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Propranolol adrenergic blockade inhibits human brain endothelial cells tubulogenesis and matrix metalloproteinase-9 secretion

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ABSTRACT

In recent clinical observation, the growth of endothelial tumors, such as hemangiomas of infancy, was repressed by the non-selective β-adrenergic antagonist propranolol possibly through targeting of the vascular endothelial compartment. As human brain microvascular endothelial cells (HBMEC) play an essential role as structural and functional components in tumor angiogenesis, we assessed whether propranolol could target HBMEC's *in vitro* angiogenic properties. We found that biopsies from human glioblastoma as well as from experimental brain tumor-associated vasculature expressed high levels of the β2-adrenergic receptor, suggesting adrenergic adaptative processes could take place during tumor vascularization. We observed that *in vitro* tubulogenesis was significantly reduced by propranolol when HBMEC were seeded on Matrigel. Propranolol, as much as 100 μM, did not reduce cell viability and did not alter HBMEC migration as assessed with Boyden chambers. Secretion of the key angiogenic and extracellular matrix degrading enzymes MMP-2 and MMP-9 was assessed by zymography. Propranolol significantly reduced MMP-9 secretion upon treatment with the tumor-promoting agent phorbol 12-myristate 13-acetate, while secretion of MMP-2 remained unaffected. This was correlated with a decrease in MMP-9 gene expression which is, in part, explained by a decrease in the nucleocytoplasmic export of the mRNA stabilizing factor HuR. Our data are therefore indicative of a selective role for propranolol in inhibiting MMP-9 secretion and HBMEC tubulogenesis which could potentially add to propranolol's anti-angiogenic properties.

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1. Introduction

Human brain microvascular endothelial cells (HBMEC) play an essential role as structural and functional components of the blood–brain barrier (BBB). While disruption of the BBB by the brain tumor-secreted matrix metalloproteinase-9 (MMP-9) favors tumor invasion [1], the role and regulation of MMP-9 secretion by HBMEC themselves in response to carcinogens has received little attention. Recent studies delineated a unique brain endothelial phenotype in which MMP-9 secretion in HBMEC was increased by the tumor-promoting agent phorbol 12-myristate 13-acetate [2,3]. MMP-9 is required for endothelial cell migration and tube formation, and inhibition of MMP-9 secretion was demonstrated to reduce both in vitro invasion and angiogenesis in human microvascular endothelial cells [4]. Recently, formation of new blood vessels through MMP-9-mediated events was attributed to circulating bone marrow-derived endothelial progenitor cells, which also partly contribute to neovascularization during the proliferative phase of infantile hemangioma [5]. Any therapeutic strategies leading to specific targeting of endothelial MMP-9 is therefore likely to be of utility in treating brain tumor-associated angiogenesis or common endothelial tumors such as hemangiomas of infancy.

The efficacy of β-blockers such as propranolol was recently reported against infantile capillary hemangiomas [6], and the physiopathological significance and therapeutic consequences was described [7]. Hemangiomas of infancy differ from vascular malformations in both tissue architecture and biological properties. To date, the most authoritative theories focus on angioblast origins, trophoblast origins, mutations in cytokine regulatory pathways, and field defects as the cause of the deranged angiogenesis of hemangiomas [8]. A possible involvement of the β2-adrenergic receptor/cAMP pathway in capillary function (including BBB integrity) was highlighted in inflammatory tumor necrosis factor (TNF)-α-stimulated HBMEC [9]. Interestingly, TNF-α is also a well-established MMP-9 secretion inducer [10], although not in HBMEC [2]. This suggests that adrenergic mediation in HBMEC may

Abbreviations: BBB, blood–brain barrier; HBMEC, human brain microvascular EC; MMP-9, matrix metalloproteinase-9; PMA, phorbol 12-myristate 13-acetate.

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regulate alternate endothelial functions, such as those involved in angiogenesis. Given that HBMEC play an essential role as structural and functional components in tumor angiogenesis, we questioned in this study whether propranolol could possibly target HBMEC's angiogenic properties including MMP-9 secretion, cell migration and *in vitro* tubulogenesis.

2. Materials and methods

2.1. Materials

Propranolol, sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from PerkinElmer (Waltham, MA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). All other reagents were from Sigma–Aldrich, Canada.

2.2. Human brain tumor biopsies

Five glioblastomas were obtained from Notre-Dame Hospital, Centre Hospitalier de l'Université de Montréal (Montreal, QC, Canada) through ethical consent procedures approved by the Research and the Ethics Committees of the Centre Hospitalier de l'Université de Montréal. Tumors were put into TRIzol reagent (Invitrogen, Burlington, ON) in order to extract total RNA as described below. Total RNA from healthy human brain tissue was from Stratagene (La Jolla, CA) and used as control.

2.3. Intracranial tumor model

All animal experiments were evaluated and approved by the Institutional Committee for Good Animal Practices (UQAM, QC). Intracerebral tumor implantation was performed as previously described, and validated [11,12]. Anesthetized 5–10-week-old Crl:CD-1®-nuBR female nude mice (Charles River, QC) were placed in a stereotaxic frame; viable U87 glioblastoma cells (5×10^4) in 5 μl of medium without serum were then implanted into the right corpus striatum at a depth of 3.5 mm at a point 2.5 mm lateral to the midline and 1.5 mm anterior to the bregma using a Hamilton syringe. The syringe was removed after 5 min and the wound was closed with sutures. Animals were sacrificed between 28 and 30 days post-implantation.

2.4. Cell culture

Human brain microvascular endothelial cells (HBMEC) were characterized and generously provided by Dr. Kwang Sik Kim of the Johns Hopkins University School of Medicine (Baltimore, MD). These cells were positive for factor VIII-Rag, carbonic anhydrase IV and Ulex Europeus Agglutinin I; they took up fluorescently labelled, acetylated low-density lipoprotein and expressed gamma glutamyl transpeptidase, demonstrating their brain EC-specific phenotype [13]. HBMEC were immortalized by transfection with simian virus 40 large T antigen and maintained their morphological and functional characteristics for at least 30 passages [14]. HBMEC were maintained in RPMI 1640 (Gibco, Burlington, ON) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (iFBS) (HyClone Laboratories, Logan, UT), 10% (v/v) NuSerum (BD Bioscience, Mountain View, CA), modified Eagle's medium nonessential amino acids (1%) and vitamins (1%) (Gibco), sodium pyruvate (1 mM) and EC growth supplement (30 μg/ml). Culture flasks were coated with 0.2% type-I collagen to support the growth of HBMEC monolayers. Cells were cultured at 37 °C under a humidified atmosphere containing 5% CO_2. All experiments were performed using passages 3–28.

2.5. cDNA synthesis and real-time quantitative RT-PCR

Total RNA was extracted from cultured HBMEC using TRIzol reagent. For cDNA synthesis, ∼1 μg total RNA was reverse-transcribed into cDNA using an oligo dT primer and the iScript reverse transcriptase cDNA synthesis kit (Bio-Rad, Mississauga, ON). cDNA was stored at −20 °C for PCR (Applied Biosystems, Inc., Foster City, CA). MMP-9 gene expression was measured by real-time quantitative RT-PCR using QuantiTect SYBR Green dye (Qiagen, Valencia, CA). DNA amplification was carried out using an Icycler (Bio-Rad, Hercules, CA), and the detection was performed by measuring the binding of the fluorescence dye SYBR Green I to double-stranded DNA. Primers for MMP-9 (QT00040040) and for HuR (forward: 5′-TCGCAGCTGTACCACTCGCCAG-3′; reverse: 5′-CCAAACATCTGCCAGAGGATC-3′) were derived from human sequences [15], while murine primers for β1-(QT00258692), β2-(QT00253967), and β3-(QT01756160) adrenergic receptors were from QIAGEN. The relative quantities of target gene mRNA against an internal control, 18S ribosomal RNA, was performed by following a ΔC_T method. An amplification plot comparing fluorescence signal *vs.* cycle number was drawn. The difference (ΔC_T) between the mean values in the duplicated samples of target gene and those of 18S ribosomal RNA were calculated by Microsoft Excel and the relative quantified value (RQV) was expressed as $2^{-\Delta C_T}$. Semi-quantitative RT-PCR analysis was performed starting with one microgram of total RNA for first strand cDNA synthesis, followed by specific gene product amplification with the One-Step RT-PCR Kit (Invitrogen, Burlington, ON). PCR conditions were optimized so that the gene products were examined at the exponential phase of their amplification and the products were resolved on 1.8% agarose gels containing 1 μg/ml ethidium bromide.

2.6. Analysis of HBMEC migration

HBMEC migration was assessed using modified Boyden chambers. The lower surfaces of Transwells (8-μm pore size; Costar, Acton, MA) were pre-coated with 0.2% type-I collagen for 2 h at 37 °C. The Transwells were then assembled in a 24-well plate (Fisher Scientific Ltd., Nepean, ON). The lower chamber was filled with serum-free HBMEC medium or growth factor-enriched conditioned medium isolated from 48 h serum-starved U87 human glioblastoma cells, which exert a very potent chemoattractant effect upon endothelial cell migration [16]. Control HBMEC were collected by trypsinization, washed and resuspended in serum-free medium at a concentration of 10^6 cells/ml; 10^5 cells were then inoculated onto the upper side of each modified Boyden chamber. The plates were placed at 37 °C in 5% CO_2/95% air for 30 min after which various concentrations of propranolol were added to the lower chambers of the Transwells. Migration then proceeded for 6 h at 37 °C in 5% CO_2/95% air. Cells that had migrated to the lower surfaces of the filters were fixed with 10% formalin phosphate and stained with 0.1% crystal violet–20% methanol (v/v). Images of at least five random fields per filter were digitized (100× magnification). The average number of migrating cells per field was quantified using Northern Eclipse software (Empix Imaging, Inc., Mississauga, ON). Migration data are expressed as a mean value derived from at least four independent experiments.

2.7. Endothelial cell morphogenesis assay

Tubulogenesis was assessed using Matrigel aliquots of 50 μl, plated into individual wells of 96-well tissue culture plates (Costar, Amherst, MA) and allowed to polymerize at 37 °C for 30 min.

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After brief trypsination, HBMEC were washed and resuspended at a concentration of 10^6 cells/ml in serum-free medium. Twenty-five microlitre of cell suspension (25,000 cells/well) and 75 μl of medium with serum were added into each culture well. Cells were allowed to form capillary-like tubes at 37 °C in 5% CO_2/95% air for 20 h in the presence or absence of different propranolol concentrations. The formation of capillary-like structures was examined microscopically and pictures (100×) were taken using a Retiga 1300 camera (QImaging) and a Nikon Eclipse TE2000-U microscope. The extent to which capillary-like structures formed in the gel was quantified by analysis of digitized images to determine the thread length of the capillary-like network, using a commercially available image analysis program (Northern Eclipse) as described and validated previously [16,17]. For each experiment, four randomly chosen areas were quantified by counting the number of tubes formed. Tubulogenesis data are expressed as a mean value derived from at least three independent experiments.

2.8. Gelatin zymography

Gelatin zymography was used to assess the extent of proMMP-2 and proMMP-9 activity as previously described [18]. Briefly, an aliquot (20 μl) of the culture medium was subjected to SDS-PAGE in a gel containing 0.1 mg/ml gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled H_2O. Gels were further incubated at 37 °C for 20 h in 20 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35, 50 mM Tris–HCl buffer, pH 7.6, then stained with 0.1% Coomassie Brilliant blue R-250 and destained in 10% acetic acid, 30% methanol in H_2O. Gelatinolytic activity was detected as unstained bands on a blue background.

2.9. Immunoblotting procedures

Cytosolic proteins from control and treated cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred onto polyvinylidene difluoride membranes which were then blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary polyclonal anti-HuR antibody (1/1000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) in TBST containing 3% bovine serum albumin, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/2500 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfée, QC).

2.10. Cell survival assay

Cell survival was assessed by the fluorometric caspase-3 activity. HBMEC were grown to 60% confluence and treated with different concentrations of propranolol. Cells were collected and washed in ice-cold PBS pH 7.0. Cells were subsequently lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for 1 h at 4 °C and the lysates were clarified by centrifugation at 16,000 × g for 20 min. Caspase-3 activity was determined by incubation with 50 μM of the caspase-3-specific fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) in assay buffer [50 mM Hepes–NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM DTT and 1 mM EDTA] in 96-well plates [19]. The release of AFC was monitored for at least 30 min at 37 °C on a fluorescence plate reader (Molecular Dynamics) (λ_{ex} = 400 nm; λ_{em} = 505 nm). The cleavage of the tetrazolium salt WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} by mitochondrial dehydrogenases (Roche Diagnostics, Laval, QC) was also used to assess cell viability [20].

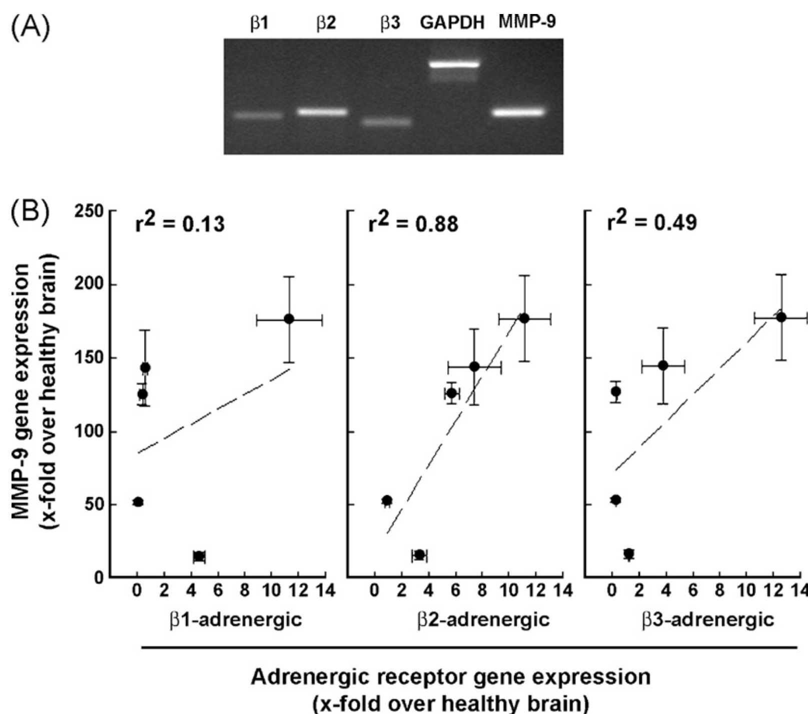

Fig. 1. β-Adrenergic receptors expression in human brain tumor specimens. Total RNA was extracted from 5 human glioblastoma biopsies obtained from operated patients. (A) Single cDNA amplicon product obtained from healthy brain total RNA was validated for each amplified gene on an agarose gel stained with ethidium bromide. (B) Total RNA was extracted from 5 glioblastoma specimens and relative gene expression levels for MMP-9, GAPDH, β1-, β2-, and β3-adrenergic receptors were assessed by qRT-PCR.

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3. Results

3.1. β-Adrenergic receptors expression in human brain tumor specimens

β1-, β2-, and β3-adrenergic receptors gene expression was first assessed in human clinical glioblastoma specimens obtained from operated patients. Total RNA was extracted from tumors and used in qRT-PCR experiments. The design of primers enabled measurement of the specific expression levels of individual human β-adrenergic genes and of the matrix metalloproteinase (MMP)-9, a crucial MMP known to be involved in BBB opening and which expression is correlated with cancer invasiveness [21]. This was validated by visualisation of a single cDNA amplicon product obtained from healthy human brain total RNA by semi-quantitative RT-PCR on an agarose gel (Fig. 1A). When qRT-PCR was then performed in glioblastoma specimens, the expression of each of the β-adrenergic receptor gene expression was compared to that of MMP-9. We found a good correlation between β2-adrenergic receptor and MMP-9 (r^2 = 0.88, Fig. 1B) but not for the other β-adrenergic receptors tested. This suggests that adaptative β-adrenergic receptors expression takes place during tumor development and correlates with the aggressiveness of the tumor.

3.2. Adaptative β-adrenergic receptors expression during experimental brain tumor development

In an attempt to assess the contribution of β-adrenergic receptors expressed in the tumor vasculature, experimental brain tumor growth was induced. We used U87 glioblastoma cell suspensions that were implanted by stereotaxis within the brains of nude mice and left to develop for several weeks as described in Section 2. Intracerebral growth of U87 tumors was routinely found to occur within the first 3–4 weeks as previously validated by us [11,12]. Total RNA was extracted from healthy brain and from brain tumors, and β-adrenergic receptors gene expression was assessed by qRT-PCR. The design of primers that enabled measurement of the specific expression levels of individual murine β-adrenergic genes was validated as shown in Fig. 2A. Only cDNA of these specific murine receptors was amplified from *in vivo* U87 tumors, but not from *in vitro* human U87 cell cultures (Fig. 2A), therefore allowing us to ignore the presence of human β-adrenergic receptor mRNA from the implanted human cancer cells. When qRT-PCR was performed with experimental brain tumors, we found that expression of β1-and β3-adrenergic receptors were, respectively, low and undetectable (ND) in comparison to healthy brain tissue, while expression of β2-adrenergic receptors was increased from ∼2 to 3-fold over the level seen in healthy brain tissue (Fig. 2B, black boxes). These results suggest that adrenergic adaptative processes could take place during tumor vascularization, but do not exclude the possible contribution of host-derived murine circulating cells to the β-adrenergic receptor gene expression which can include, among many others, endothelial progenitor cells, mesenchymal stem cells, hematopoietic stem cells, as well as non-endothelial cells.

3.3. Propranolol inhibits in vitro capillary-like structure formation but not basal HBMEC migration

Given the increased expression of β2-adrenergic receptors in experimental brain tumors, we next tested the effects of the β-blocker propranolol against the angiogenic properties of HBMEC. Cells were seeded on top of Matrigel and left to adhere as described in Section 2. Various concentrations of propranolol were then added, and capillary formation was left to proceed for 18 h. While a well defined capillary-like network formed in vehicle-treated cells (Fig. 3A), tubulogenesis was inhibited by propranolol with

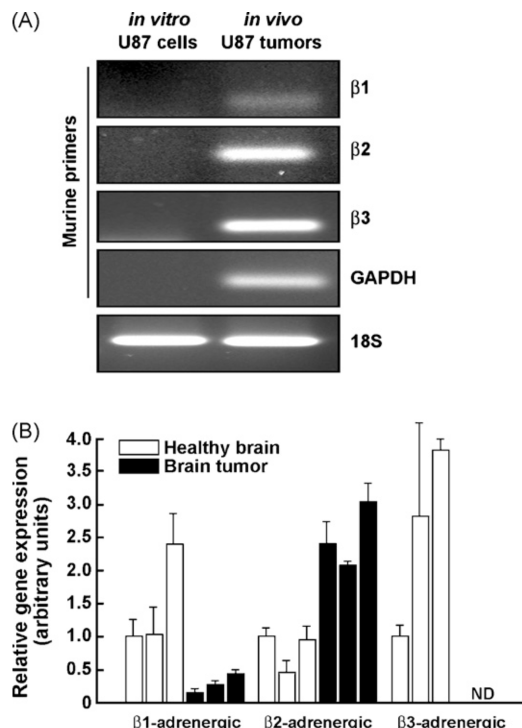

Fig. 2. Adaptative β-adrenergic receptors expression during experimental brain tumor development. Total RNA was extracted from human U87 cells, healthy mouse brain, or from U87 experimental tumors, and semi-quantitative RT-PCR performed as described in Section 2. (A) Primer design was validated to distinguish human- from murine-derived β-adrenergic receptors gene expression. Primers for 18S were designed to recognize both human and murine genes. (B) Relative gene expression levels for β1-, β2-, and β3-adrenergic receptors were assessed by qRT-PCR using murine primers and total RNA extracted from healthy murine brain (white box) and implanted experimental U87 tumors (black box) (n = 3; ND, not detectable).

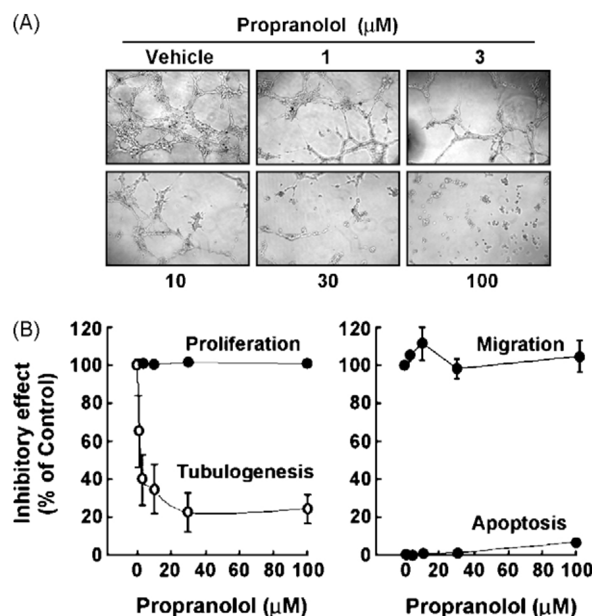

Fig. 3. Propranolol inhibits *in vitro* capillary-like structure formation but not basal HBMEC migration. (A) In order to assess *in vitro* tubulogenesis, HBMEC were seeded on top of Matrigel as described in Section 2, and then treated with various concentrations of propranolol for 16 h. Representative phase contrast pictures were taken. (B) The extent of three-dimensional capillary-like structure formation (open circles) and of cell survival (closed circles) was assessed as described in Section 2. HBMEC migration (B, right panel) was assessed in modified Boyden chambers. Cells were harvested and 10^6 cells seeded on top of gelatin-coated filters. Migration proceeded for 6 h in the presence of various concentrations of propranolol in the lower chambers.

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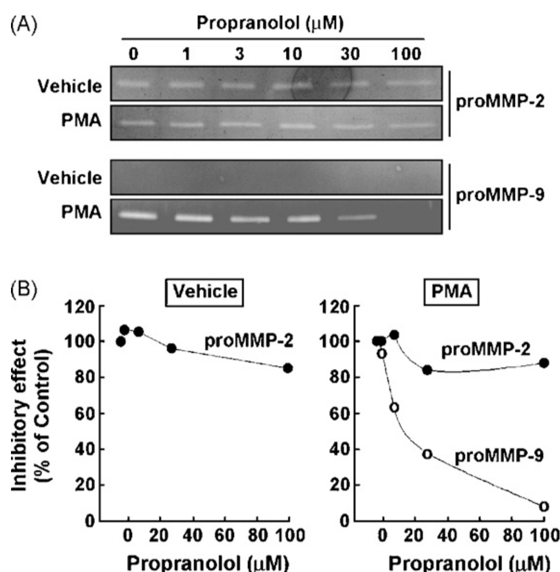

Fig. 4. Propranolol inhibits PMA-induced MMP-9 secretion in HBMEC. HBMEC were serum-starved in the presence of various concentrations of propranolol in combination with 1 μM PMA for 18 h. (A) Conditioned media were then harvested and gelatin zymography was performed in order to detect proMMP-9 and proMMP-2 hydrolytic activity as described in Section 2. (B) Scanning densitometry was used to quantify the extent of either basal proMMP-2 gelatin hydrolysis (left panel), or of proMMP-2 and proMMP-9 in PMA-treated cells (right panel). Data shown are representative of two independent experiments.

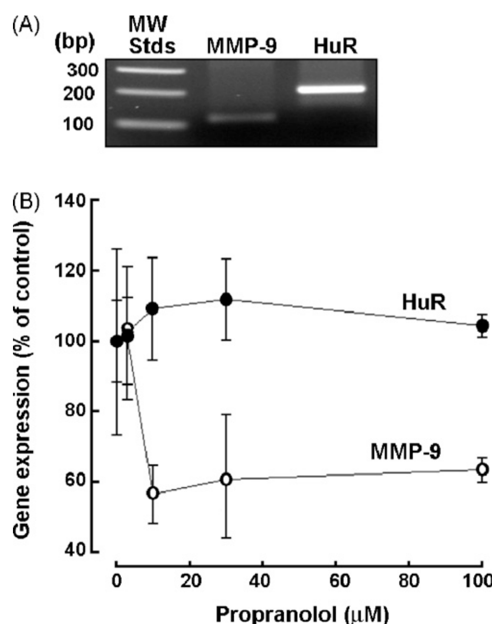

Fig. 5. Propranolol inhibits MMP-9, but not HuR, gene expression levels in HBMEC. Total RNA was extracted from HBMEC as described in Section 2. (A) Validation was performed by semi-quantitative RT-PCR to confirm the presence of a single amplicon for MMP-9 and for HuR. (B) Relative gene expression levels for MMP-9 and HuR were assessed by qRT-PCR using total RNA extracted from HBMEC treated with 1 μM PMA and various concentrations of propranolol. Data are representative of three independent experiments.

an IC_{50} approximating 1.7 μM (Fig. 3B). We also assessed propranolol's ability to affect HBMEC migration, which is a crucial event in angiogenesis. Basal HBMEC migration was unaffected by propranolol (Fig. 3B, right panel). Lack of any effect on migration, combined with the inhibition of structure formation within Matrigel, prompted us to investigate whether any extracellular matrix (ECM) degrading events were involved in propranolol inhibition. Cell viability, based on the cleavage of the tetrazolium salt WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} by mitochondrial dehydrogenases (Roche Diagnostics, Laval, Quebec, Canada), was not significantly affected by propranolol (Fig. 3A).

3.4. Propranolol inhibits PMA-induced MMP-9 secretion in HBMEC

Among the secreted enzymes involved in ECM degradation, matrix metalloproteinases (MMP) are well-documented as being involved in cell migration and tubulogenesis [2,22]. More specifically, MMP-2 and MMP-9 are secreted by numerous cell types and their presence is often representative of angiogenesis [23,24]. HBMEC were serum-starved, treated for 18 h with propranolol and the conditioned media were harvested to measure the levels of MMP-2 and of MMP-9 in control and in PMA-treated cells by gelatin zymography. While MMP-2 extracellular levels remained unaffected by propranolol, those of MMP-9 were undetectable in basal conditions but were significantly increased in PMA-treated cells (Fig. 4A, upper panel). When HBMEC were treated with combined PMA and propranolol, MMP-9 was significantly inhibited with an IC_{50} of ~8.1 μM (Fig. 4B). Collectively, these results suggest that propranolol selectively inhibits MMP-9 in response to tumor promoting conditions.

3.5. Propranolol inhibits PMA-induced cytosolic levels of HuR protein in HBMEC

Although most published studies have focused on the transcriptional control of MMP-9 expression, there is increasing evidence that its expression can also be regulated at the levels of mRNA stability, translation and protein secretion. Among the factors shown to stabilize MMP-9 mRNA and to augment its expression [25], HuR has been ascribed a pivotal role in the development of tumors [26] and been found to be a key mediator during macrophage activation in PMA-differentiated HL-60 cells [15]. We therefore tested whether propranolol affected MMP-9 or HuR transcript levels. Total RNA was isolated from PMA-treated HBMEC, and HuR and MMP-9 gene expression validated by the presence of a single cDNA amplicon (Fig. 5A), then levels of expression were assessed by quantitative RT-PCR. PMA was found to increase MMP-9 gene expression (not shown), while propranolol was able to significantly antagonize that increase without affecting HuR gene expression levels (Fig. 5B). We next assessed effects on HuR subcellular compartmentation by isolation of a cytoplasmic fraction depleted from the nuclear compartment. The protein expression of HuR was found to be significantly and transiently increased by PMA within 30 min (Fig. 6A). Propranolol prevented PMA-mediated HuR translocation into the cytosol where it has been reported to exert MMP-9 mRNA stabilization (Fig. 6B), while the expression of cytosolic GAPDH remained unaffected under all conditions. A time-course of MMP-9 secretion further confirmed that propranolol efficiently inhibited MMP-9 secretion in PMA-treated HBMEC (Fig. 6C), and this was observed as fast as within the first 4 h of secretion (Fig. 6D). The anti-MMP-9 effect of propranolol may, in part, involve downregulation of HuR which may ultimately decrease MMP-9 mRNA stability and subsequent synthesis/secretion.

4. Discussion

Propranolol is a non-selective β-adrenergic antagonist that crosses the blood–brain barrier (BBB) and that is widely used clinically for various conditions, including hypertension, anxiety, and excessive sympathetic responses that often characterize patients during the perioperative period [27]. Clinical benefits have been observed in operated cancer patients and in combination

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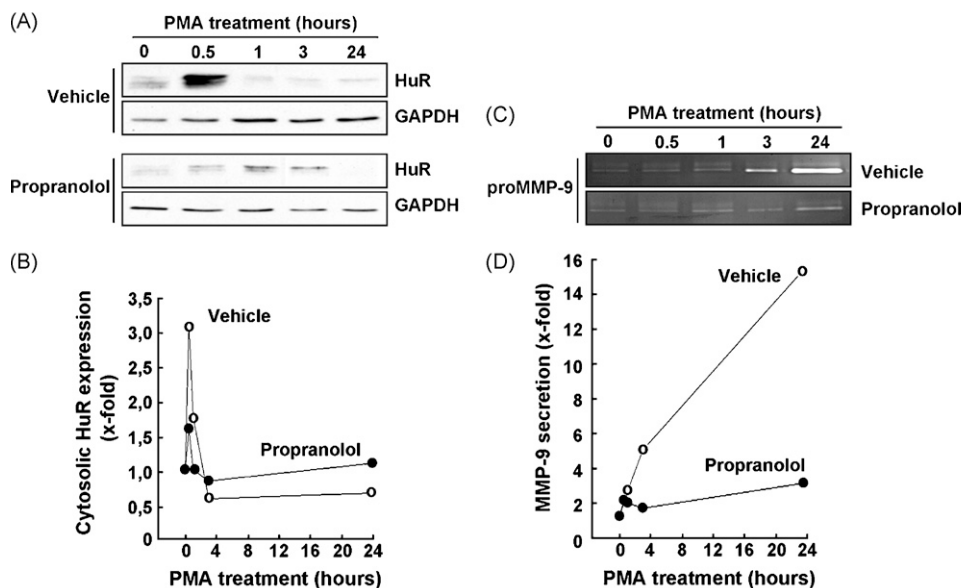

Fig. 6. Propranolol inhibits PMA-induced cytosolic levels of HuR protein in HBMEC. (A) Lysates of the cytosolic fraction of serum-starved PMA-treated HBMEC were isolated after various treatment times, electrophoresed via SDS-PAGE and immunodetection of HuR and of GAPDH proteins was performed as described in Section 2. (B) Quantification was performed by scanning densitometry of the autoradiograms. (C) Conditioned media was collected from (A) and gelatin zymography performed as described in Section 2. (D) Quantification was performed by scanning densitometry of the zymograms. Data were expressed as the *x*-fold induction over untreated basal conditions.

with COX-2 inhibitors, where perioperative treatment resulted in improved immune competence and in reduced risk of tumor metastasis [28]. It is therefore suggested that blockade of β-adrenergic receptors may impact on tumor development, an effect that was confirmed in the inhibition of experimentally-induced pulmonary adenocarcinoma development [29]. Whether any contribution of β-adrenergic receptors occurs in the potential anti-angiogenic effects of β-blockers is currently unknown. Moreover, no information is available on the expression of β-adrenergic receptors in tumor-associated endothelium. In this study, we have provided evidence for increased expression of β2-adrenergic receptors in the brain tumor-derived vascular compartment. Adrenergic blockade with propranolol also results in anti-angiogenic effects as reflected by the inhibition of HBMEC tubulogenesis and carcinogen-induced MMP-9 secretion. Targeting tumor-associated endothelial cells functions with β-blockers, as part of cancer treatments, may therefore become an appealing prospect to be further investigated.

To date, there is no human clinical study that documents the sole chemotherapeutic effect of the clinically-established propranolol in anti-cancer therapy. *In vivo* clinical data will therefore provide definitive proofs on the therapeutic efficacy of propranolol in anti-cancer modalities, and would further benefit from our *in vitro* demonstration and molecular mechanism elucidation. Among the strongest evidence and, perhaps, the best *in vivo* study that supports our current study is the demonstration that MMP-9 and the pro-angiogenic factor VEGF are both inhibited by propranolol in nasopharyngeal carcinoma tumor cells [30]. Several other *in vivo* approaches have, furthermore, already shed light on the chemopreventive action of propranolol in reducing pancreatic ductal adenocarcinoma growth in animal models [31], and in reducing metastasis development of PC-3 prostate cancer in nude mice [32]. Such published data are strongly suggestive of a potent anti-cancer action in line with those we infer in our study using the best surrogate model that approximates tumor-derived endothelial cells.

In particular, the expression of matrix metalloproteinase-9 (MMP-9) is significantly increased during tumor progression and is associated with the opening of the BBB [21]. Adenoviral-mediated MMP-9 downregulation inhibited human dermal microvascular endothelial cell migration in cell wounding and spheroid migration assays, and reduced capillary-like tube formation, demonstrating the key role of MMP-9 in endothelial cell network organization [4]. Recently, MMP-9 was among the increased hypoxia-induced mediators found in stem/progenitor cells in children with hemangioma [5]. Although propranolol was recently used to efficiently inhibit the growth of hemangiomas [6,7], the effects of such treatment on MMP-9 expression remain undocumented. However, MMP-9 is known to be required for tumor vasculogenesis [33], an alternate pathway for neovascularization that is increasingly being evoked in a variety of states characterized by vascular growth such as hemangioma. Therefore, among all MMP, the MMP-9 secreted from brain endothelial cells may be of importance in brain tumor-associated neovascularization and become a key therapeutic target.

Brain tumors are highly vascularized and, at early stages of development, tumor-associated vessels possess a phenotype similar to that in their normal brain environment. This phenotype can, in fact, explain why it is difficult to detect gliomas at early stages by MRI analysis [34]. Anti-angiogenic agents have thus far been used with some success [35,36], but mostly in combination with ionizing radiation (IR) to increase their therapeutic efficacy [37,38]. Until recently, most of the anti-angiogenic or anti-cancerous studies performed on endothelial cells *in vitro* involved macrovascular endothelial models such as human umbilical vein endothelial cells (HUVEC) or bovine aortic endothelial cells (BAEC). However, microvascular endothelial cells, such as brain endothelial cells, display a selective phenotype which differs from macrovascular endothelial cells. The availability of a stable human model of brain endothelial cells has only very recently allowed the emergence of studies which can now more closely represent cerebral endothelial microvasculature and mimic the impact of anti-cancerous treatments on the brain vasculature [39]. Our study is thus among the pioneering ones that are being reported. Currently, the HBMEC model is the best surrogate model that approximates tumor-derived endothelial cells available for long-term *in vitro* studies [13,16,40]. Although some groups have studied human endothelial cells isolated from glioblastoma specimens, these cells are not ideal targets for long-term *in vitro* studies as it is believed that they undergo some de-differentiation in culture and have inherently limited proliferative potential before senescence [41].

Only recently have brain endothelial cells been considered not only as target for anti-angiogenic agents but also for IR, there-

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fore representing a powerful new potential treatment target in highly vascularized tumors such as glioblastoma [16,42]. Interestingly, among naturally-occuring chemopreventive agents shown to inhibit MMP-9 secretion, the green tea polyphenol epigallocatechin gallate and the isothiocyanate sulforaphane selectively inhibited the secretion of MMP-9 in part through a decrease in expression of the MMP-9 mRNA stabilizing factor HuR [2,15]. Given that HuR levels are elevated in cancer [43], and have a pivotal role in promoting angiogenesis [44,45], we show in this study, in line with the anti-MMP-9 gene expression effect of propranolol, that inhibition of cytoplasmic HuR expression efficiently suppressed PMA-induced MMP-9 secretion.

β1- and β2-adrenergic receptors are expressed by several human tumor lines [46,30], and the application of a β-blocker was reported to abolish the direct prometastatic effects of stress and norepinephrine on human tumor cells implanted in nude mice [47]. Native β-adrenergic receptors occurring in C6 glioma cells were also recently shown to be constitutively active [48]. Interestingly, β1-adrenergic receptor mRNAs are post-transcriptionally regulated at the level of mRNA stability in part through the interaction of their 3′ untranslated region with RNA binding proteins including HuR [49]. Since HuR was also identified as a β-adrenergic receptor mRNA-binding protein [50], one can safely predict a similar regulation by propranolol that would also affect the expression of β-adrenergic receptors within tumors. It is thus tempting to suggest that inhibiting HuR-regulated expression of markers such as MMP-9 by propranolol could be potential targets of future anticancer therapies.

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References

[1] Lakka SS, Gondi CS, Rao JS. Proteases and glioma angiogenesis. Brain Pathol 2005;15:327–41.
[2] Annabi B, Rojas-Sutterlin S, Laroche M, Lachambre MP, Moumdjian R, Béliveau R. The diet-derived sulforaphane inhibits matrix metalloproteinase-9-activated human brain microvascular endothelial cell migration and tubulogenesis. Mol Nutr Food Res 2008;52:692–700.
[3] Roomi MW, Monterrey JC, Kalinovsky T, Rath M, Niedzwiecki A. Distinct patterns of matrix metalloproteinase-2 and -9 expression in normal human cell lines. Oncol Rep 2009;21:821–6.
[4] Jadhav U, Chigurupati S, Lakka SS, Mohanam S. Inhibition of matrix metalloproteinase-9 reduces in vitro invasion and angiogenesis in human microvascular endothelial cells. Int J Oncol 2004;25:1407–14.
[5] Kleinman ME, Greives MR, Churgin SS, Blechman KM, Chang EI, Ceradini DJ, et al. Hypoxia-induced mediators of stem/progenitor cell trafficking are increased in children with hemangioma. Arterioscler Thromb Vasc Biol 2007;27: 2664–70.
[6] Léauté-Labrèze C, Dumas de la Roque E, Hubiche T, Boralevi F, Thambo JB, Taïeb A. Propranolol for severe hemangiomas of infancy. N Engl J Med 2008;358:2649–51.
[7] Siegfried EC, Keenan WJ, Al-Jureidini S. More on propranolol for hemangiomas of infancy. N Engl J Med 2008;359:359–2846 [author reply, 2846–2847].
[8] Bauland CG, van Steensel MA, Steijlen PM, Rieu PN, Spauwen PH. The pathogenesis of hemangiomas: a review. Plast Reconstr Surg 2006;117:29e–35e.
[9] Ohara Y, McCarron RM, Hoffman TT, Sugano H, Bembry J, Lenz FA, et al. Adrenergic mediation of TNF alpha-stimulated ICAM-1 expression on human brain microvascular endothelial cells. Acta Neurochir Suppl 2000;76:117–20.
[10] Hetzel M, Walcher D, Grüb M, Bach H, Hombach V, Marx N. Inhibition of MMP-9 expression by PPARgamma activators in human bronchial epithelial cells. Thorax 2003;58:778–83.
[11] Annabi B, Rojas-Sutterlin S, Laflamme C, Lachambre MP, Rolland Y, Sartelet H, et al. Tumor environment dictates medulloblastoma cancer stem cell expression and invasive phenotype. Mol Cancer Res 2008;6:907–16.
[12] Annabi B, Lachambre MP, Plouffe K, Sartelet H, Béliveau R. Modulation of invasive properties of CD133(+) glioblastoma stem cells: a role for MT1-MMP in bioactive lysophospholipid signaling. Mol Carcinog; in press.
[13] Stins MF, Gilles F, Kim KS. Selective expression of adhesion molecules on human brain microvascular endothelial cells. J Neuroimmunol 1997;76:81–90.
[14] Greiffenberg L, Goebel W, Kim KS, Weiglein I, Bubert A, Engelbrecht F, et al. Interaction of Listeria monocytogenes with human brain microvascular endothelial cells: InlB-dependent invasion, long-term intracellular growth, and spread from macrophages to endothelial cells. Infect Immun 1998;66:5260–7.
[15] Annabi B, Currie JC, Moghrabi A, Béliveau R. Inhibition of HuR and MMP-9 expression in macrophage-differentiated HL-60 myeloid leukemia cells by green tea polyphenol EGCg. Leuk Res 2007;31:1277–84.
[16] McLaughlin N, Annabi B, Sik Kim K, Bahary JP, Moumdjian R, Béliveau R. The response to brain tumor-derived growth factors is altered in radioresistant human brain endothelial cells. Cancer Biol Ther 2006;5:1539–45.
[17] Lamy S, Gingras D, Béliveau R. Green tea catechins inhibit vascular endothelial growth factor receptor phosphorylation. Cancer Res 2002;62:381–5.
[18] Sina A, Lord-Dufour S, Annabi B. Cell-based evidence for aminopeptidase N/CD13 inhibitor actinonin targeting of MT1-MMP-mediated proMMP-2 activation. Cancer Lett 2009;279:171–6.
[19] Fortier S, Touaibia M, Lord-Dufour S, Galipeau J, Roy R, Annabi B. Tetra- and hexavalent mannosides inhibit the pro-apoptotic, antiproliferative and cell surface clustering effects of concanavalin-A: impact on MT1-MMP functions in marrow-derived mesenchymal stromal cells. Glycobiology 2008;18:195–204.
[20] Annabi B, Pilorget A, Bousquet-Gagnon N, Gingras D, Béliveau R. Calmodulin inhibitors trigger the proteolytic processing of membrane type-1 matrix metalloproteinase, but not its shedding in glioblastoma cells. Biochem J 2001;359:325–33.
[21] Shigemori Y, Katayama Y, Mori T, Maeda T, Kawamata T. Matrix metalloproteinase-9 is associated with blood–brain barrier opening and brain edema formation after cortical contusion in rats. Acta Neurochir Suppl 2006;96:130–3.
[22] Abécassis I, Olofsson B, Schmid M, Zalcman G, Karniguian A. RhoA induces MMP-9 expression at CD44 lamellipodial focal complexes and promotes HMEC-1 cell invasion. Exp Cell Res 2003;291:363–76.
[23] Handsley MM, Edwards DR. Metalloproteinases and their inhibitors in tumor angiogenesis. Int J Cancer 2005;115:849–60.
[24] Noël A, Jost M, Maquoi E. Matrix metalloproteinases at cancer tumor–host interface. Semin Cell Dev Biol 2008;19:52–60.
[25] Akool el-S, Kleinert H, Hamada FM, Abdelwahab MH, Förstermann U, Pfeilschifter J, et al. Nitric oxide increases the decay of matrix metalloproteinase 9 mRNA by inhibiting the expression of mRNA-stabilizing factor HuR. Mol Cell Biol 2003;23:4901–16.
[26] Audic Y, Hartley RS. Post-transcriptional regulation in cancer. Biol Cell 2004;96:479–98.
[27] Emilien G, Maloteaux JM. Current therapeutic uses and potential of beta-adrenoceptor agonists and antagonists. Eur J Clin Pharmacol 1998;53:389–404.
[28] Benish M, Bartal I, Goldfarb Y, Levi B, Avraham R, Raz A, et al. Perioperative use of beta-blockers and COX-2 inhibitors may improve immune competence and reduce the risk of tumor metastasis. Ann Surg Oncol 2008;15:2042–52.
[29] Park PG, Merryman J, Orloff M, Schuller HM. Beta-adrenergic mitogenic signal transduction in peripheral lung adenocarcinoma: implications for individuals with preexisting chronic lung disease. Cancer Res 1995;55:3504–8.
[30] Yang EV, Sood AK, Chen M, Li Y, Eubank TD, Marsh CB, et al. Norepinephrine up-regulates the expression of vascular endothelial growth factor, matrix metalloproteinase (MMP)-2, and MMP-9 in nasopharyngeal carcinoma tumor cells. Cancer Res 2006;66:10357–64.
[31] Al-Wadei HA, Al-Wadei MH, Schuller HM. Prevention of pancreatic cancer by the beta-blocker propranolol. Anticancer Drugs; in press.
[32] Palm D, Lang K, Niggemann B, Drell 4th TL, Masur K, Zaenker KS, et al. The norepinephrine-driven metastasis development of PC-3 human prostate cancer cells in BALB/c nude mice is inhibited by beta-blockers. Int J Cancer 2006;118:2744–9.
[33] Ahn GO, Brown JM. Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: role of bone marrow-derived myelomonocytic cells. Cancer Cell 2008;13:193–205.
[34] Leenders WP, Küsters B, de Waal RM. Vessel co-option: how tumors obtain blood supply in the absence of sprouting angiogenesis. Endothelium 2002;9:83–7.
[35] Reardon DA, Wen PY. Therapeutic advances in the treatment of glioblastoma: rationale and potential role of targeted agents. Oncologist 2006;11:152–64.
[36] Simpson L, Galanis E. Recurrent glioblastoma multiforme: advances in treatment and promising drug candidates. Expert Rev Anticancer Ther 2006;6:1593–607.
[37] Schuuring J, Bussink J, Bernsen HJ, Peeters W, van Der Kogel AJ. Irradiation combined with SU5416: microvascular changes and growth delay in a human xenograft glioblastoma tumor line. Int J Radiat Oncol Biol Phys 2005;61:529–34.
[38] Stupp R, Weber DC. The role of radio- and chemotherapy in glioblastoma. Onkologie 2005;28:315–7.
[39] Demeule M, Régina A, Annabi B, Bertrand Y, Bojanowski MW, Béliveau R. Brain endothelial cells as pharmacological targets in brain tumors. Mol Neurobiol 2004;30:157–83.
[40] McLaughlin N, Annabi B, Lachambre MP, Kim KS, Bahary JP, Moumdjian R, et al. Combined low dose ionizing radiation and green tea-derived epigallocatechin-3-gallate treatment induces human brain endothelial cells death. J Neurooncol 2006;80:111–21.

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[41] Bian C, Zhao K, Tong GX, Zhu YL, Chen P. Immortalization of human umbilical vein endothelial cells with telomerase reverse transcriptase and simian virus 40 large T antigen. J Zhejiang Univ Sci B 2005;6:631–6.
[42] Yuan H, Gaber MW, Boyd K, Wilson CM, Kiani MF, Merchant TE. Effects of fractionated radiation on the brain vasculature in a murine model: blood–brain barrier permeability, astrocyte proliferation, and ultrastructural changes. Int J Radiat Oncol Biol Phys 2006;66:860–6.
[43] López de Silanes I, Fan J, Galbán CJ, Spencer RG, Becker KG, Gorospe M. Global analysis of HuR-regulated gene expression in colon cancer systems of reducing complexity. Gene Expr 2004;12:49–59.
[44] Levy AP. Hypoxic regulation of VEGF mRNA stability by RNA-binding proteins. Trends Cardiovasc Med 1998;8:246–50.
[45] Sheflin LG, Zou AP, Spaulding SW. Androgens regulate the binding of endogenous HuR to the AU-rich 3′UTRs of HIF-1alpha and EGF mRNA. Biochem Biophys Res Commun 2004;322:644–51.
[46] Masur K, Niggemann B, Zanker KS, Entschladen F. Norepinephrine-induced migration of SW 480 colon carcinoma cells is inhibited by beta-blockers. Cancer Res 2001;61:2866–9.
[47] Sood AK, Bhatty R, