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Down-Regulation of Caveolin-1 in Glioma Vasculature: Modulation by Radiotherapy

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Primary brain tumors, particularly glioblastomas (GB), remain a challenge for oncology. An element of the malignant brain tumors' aggressive behavior is the fact that GB are among the most densely vascularized tumors. To determine some of the molecular regulations occuring at the brain tumor endothelium level during tumoral progression would be an asset in understanding brain tumor biology. Caveolin-1 is an essential structural constituent of caveolae that has been implicated in mitogenic signaling, oncogenesis, and angiogenesis. In this work we investigated regulation of caveolin-1 expression in brain endothelial cells (ECs) under angiogenic conditions. In vitro, brain EC caveolin-1 is down-regulated by angiogenic factors treament and by hypoxia. Coculture of brain ECs with tumoral cells induced a similar down-regulation. In addition, activation of the p42/44 MAP kinase is demonstrated. By using an in vivo brain tumor model, we purified ECs from gliomas as well as from normal brain to investigate possible regulation of caveolin-1 expression in tumoral brain vasculature. We show that caveolin-1 expression is strikingly down-regulated in glioma ECs, whereas an increase of phosphorylated caveolin-1 is observed. Whole-brain radiation treatment, a classical way in which GB is currently being treated, resulted in increased caveolin-1 expression in tumor isolated ECs. The level of tumor cells spreading around newly formed blood vessels was also elevated. The regulation of caveolin-1 expression in tumoral ECs may reflect the tumoral vasculature state and correlates with angiogenesis kinetics. © 2003 Wiley-Liss, Inc.

Key words: caveolin-1; angiogenesis; brain tumors; endothelial cells; irradiation

Under normal conditions, brain vasculature constitutes a highly differentiated structure, the blood–brain barrier (BBB), that allows maintenance of brain homeostasis and protects the brain from potentially toxic molecules. Brain tumor progression is accompanied by intensive angiogenesis, the formation of new blood vessels first described by Folkman (1995). Characterization of the molecular events occurring in angiogenic brain tumor vasculature may be a determinant in aggressive behavior and invasiveness. Several lines of evidence suggest that caveolin-1 is a tumor suppressor gene; it is often down-regulated in human tumors (Galbiati et al., 1998). In this study, we investigated caveolin-1 regulation in brain capillary endothelial cells (ECs) under angiogenic conditions. Caveolae are flask-shaped plasma membrane invaginations involved in many cellular events, such as signal transduction, lipid and protein sorting, endocytosis, and potocytosis. Caveolins are the principal protein components of caveolae. The caveolin gene family consists of caveolin-1, -2, and -3. Caveolin-1 and -2 are coexpressed particularly in adipocytes, ECs, and epithelial cells as well as in fibroblasts (Shaul and Anderson, 1998). In the brain, the three caveolin subtypes have been detected in vivo by affinity purification and immunohistochemistry. Caveolin-1 and -2 are expressed in microvessels, predominantly in ECs but also in pericytes and astrocytes, whereas caveolin-3 expression is restricted to astrocytes (Ikezu et al., 1998; Virgintino et al., 2002). Terminally differentiated cells expressed a higher level of caveolin, in contrast to transformed cells and some tumoral cells, in which caveolin-1 is down-regulated (Koleske et al., 1995; Okamoto et al., 1998). The involvement of caveolin-1 in angiogenesis remains subject to controversy. Angiogenesis activators have been shown to down-regulate EC's caveolin-1 expression (Liu et al., 1999), and knock-down of caveolin-1 by antisense oligonucleotides impairs angiogenesis (Griffoni et al., 2000). However, caveolin-1 expression also enhances endothelial capillary tubule formation (Liu et al., 2002). Those results suggest that caveolin-1 may act at

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different levels during the angiogenesis process, which contains several stages with fine regulation.

The phenotypes of ECs that make up capillaries are known to differ as a result of the microenvironment to which they are exposed. Brain ECs are particularly responsive to such regulation. In the present study we investigated potential regulation of caveolin-1 expression at the level of the brain tumoral vasculature. We first used brain ECs cultured under angiogenic conditions to show that such a regulation was occurring. We then used an EC-isolation approach to study caveolin-1 expression and the effect of irradiation on the tumoral vasculature of rat gliomas. This magnetic cell sorting (MACS) approach allows isolation of enriched EC populations from tissues. We have already with this approach demonstrated phenotypical differences among ECs from lung, kidney, and brain (Demeule et al., 2001) and between ECs from normal and from tumoral vasculature (Régina et al., 2003). The results reported here identify a new molecular regulation at the tumoral vasculature by showing that caveolin-1 expression is down-regulated in tumoral brain ECs. We also demonstrate that irradiation treatment exerts the opposite effects on this regulation.

