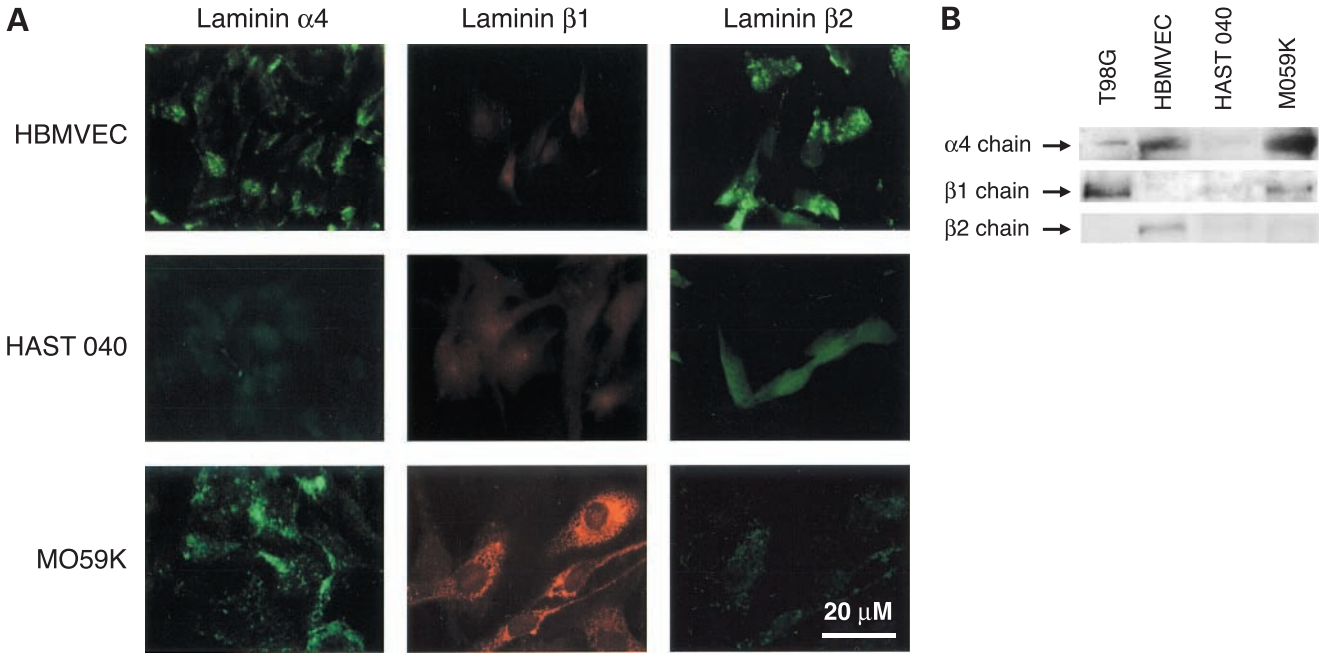

Figure 2. Laminin α4, β1, and β2 chain expression in cells and conditioned media of pure cultures. **A,** immunolocalization of laminin chains in cells. Normal brain endothelium (*HBMVEC*) expresses α4 and β2 chains (consistent with laminin-9, α4β2γ1), whereas astrocytes (*HAST 040*) do not express these laminin chains. M059K glioma cells, however, express α4 and β1 chains, consistent with laminin-8 (α4β1γ1). Indirect immunofluorescence. **B,** Western blot analysis of conditioned media. Endothelial cells (*HBMVEC*) secrete chains of laminin-9 (*α4* and *β2*), astrocytes (*HAST 040*) show little to no secretion of any studied chains, and M059K glioma cells secrete chains of laminin-8 (*α4* and *β1*). *T98G,* lysate of T98G glioma cells expressing laminin-8 chains only (*α4* and *β1*), was used as positive control. Equal amounts of conditioned media protein were applied to each lane. Note complete agreement between the results of immunostaining (**A**) and Western blotting (**B**).

Immunohistochemistry of Laminin Chain Expression in Antisense-Treated Cultures

Because glioma-endothelial cocultures mostly expressed laminin-8 α4 and β1 chains (but not laminin-9 β2 chain), antisense oligos were used only to block laminin-8 expression. Treatment with α4 antisense resulted in markedly decreased staining for this chain and a reduction of staining for the β1 chain (Fig. 5). The same result was seen with β1 antisense treatment, compatible with the role of this chain in laminin trimer assembly. A combination of the two oligos dramatically reduced staining for α4 and β1 chains at all time points.

Western Blot Analysis of Pure Cultures and Cocultures

In lysates of cultures and cocultured cells, the signals for laminin α4 and β1 chains were very weak and detectable only on days 5–7 of culture or coculture (data not shown). Therefore, the amounts of these chains were further analyzed in conditioned media after their substantial and equal fold concentration and normalization by total protein and fibronectin content.

As shown in Fig. 6, both α4 and β1 chains could be detected in sense-treated cultures at days 3–6 as well as in a positive control [T98G glioma cell lysate (14)]. Antisense treatment to either chain resulted in a decreased signal for both chains. Again, maximum inhibition for both chains was achieved by a combined α4 + β1 antisense treatment in a concentration of 0.25 mM for each oligo. These results were in complete agreement with cell immunostaining data.

Matrigel Invasion Assay

Matrigel invasion assay was used to study the influence of antisense oligos to laminin-8 α4 and β1 chains on the invasive parameters of cocultures. Corresponding sense oligos were used in control chambers. Another set of controls included endothelial or glioma cells alone.

Two glioma cell lines, U-87MG and M059K, alone had 91% and 76%, respectively, of invasion potential with or without treatment with either single or combined sense oligos against α4 and β1 chains. HBMVEC cells demonstrated only 11% invasion. Each experiment was repeated thrice in triplicate.

In the next set of experiments, cocultures of glioma and endothelial cells were treated for 3 days with α4 and β1 antisense oligos, alone or in combination. Each antisense used in this study significantly inhibited invasion of two different coculture types (Fig. 7). In this study, 842 microscopic fields with a total of 64,276 cells were evaluated. Specific endothelial staining has demonstrated that both endothelial and glioma cells migrated through Matrigel, with clear prevalence of glioma cells (data not shown). Cocultures treated with sense oligos to the laminin-8 α4 and β1 chains were considered as controls equal to 100%. When cocultures were treated with α4 antisense oligo, invasion was blocked by 40% for U-87MG (Fig. 7, right; $P < 0.02$ *versus* control) and by 41% for M059K (Fig. 7, left; $P < 0.03$) cell lines compared with cultures treated with sense oligos (taken as 100%). β1 antisense oligo also blocked the invasion by 40% for U-87MG ($P < 0.04$)

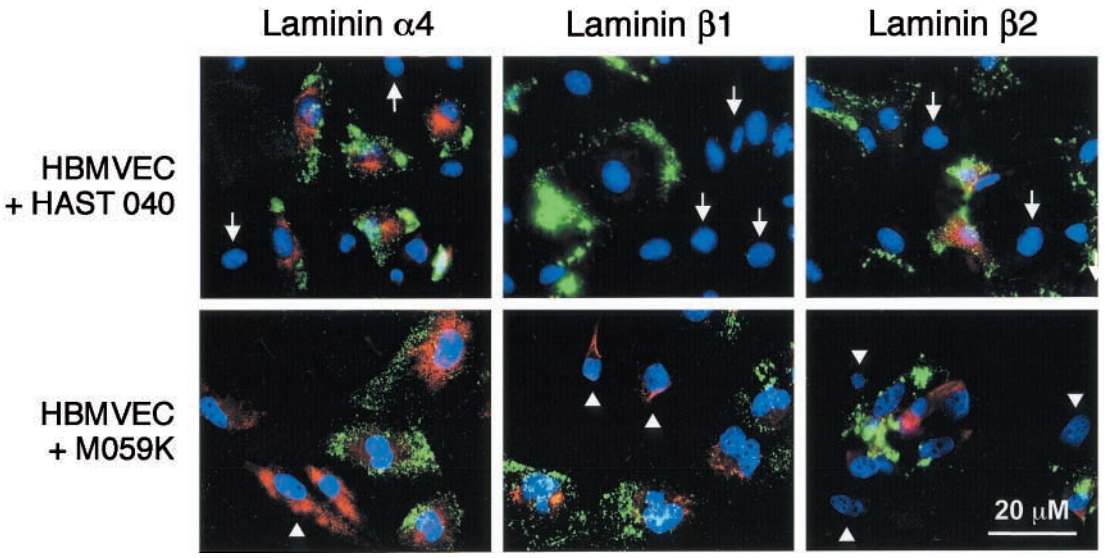

Figure 3. Laminin α4, β1, and β2 chain staining of cocultures. Live cocultures were exposed to Ac-LDL (*green color*) to reveal endothelial cells and then fixed and simultaneously stained for select laminin chains (*red color*) and nuclei (DAPI; *blue color*). In endothelial-astrocyte cocultures (*HBMVEC + HAST 040*), α4 and β2 chains are expressed in Ac-LDL-positive endothelial cells only but not in Ac-LDL-negative astrocytes (*arrows*). β1 chain is largely absent. In endothelial-glioma cocultures (*HBMVEC + M059K*), α4 chain is expressed by both cell types and β2 chain is expressed only by endothelial cells. Importantly, β1 chain is now expressed not only by Ac-LDL-negative glioma cells (*arrowheads*) but also by Ac-LDL-positive endothelial cells.

and by about 47% for M059K ($P < 0.001$) cocultures. When cocultures were treated with both antisense oligos against α4 and β1 chains, invasion was reduced on average by 62% for U-87MG ($P = 0.0005$) and by 53% for M059K ($P < 0.0001$) cocultures. In two of five experiments, the inhibition exceeded 75% (data not shown).

A combination of α4 + β1 antisense was more efficient at blocking laminin expression than α4 or β1 antisense in U-87MG cells and almost equal to β1 antisense in M059K cells. Interestingly, α4 and β1 chain expression was inhibited more efficiently with lower concentrations of antisense oligos (0.25 + 0.25 mM) than with higher ones (0.5 + 0.5 mM). This result calls for careful optimization of Morpholino oligo concentrations for future *in vitro* and *in vivo* studies.

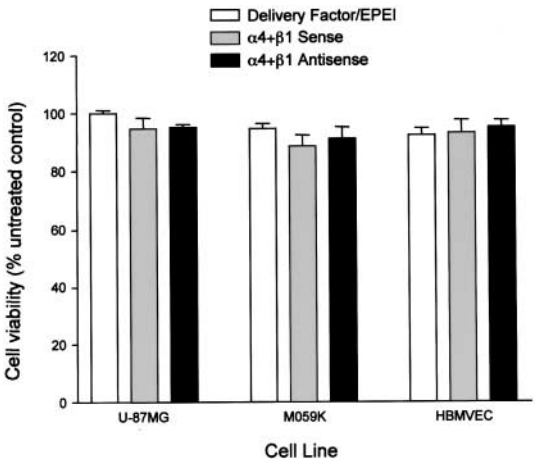

Figure 4. Cell viability assay. Viability of glioma cell lines M059K and U-87MG as well as of normal endothelial cell line HBMVEC after treatment with Morpholino sense or antisense oligos and delivery factor is higher than 90%. No significant difference from parallel untreated control cultures was detected with any treatment. Cell viability without treatment was taken as 100%. Cell numbers were determined using MTS assay.

Discussion

This is the first study to examine the role of laminin-8 in human tumor cell invasion using antisense inhibitors that block synthesis of this complex trimeric protein. We showed that normal brain endothelial cells expressed small amounts of laminin-9 α4 and β2 chains. The expression of laminin-8 β1 chain, however, was not detected. Normal astrocytes did not express any of these chains. This *in vitro* system is similar to *in vivo* normal brain, where there was a low expression of predominantly laminin-9 (4). At the same time, glioma cells expressed chains of laminin-8 in culture in accordance with our previous *in vivo* data (4). Moreover, in cocultures with glioma cells, brain endothelial cells also started expressing laminin β1 chain (compatible with laminin-8 production) in agreement with the finding of laminin-8 overexpression in GBM *in vivo* (Fig. 3).

These data clearly show that normal and tumor *in vivo* patterns of α4 chain-containing laminin isoform expression were retained in the culture setting. Therefore, we were able to validate the respective cocultures for the patterns of laminin chain expression as a system similar to that observed *in vivo*, both in normal brain tissue and during glioma growth. This system thus could be used to study further the role of laminin-8 in glioma behavior and, especially, in tumor invasiveness. In combination with several new well-characterized proteins associated with glioma progression, such as tenascin-C, matrix metalloproteinase (MMP)-2, and MMP-9 (4, 11, 24–28), laminin-8

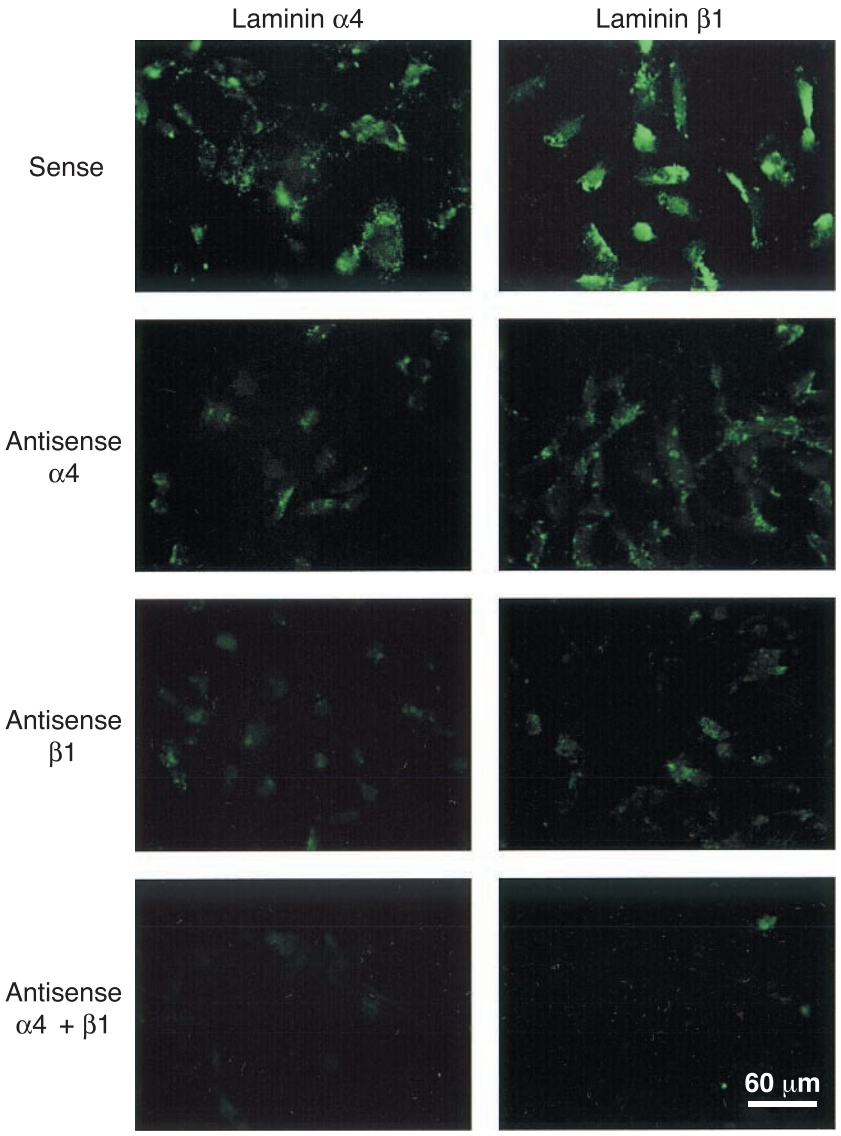

Figure 5. Laminin α4 and β1 staining of antisense-treated cocultures. When cocultures of M059K or U-87MG with HBMVEC are treated with sense oligos to laminin α4 and β1 chains for 5 days, the patterns of laminin chain expression are similar to untreated cultures (*upper row*; compare Fig. 3). Treatment with antisense oligos to either laminin α4 (*antisense α4*) or laminin β1 (*antisense β1*) chain partially inhibits both α4 and β1 chain expression (*middle rows*). Treatment with antisense oligos for both chains (*antisense α4 + β1*) abolishes staining (*lower row*). Indirect immunofluorescence.

may be an important tool for potential diagnosis or treatment of gliomas. Previously, only laminin-5 was shown to play a role in melanoma invasion (29). Our present data suggest that ''vascular'' laminin-8 also plays a significant role in glioma cell invasiveness. Because matrix-degrading proteinases are also important for glioma invasion (30), future research should explore whether proteolysis of laminin is required for glioma invasion.

To probe the role of laminin-8 in glioma invasion, we attempted to use antisense oligos to block its expression. The potential of antisense is widely recognized, but it remained largely unfulfilled since, until recently, the available oligos suffered from poor specificity, instability, and undesirable non-antisense effects (31, 32). These problems have been largely solved by the new generation of antisense oligos that offer the promise of safe and effective therapeutics for various diseases including cancer (32, 33). The most promising types of oligos are Morpholino and peptide nucleic acid (they have nucleobases attached to a neutral ''peptide-like'' backbone) oligos (31, 33). Morpholino oligos function independently of RNase H and are soluble in aqueous solutions. They work well in the presence or absence of serum, are totally resistant to nucleases, and remain intact in culture medium and in cells indefinitely. Morpholino oligos have a high affinity for RNA and efficiently invade even quite stable secondary structures in mRNAs. They have the highest sequence specificity of all antisense types over a very broad concentration range and appear to be free of non-antisense effects (33, 34). They have high activity in a cell-free translation system and can block target protein production in cultured cells (35). Morpholino are also effective *in vivo* (36). Given these properties, Morpholino oligos have been chosen here to inhibit the expression of laminin-8 chains in culture. Special experiments have shown that Morpholino treatment did not affect the viability of any cell line used.

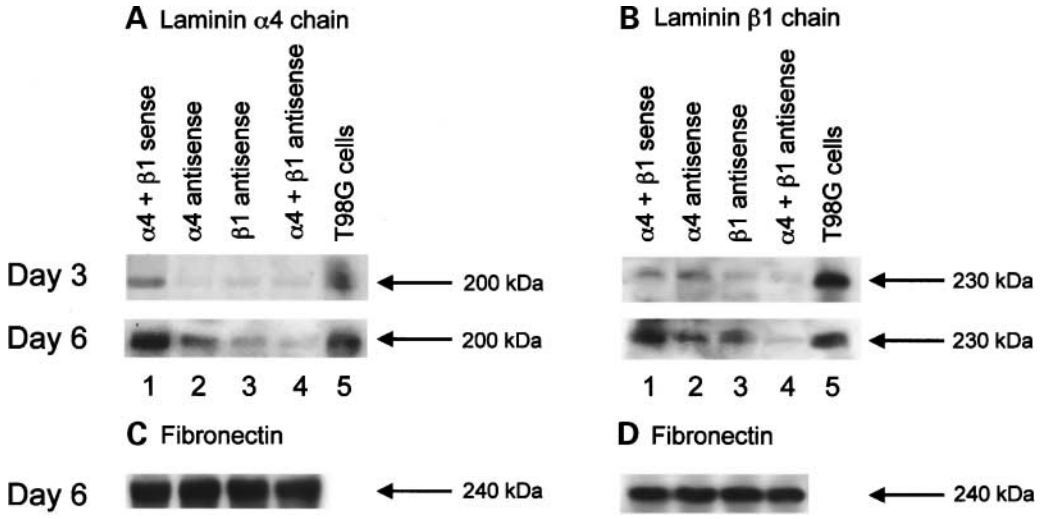

Figure 6. Western blot analysis of laminin-8 α4 and β1 chains in conditioned media of cocultured M059K and HBMVEC cells. Incubation with Morpholino sense and antisense oligos was for 3 or 6 days. **A,** a 200-kDa band corresponding to laminin α4 chain in coculture on days 3 and 6. The amount of immunoreactive laminin α4 was diminished by antisense oligos to either α4 or β1 or, especially, α4 + β1. **B,** a 230-kDa band corresponding to laminin β1 chain in cocultures on days 3 and 6. The combination of antisense oligos (α4 + β1) was also most efficient in decreasing the amount of immunoreactive β1 chain band at both time points. **C** and **D,** Western blots of fibronectin (240-kDa band) on day 6 after stripping the respective membranes from α4 and β1 chain detection and reprobing them for fibronectin. These lanes are shown for loading control purpose. Only human (but not serum) fibronectin was detected by this antibody. *Lane 1,* sense oligos for α4 + β1 chains; *lane 2,* antisense oligo for α4 chain; *lane 3,* antisense oligo for β1 chain; *lane 4,* antisense oligos for α4 + β1 chains. T98G, cell lysate of a laminin-8 expressing GBM cell line T98G, was used as positive control. Very similar results were obtained using coculture of HBMVEC with cells of another glioma line, U-87MG (data not shown).

Recently, promising data on the use of antisense technology in glioma cells were obtained. The blocking of MMP-9 reduced the invasiveness of glioma cells *in vitro* (30, 37). Glioma growth *in vitro* and *in vivo* (as xenotransplants in nude mice) could be inhibited by antisense to telomerase (38). A recent pilot study showed that antisense to the insulin-like growth factor-I receptor induced glioma cell apoptosis and resulted in clinical improvement in patients (39). Several clinical trials are currently using antisense oligos for the treatment of other cancers (40).

To examine the involvement of laminin-8 in glioma invasion, we needed reliable systems where it was possible to quantify invasion rates and to optimize the dosage of antisense laminin oligos. We used a cell culture system to meet these important needs. One could potentially use glioma cultures. To better mimic the *in vivo* situation, however, and because laminin-8 seems to be produced by both glioma and endothelial cells (14), we needed to combine glioma cells with brain endothelium in a coculture (43). In such a situation, endothelial cells can develop capillary-like structures, and this process is faster

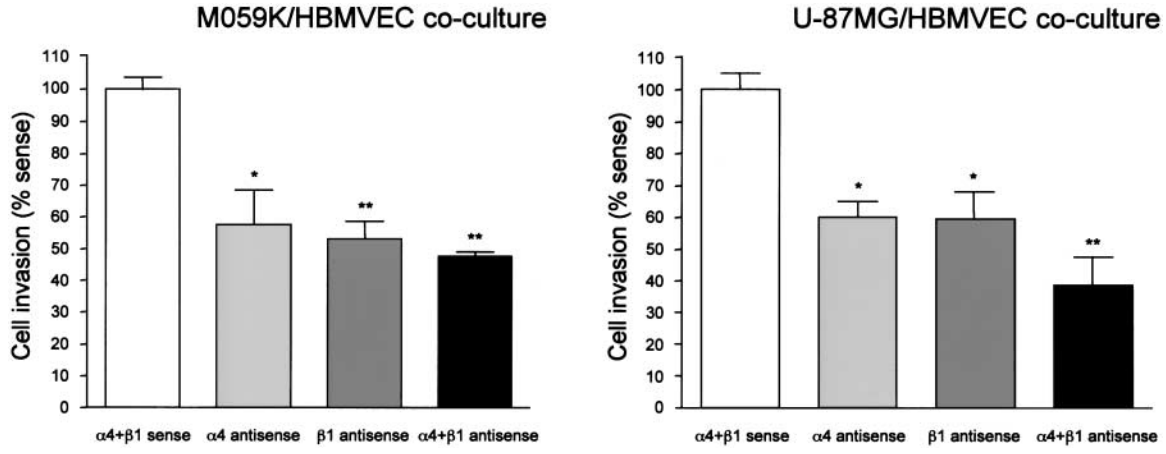

Figure 7. Measurement of invasion in cocultures after antisense treatment. The Matrigel invasion assay was carried out as described in ''Materials and Methods.'' Note significant decrease in the fraction of cells that invaded through Matrigel in antisense-treated cultures. A more pronounced effect is seen with a combination of antisense oligos. Similar results were obtained with M059K and U-87MG glioma cell lines. *, $P < 0.02$; **, $P < 0.001$ by ANOVA. Invasion in sense-treated cultures was taken as 100%.

when endothelial cells are cultured with tumor astrocytes than with normal embryonic brain astrocytes (44). We hypothesized that in glioma-endothelium cocultures there would be more laminin-8 produced and that this laminin might increase glioma invasion in a Matrigel assay. Research into these issues could facilitate GBM diagnosis and prognosis and increase survival of brain cancer patients.

Matrigel invasion assay was developed for quantitative measurement of the invasiveness of tumor cells through a BM matrix. Most tested cells characterized as invasive and metastatic *in vivo* are able *in vitro* to invade Matrigel, which is a BM-like material from the mouse Engelbreth-Holm-Swarm tumor (20, 21).

When glioma-endothelial cocultures were treated by antisense, the inhibition of invasiveness on Matrigel was 62% for U-87MG + HBMVEC and 53% for M059K + HBMVEC of that seen in the control cells treated with corresponding sense oligos. In our experiments, α4 and β1 expression was inhibited more efficiently with a lower concentration of antisense oligos (0.25 + 0.25 mM) than with a higher concentration (0.5 + 0.5 mM), although no apparent toxicity was noticed at either concentration. These data may be explained by previous findings, where oligo receptors on membranes of HepG2 cells were blocked. It was shown that at relatively high oligo concentrations, these receptors were saturated and the pinocytotic process assumed larger importance (45). A similar mechanism may occur in our system, which would explain the obtained results.

The use of antisense technology *in vivo* may offer an effective future tumor treatment because of its efficiency, specificity, and ease of delivery to tumor cells (41, 42). This technology is being continuously developed and refined not only for the drug validation and diagnostic purposes but also for the development of future treatments. The present data emphasize the feasibility of antisense approach using laminin-8 as a target for treatment of brain gliomas. Reduction of tumor invasion by antisense to laminin-8 may slow the growth and spread of aggressive GBMs. In combination with other treatment methods or with blocking of other targets as well (epidermal growth factor receptor and MMPs), it may prolong disease-free periods and increase survival of glioma patients. Future developments of laminin-8 blocking for therapeutic purposes may also include the use of specific mAbs and/or small interfering RNA that is an emerging and very promising approach for gene silencing.

It remains to be established how laminin-8 promotes glioma invasiveness. One possible mechanism may be stimulation of cell migration. It was previously shown that at least one form of laminin-8 containing α4A splice variant rather weakly supported cell adhesion and spreading compared with laminin-5 or laminin-10/11 (14, 46). At the same time, laminin-8 stimulated cell migration better than several other laminin isoforms (14). Increased expression of laminin-8 in both glioma cells and glioma-adjacent capillary endothelial cells (4, 14; this report) may reduce glial cell adhesion and enhance migration, which is necessary for local tumor invasiveness.

In summary, we developed a glioma-endothelial coculture model suitable for studying laminin-8 expression and its inhibition *in vitro* by antisense oligos. Morpholino proved to be efficient inhibitors of laminin-8 expression in cocultures. Antisense oligos to laminin-8 chains also significantly inhibited invasion of two different glioma cell lines *in vitro*. The results suggest that laminin-8 may play an important role in glioma invasion. Morpholino oligos may provide an efficient method to block laminin-8 expression for future therapeutic purposes.

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