MATERIALS AND METHODS

Chemicals

MACS was performed with microbeads, a MidiMacs separation unit, and positive-selection MACS columns from Miltenyi Biotec (Auburn, CA). Mouse anti-PECAM-1 antibody (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) that was linked to microbeads is specific to rat PECAM-1 (clone No. TLD-3A12). Goat anti-PECAM-1 antibody, used in Western blotting analysis, was from Santa Cruz Biotechnology (Santa Cruz, CA) and cross-reacts with rat tissue according to the manufacturer's product information. The monoclonal antibody directed against the endothelial nitric oxide synthase (eNOS; Transduction Laboratories, Mississauga, Ontario, Canada) was raised against the C-terminal portion of the protein and cross-reacts with human, rat, and mouse tissues. Erk1/2 antibody (p44/42 MAP kinase antibody) was from Cell Signaling (Beverly, MA) and was raised against a synthetic peptide derived from the sequence of rat p42 MAP kinase. Phospho-ERK1/2 antibody (Cell Signaling) was raised against a synthetic phosphopeptide corresponding to residues around Thr202/Tyr204 of human p44 MAP kinase and cross-reacts with human, rat, and mouse tissues. Antibody directed against caveolin-1 (Transduction Laboratories, Lexington, KY) was generated against a fragment of human caveolin-1 and cross-reacts with human, rat, dog, mouse, and chick tissues according to the manufacturer's product information. Caveolin (PY14) phosphospecific antibody (BD Biosciences Pharmingen, Mississauga, Ontario, Canada) was generated from human caveolin-1 and cross-reacts with rat tissue. Electrophoresis reagents were purchased from Bio-Rad (Mississauga, Ontario, Canada). IgG horseradish peroxidase-linked whole antibody was from Jackson Immunoresearch Laboratories (West Grove, PA), and the chemiluminescence (ECL) reagent was purchased from Amersham-Pharmacia Biotech (Baie-d'Urfé, Québec, Canada). Cell culture media were from Gibco BRL (Burlington, Ontario, Canada). Others reagents were from Sigma-Aldrich (Oakville, Ontario, Canada).

Cell Culture

The CNS-1 murine glioma cell line, kindly provided by Dr. W.F. Hickey (Hanover, NH), was cultivated in RPMI 1640 supplemented with 10% fetal calf serum (FCS). RBE4 immortalized rat brain microvessel ECs (Roux et al., 1994) were maintained in α-MEM/Ham's F-10 (1:1) supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 300 μg/ml geneticin (G418), and 1 ng/ml basic fibroblast growth factor (bFGF).

Isolation of rat astrocytes and the establishment of an in vitro model of the BBB were performed as previously described (Fenart et al., 1999; Cecchelli et al., 1999). Bovine brain capillary endothelial cells (BBCEC) were plated onto small culture plate inserts (Millicell PC; Millipore Corp., Bedford, MA) and set into six-well dishes containing astrocytes. The coculture medium was composed of DMEM supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 50 μg/ml gentamycin, and 1 ng/ml bFGF. BBCEC formed a confluent monolayer after 5 days, and experiments were performed 7 days following confluence.

Brain Tumor Implantation

All animal experiments were evaluated and approved by the Institutional Comittee for Good Animal Practices (UQAM, Montréal, Québec, Canada). Intracerebral tumor implantation was performed as previously described (Régina et al., 2003). Anesthetized 250–280-g male Lewis rats (Charles River, Québec, QC, Canada) were placed in a stereotaxic frame; then, a burr hole was drilled into the right frontal cortex, and viable CNS-1 glioma cells (5×10^4) suspended in 5 μl of RPMI 1640 were injected at a depth of 4 mm at a point 3 mm lateral to the midline and 2 mm anterior to the bregma.

Treatment Schedule

In Vivo Treatment. In total, 28 rats were divided into four groups (n = 7). Five days after implantation, two groups were exposed to radiotherapy in the form of whole-head irradiation by using a single 15-Gy dose delivered with an Elekta SL75 linear accelerator with a 6-MV photon beam. Animals from one control and one irradiated group were sacrificed when rats from the control group began to lose weight. Two brains from each rat group were recovered, fixed in 10% buffered formalin, and embedded in paraffin for histopathological evaluation. Five brains were used for EC isolation. A survival study was performed with animals from the two remaining groups.

In Vitro Treatment. Confluent RBE4 cells in serum-free medium were irradiated using the same apparatus at lower single dose of 3 Gy. Cells were analyzed 24 hr posttreatment. Moreover, RBE4 cells and BBCEC were exposed to hypoxia during 24 hr in a controlled-atmosphere culture chamber, an airtight incubation apparatus into which a gas mixture containing 1% O_2, 5% CO_2, 94% N_2 was flushed. A compact gas O_2 controller (Proox model 110) was used to maintain a constant gas composition. Control cells were cultivated under normoxic conditions (20% O_2, 5% CO_2, and 75% N_2).

Isolation of EC Fractions

From normal brain, meninges-free cortex was homogenized in Ringer's solution, mixed with dextran T-70 (31%), and centrifuged at 4°C to remove myelin. Intracerebral gliomas were dissected from the tumor-injected animals and minced. Isolation of ECs was performed as previously described (Demeule et al., 2001; Régina et al., 2003) with a MidiMacs separation unit and positive selection using MACS columns from Miltenyi Biotec. Briefly, samples were incubated with collagenase A (1 mg/ml), passed through Nitex filters (180 and 30 μm), and incubated with microbeads linked to the anti-PECAM-1 antibody (20 $\mu l/10^7$ cells). ECs bound by the magnetic microbeads were selected with the separation unit. Cell fractions retained by the column were washed with phosphate-buffered saline (PBS), followed by centrifugation at 600*g* for 5 min. The final pellets containing ECs were kept at −80°C until use.

Western Blot Analysis

Normal brain and brain tumor tissue samples, isolated ECs, RBE4, and BBCEC were homogenized in lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM Tris HCl, pH 7.4, containing protease inhibitors (Boehringer Mannheim, Mannheim, Germany). Lysates were then centrifuged at 12,000*g* for 10 min to remove insoluble debris, resuspended in Laemmli electrophoresis buffer, heated for 5 min at 95°C, and separated on SDS-PAGE gels. After electrophoresis, proteins were transferred onto PVDF membranes, and the immunodetection of PECAM-1, caveolin-1, pCav, eNOS, ERK and pERK was performed with specific antibodies. Horseradish peroxidase (HRP)-conjugated anti-IgG antibodies were used as secondary antibodies, and immunoreactive proteins were revealed with ECL reagents (Amersham-Pharmacia Biotech). Protein concentrations were determined with the micro-BCA protein assay kit from Pierce (Rockford, IL). PVDF membranes were further stained with Coomassie blue to ensure that equal gel protein loading was accomplished.

Assessment of Tumor Vascularization

Histopathological examination of hematoxylin-eosin-stained rat tumor brain sections was performed. Tumor vascularization was assessed by immunohistochemistry with a polyclonal rabbit antibody to human von Willebrand factor (factor VIII; Dako, Carpinteria, CA) as a marker for the cytoplasm of vascular ECs. Four-micrometer-thick brain tumor slices were treated with pronase, washed, and incubated with the antiserum to factor VIII (1/1500 dilution), followed by incubation with a biotinylated goat anti-rabbit antibody. Sections were then incubated with streptavidin-HRP and finally developed with 3,3′-diaminobenzidine (DAB^+) substrate chromogen (Dako).

RESULTS

In Vitro Regulation of Brain EC Caveolin-1 Expression by Angiogenic Factors and Hypoxia

Brain capillary ECs expressed caveolin-1 (Ikezu et al., 1998; Virgintino et al., 2002). However, conditions that regulate caveolin-1 expression at the BBB remain unexplored. We first evaluated the effects of angiogenic factors and hypoxia on caveolin-1 expression in RBE4 immortalized rat brain microvessel ECs. Caveolin-1 expression was down-regulated in RBE4 cells after a 24-hr treatment with VEGF, bFGF, or glioma cell-conditioned medium (Fig. 1A). Moreover, 24 hr of hypoxia decreased caveolin-1 expression in RBE4 cells. An increase in hypoxia inducible factor 1-α (HIF-1) protein expression could be observed after only 4 hr of hypoxia in RBE4 cells (data not shown), showing that hypoxia-responsive genes are induced, as demonstrated by Turcotte et al. (2003) using CAKI-1 cells. However, those conditions did not cause EC injury, insofar as no cell cytotoxicity was measured when using a WST-1 assay (data not shown). In contrast, serum deprivation induced an up-regulation of caveolin-1 expression in RBE4 cells. Under normal conditions, astrocytes surrounding brain ECs exert a paracrine regulation on EC differenciation. We studied caveolin-1 regulation using BBCEC growing on filters in coculture with astrocytes, which is an accurate BBB model. BBCEC caveolin-1 expression increased by 35% in coculture con-

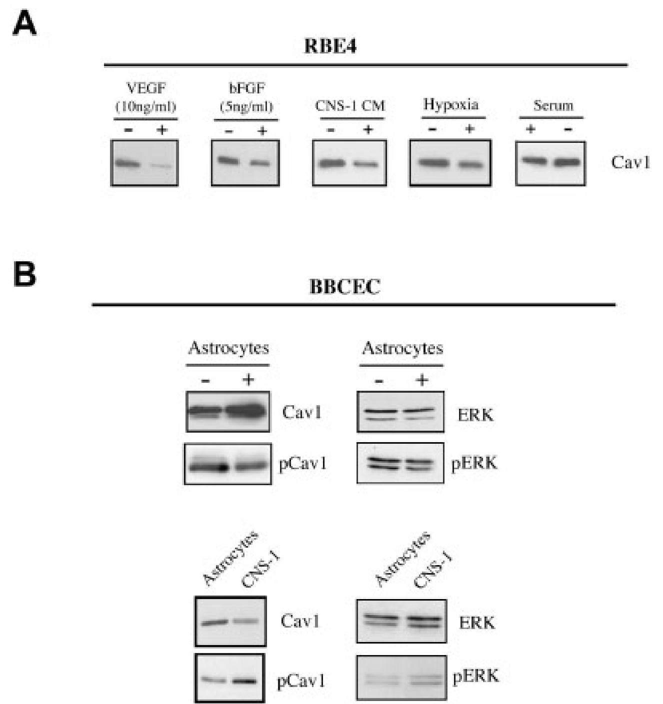


Fig. 1. Regulation of caveolin-1 expression in cultured brain ECs by angiogenic factors, serum deprivation, coculture conditions, and hypoxia. **A:** Caveolin-1 was detected in RBE4 cells treated with VEGF (10 ng/ml), bFGF (5 ng/ml), and glioma cell-conditioned medium (CNS-1 CM) subjected to normal (N) and hypoxic (H) conditions or to serum deprivation for 24 hr. **B:** BBCEC were grown with or without astrocytes as described in Materials and Methods. In addition, BBCEC cocultured with astrocytes were placed for 48 hr in coculture with CNS-1 cells grown in six-well plates. Immunodetection of caveolin-1, pCav-1, ERK, and pERK in BBCEC was performed. One representative experiment of three is shown.

ditions compared with monoculture conditions (Fig. 1B). We also measured the phosphorylation of caveolin-1 by Western blot analysis, using an antibody that detected the phosphorylation of caveolin-1 at tyrosine 14. Caveolin-1 phosphorylation decreased by 50% in BBCEC cocultured with astrocytes. Thus, the ratio pCav/caveolin in BBCEC grown in th epresence of astrocytes decreased (0.4) compared with the monoculture (1.0). Western blot analysis with a phosphospecific antibody that selectively recognizes only the activated/phosphorylated form of ERK1/2 and a phosphoindependent ERK1/2 antibody revealed that the ratio pERK/ERK was unaffected (1.0) in BBCEC cocultured with astrocytes compared with monoculture conditions (Fig. 1B).

We reproduced the change in the EC cellular environment that occurs during tumor progression by coculturing BBCEC with glioma cells (CNS-1). BBCEC filters that were first maintained in coculture with astrocytes were placed in coculture with CNS-1 cells for 48 hr. Under this condition, a down-regulation of caveolin-1 expression and an up-regulation of pCav were observed in BBCEC (Fig. 1B). The ratios pCav/Cav (2.0) and pERK/ERK (1.2) was increased in BBCEC exposed to CNS-1 cells. In addition, down-regulation of caveolin-1 occurs in BBCEC treated with 10 ng/ml VEGF or submitted to 24 hr of hypoxia (data not shown) as observed in RBE4 cells.

Regulation of Caveolin-1 Expression in Normal Brain and Brain Tumor Isolated ECs

Because caveolin-1 expression in BBB in vitro models was down-regulated by angiogenic factors, we investigated vascular caveolin-1 expression in an intracerebral brain tumor model. ECs from normal and tumoral rat brains were isolated by magnetic positive selection with a monoclonal antibody directed against the endothelial marker PECAM-1, or CD31, as previously reported (Demeule et al., 2001; Régina et al., 2003). Caveolin-1 expression was measured in normal brain and brain tumor homogenates (25 μg protein) and in the final, isolated EC fractions (5 μg proteins). Figure 2A shows that caveolin-1 was immunodetected at a very high level in isolated ECs from normal brain, whereas it was almost absent from isolated ECs of brain tumors. Even a fivefold increase in the amount of protein loaded was not sufficient to immunodetect caveolin-1 in the homogenates, reflecting the high level of caveolin expression in normal-brain ECs. In isolated ECs of brain tumors, caveolin-1 expression and caveolin-1 phosphorylation decreased by 75% and 44% respectively, compared with isolated ECs from normal brain. Thus, the ratio pCav/Cav was highly increased in tumoral brain ECs (2.2) compared with normal brain ECs (1.0), suggesting that the remaining caveolin-1 is highly phosphorylated. In addition, the ratio pERK/ERK in brain tumor ECs is increased (1.4) compared with normal brain ECs (1.0), indicating that ERK1/2 is activated in isolated tumor ECs (Fig. 2B). PECAM-1 immunodetection in the same samples shows that high, equivalent enrichments in PECAM-1 expression were obtained in

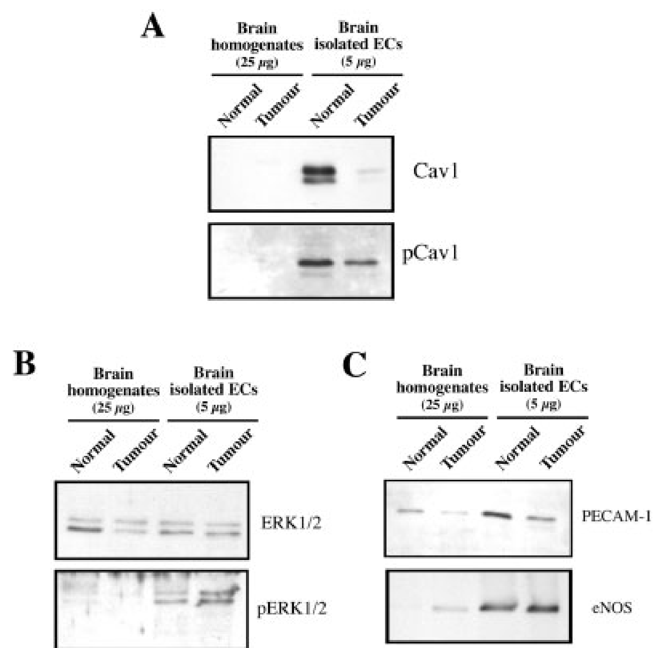


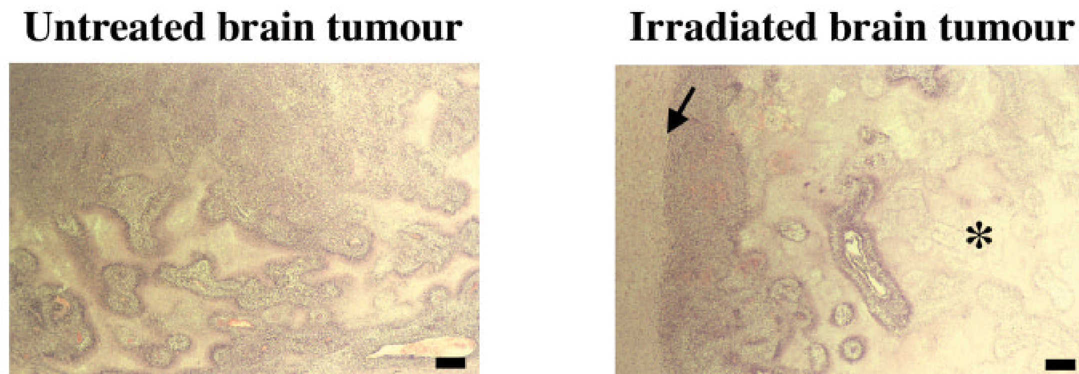
Fig. 2. Western blot analysis of caveolin-1, pCav-1, ERK, pERK, and EC marker expression in homogenates and isolated ECs from normal brain and brain tumor. Twenty-five micrograms of protein from normal brain (NB) and brain tumor (BT) homogenates and 5 μg of protein from normal brain and brain tumor-isolated ECs were separated on a 12.5%polyacrylamide gel for caveolin-1/pCav-1 (**A**) and ERK/pERK detection (**B**) and a 7.5% gel for eNOS and PECAM-1 detecion (**C**). One representative experiment of three is shown.

both EC fractions compared with their homogenates. We examined, in addition to that of PECAM-1, the expression level of eNOS in ECs, as an endothelial cell marker. Highly enriched eNOS expression levels were seen in ECs isolated from both samples compared with their homogenates. No differences in the expression levels of eNOS were observed between EC fractions isolated from normal brain or from brain tumor (Fig. 2C).

Effect of Radiotherapy in Glioma Tumor Model

Because radiotherapy is a current treatment for glioma, we investigated the effect of irradiation on the molecular regulation identified in brain tumoral endothelium. First, hematoxylin-eosin staining was performed on brain tumor slices from untreated and irradiated rat brains. Irradiation induced a large increase in the necrotic area center of the tumor, as can be clearly observed in Figure 3A. Survival of rats with intracerebral glioma exposed or not to a single 15-Gy dose of radiotherapy was measured (Fig. 3B). The median survival time for the untreated rat group was 20.3 ± 1.5 days. Irradiation treatment resulted in a very significant increase in survival, with a median survival time of 29.2 ± 5.1 days.

In addition, factor VIII immunohistochemistry was performed to assess vascularization at the tumor–brain interface, indicative of tumor invasion and tumoral



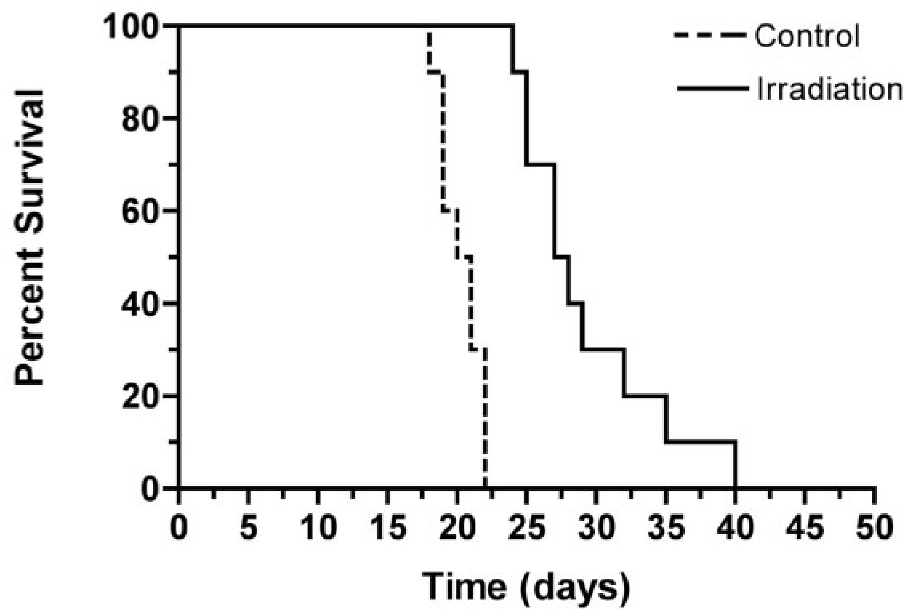


Fig. 3. Effect of radiotherapy on histopathological appearance of CNS-1 tumors and survival of rats with intracranial CNS-1 tumors. **A:** Hematoxylin-eosin staining of untreated and irradiated tumor. Irradiation induced a large increase in necrotic areas (asterisk) in the tumor center. The interface between normal brain and brain tumor is indicated by the arrow. **B:** Kaplan-Meir curve showing length of survival of animals implanted with 5×10^4 CNS-1 cells as described in Materials and Methods. Each group represents n = 7 rats. Irradiation significantly increased survival (median survival 29 days vs. 20 days, $P <$ .0001). Scale bars = 100 μm. Figure can be viewed in color online via www.interscience.wiley.com.

perivascular spreading. Compared with the case in normal brain endothelium, an increase in vascularization could be observed in tumor control animals, with hyperplasia of ECs. An increase in tumoral vascular spreading and stronger factor VIII cytoplasmic staining were observed after irradiation treatment. After irradiation, ECs were clearly surrounded by more numerous tumoral cells (Fig. 4A).

In Vitro and In Vivo Effects of Irradiation on Caveolin-1 Expression

Finally, regulation of caveolin-1 expression in tumoral vasculature following irradiation was investigated. Immunodetection of caveolin-1 was performed in homogenates and isolated ECs from brain tumors exposed or not to irradiation. Compared with the case in untreated brain tumor, an increase in caveolin-1 expression was seen both in tumor homogenate and in isolated tumor ECs from animals treated with irradiation. However, the level of caveolin-1 expression in isolated tumor ECs following irradiation remains lower than that in normal brain isolated ECs. Moreover, caveolin-1 was immunodetected in RBE4 cells exposed to irradiation (3 Gy). Irradiation treatment induced an increase in caveolin-1 expression (Fig. 4B).

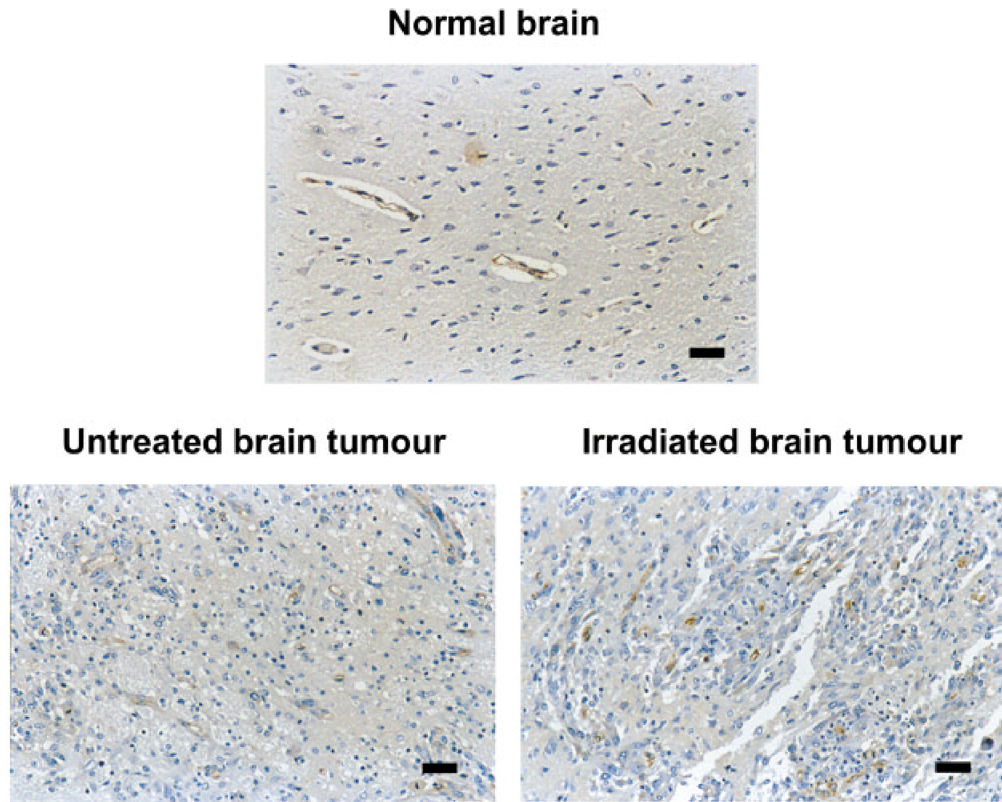
DISCUSSION

Brain tumors, and in particular glioblastomas, are among the most angiogenic of all human tumors (Lopes, 2003). In a continuing attempt to define systematically the unique molecular profile of ECs under pathophysiologic conditions, we have already demonstrated phenotypic differences between normal brain and tumoral brain ECs (Régina et al., 2003) in important elements in brain tumor progression, matrix metalloproteinase activities, and the multidrug resistance phenotype. We report here that a drastic decrease in caveolin-1 expression occurs in brain tumor ECs. This decrease in caveolin-1 expression could be associated with an activation of the p42/44 MAP kinase cascade, shown by increased phosphorylation of ERK1/2. When the effects of irradiation treatment were monitored, we observed a reversal of this caveolin-1 regulation.

Angiogenesis is a complex phenomenon that involves several steps by which ECs form new capillaries, an initial proliferative step and a terminal differentiation phase. In a human umbilical vein EC line (ECV304) and human microvascular ECs (HMEC-1) in vitro, caveolin-1 expression has been shown to be down-regulated by angiogenesis factors that stimulate cell proliferation (Liu et al., 1999, 2002). We observed a similar regulation of caveolin-1 expression in the brain EC line RBE4 and in bovine brain ECs that represent an in vitro model of the BBB when cocultured with astrocytes. In this model, caveolin-1 expression was increased following coculture with astrocytes and decreased after the cells were cocultured with glioma cells for 48 hr. Glioma cell-conditioned medium also induced a decrease in RBE4 caveolin-1 expression. Down-regulation of caveolin-1 was also seen in both models after 24 hr of hypoxia. Our in vitro results showing that caveolin-1 is down-regulated under angiogenic conditions led us to investigate this regulation in vivo by using a brain tumor model. CNS-1 tumors show a complex three-dimensional shape and a high degree of invasiveness. In that infiltration is a well-known feature of human glioblastoma multiforme, this model is appropriate for studying the effect of treatments on tumor spread. Furthermore, we have already shown that angiogenesis occurs in this model, demonstrating that it is suitable for studying molecular regulation of angiogenesis (Régina et al., 2003).

Caveolin-1 expression has been associated with the extent of cell differentiation (Liu et al., 2002). Caveolin-1 expression is down-regulated in rapidly dividing cells (Galbiati et al., 1998) and in many oncogenically transformed and cancerous cells (Fiucci et al., 2002). On the other hand, up-regulation of caveolin-1 expression is observed in confluent cells and in terminally differentiated cells (Fiucci et al., 2002). Our results show that caveolin-1 expression is drastically down-regulated in ECs from brain tumor compared with ECs from normal brain. This difference was not due to a difference in EC isolation between normal brain and brain tumor, in that the expression levels of the endothelial markers PECAM-1 and eNOS were similar in both cell fractions. Furthermore, we have previously reported that the cell fractions obtained are highly enriched EC populations, insofar as no contamination by astrocytes (for normal brain samples) or tumoral cells (for brain tumor samples) was observed (Régina et al., 2003). In this previous study, no morphological difference between normal and tumoral ECs was seen when they were placed in culture. However, our in vivo observations suggest that ECs in brain tumors are in a proliferative and dedifferentiated state.

Down-regulation of caveolin-1 may affect the activity of several proteins that have been reported to be closely coupled with caveolin-1. We observed increased ERK1/2 phosphorylation in ECs isolated from brain tumors. Activation of ERKs occurs in response to growth factors and phorbol esters and is associated with proliferation and differentiation (Enslen and Davis, 2001). ERK1/2 and other components of the Ras-extracellular signal-regulated kinase (ERK) mitogenic pathway are reported to be localized in caveolae. As reported for most interactions of caveolin-1 with other molecules, in vitro negative regulation of the ERK cascade by caveolin-1 has been demonstrated (Engelman et al., 1997, 1998; Galbiati et al., 1998). Demonstration of this regulation in an in vivo system has been recently provided by using the caveolin-1 null mouse model. More specifically, hyperactivation of the p42/44 MAP kinase cascade has been demonstrated in heart tissue (Cohen et al., 2003). It has been shown that, in gliomas, ERK/MAPK activation may contribute to the neoplastic glial phenotype (Mandell et al., 1998). Our results demonstrate that constitutive activation of ERK pathway also occurs in glial vascular endothelium. Furthermore, a link was observed in vitro between glioma invasion and ERK activation with a decrease in glioma cell invasion associated with down-regulation of MMP-9 after



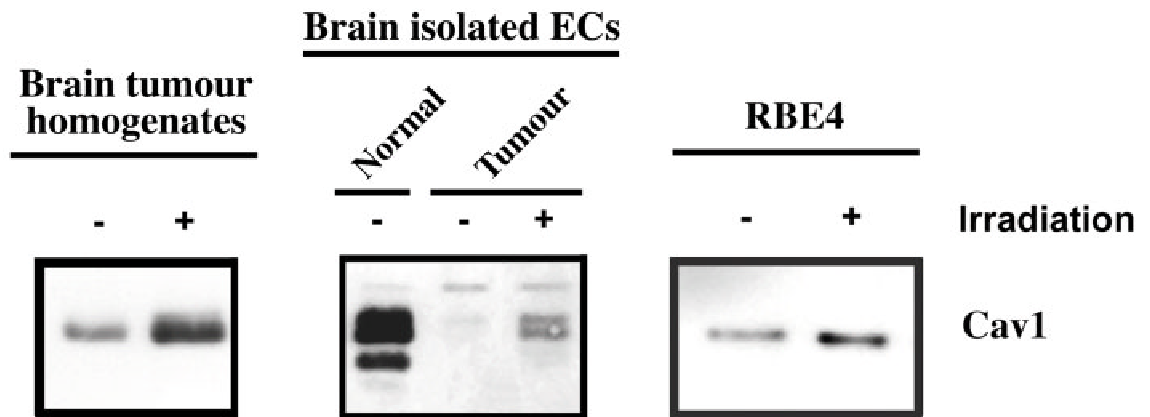


Fig. 4. Effect of irradiation on tumor vascularization and caveolin-1 expression. **A:** Factor VIII immunostaining of normal parenchyma and tumoral tissue. Photomicrographs were taken at the tumor–brain interface to evaluate tumor infiltrative growth pattern and neovascularisation level. Normal brain parenchyma shows thick vessel walls. Increased vessel number is observed in untreated tumor with EC hyperplasia compared with normal parenchyma. High perivascular tumor cell density and increased factor VIII cytoplasmic staining are observed after radiotherapy. **B:** Immunodetection of caveolin-1 in brain tumor homogenates and in ECs isolated from control and irradiated brain tumors and in the RBE4 cell line with or without irradiation, as described in Materials and Methods. One representative experiment of three is shown. Scale bars = 40 μm.

stable transfection of a mutated ERK (Lakka et al., 2002). We have previously reported an up-regulation of MMP-9 activity in ECs from brain tumors (Régina et al., 2003). Activation of the ERK pathway, as reported here, may well correlate with this MMP-9 up-regulation. Those results demonstrate again that glioma invasion is associated not only with tumoral cell behavior but also with EC modulation. In addition, our results suggest that the phosphorylation level of caveolin-1 in tumoral ECs is higher than that in normal ECs. There are not many studies of the role and function of phosphocaveolin-1. One investigation has recently shown that caveolin-1 is phosphorylated at tyrosine 14 in response to cellular stress (Sanguinetti and Mastick, 2003) and VEGF (Labrecque et al., 2003). In another study, phosphocaveolin-1 was localized at cell focal-adhesion regions, which are major sites of tyrosine kinase signaling (Brown et al., 2002). The consequences of increased phosphorylation of caveolin-1 in angiogenic ECs remain to be determined. Our results for brain tumor ECs can be put into perspective with recent results obtained with the caveolin-1 (–/–) knockout mouse model showing that lung capillaries in these mice are hyperpermeable (Schubert et al., 2002). Brain tumor capillaries are also known to be hyperpermeable, causing brain tumor-associated edema (Papadopoulos et al., 2001). The model proposed to explain this is based on tight junction opening associated with VEGF secretion by tumor cells. It was reported that VEGF receptor VEGFR-2 is localized in endothelial caveolae and associated to caveolin-1. Moreover, caveolin-1 acts as a negative regulator of VEGFR-2 activity (Labrecque et al., 2003). The loss of brain tumor EC caveolin-1 expression that we report here may certainly be one of the molecular mechanisms associated with blood–tumor hyperpermeability.

Because patients with glioma are often subjected to radiotherapy, we investigated the effects of radiation on the molecular regulation that we identified in the tumoral vasculature. Radiation treatment alone resulted in up-regulation of caveolin-1 expression both in vitro in the RBE4 cell line and in vivo in the tumor homogenates and corresponding isolated ECs. Up-regulation of caveolin-1 expression in ECs has been reported for irradiated lung parenchyma, a model of irradiation-induced lung injury (Kasper et al., 1998). In contrast to this, a study aimed at characterizing the effects of irradiation on tumor ECs found a decrease in caveolin-1 expression in tumor-isolated arterioles following irradiation (Sonveaux et al., 2002). In this study, arterioles were dissected 24 hr after irradiation, permitting analysis of the short-term impact of irradiation on tumor vasculature. In our experiments, tumor EC isolation was performed 20 days after irradiation, allowing examination of the long-term effects of irradiation on tumor vasculature. Radiotherapy treatment produces the expected increase in survival. Histopathological evaluation of tumor after irradiation showed a large tissue necrotic center. However, immunohistochemical study of the remaining tumor vascularization showed an increase in tumor cells density around newly form vessels in the parenchyma adjacent to the tumor center following irradiation. This observation is indicative of increased perivascular spreading of tumor cells, suggesting increased dissemination of the tumor. Recent reports have also shown that low-dose irradiation promotes migration and invasiveness of glioma cells (Wild-Bode et al., 2001) and stimulates angiogenesis (Mirsky et al., 2002; Sonveaux et al., 2003). In vitro, it has been shown that caveolin-1 expression level was regulated during capillary formation, with the highest expression found just before the stabilization of the vessels network (Liu et al., 2002).

In conclusion, we have investigated caveolin-1 regulation in brain endothelium in a pathological state and show that, in vitro, regulation of caveolin-1 expression occurs in brain ECs subjected to angiogenic culture conditions. By using magnetic cell sorting, we show that, in vivo, highly differentiated normal-brain ECs express a high level of caveolin-1. In tumor ECs a down-regulation of caveolin-1 occurs. After irradiation, caveolin-1 expression in tumor ECs tends to return to the level in normal-brain ECs. This observation suggests that irradiation might have stimulated the maturation of the remaining tumoral capillary network. Thus caveolin-1 expression could be a marker for vasculature state at a defined time in the angiogenic process.

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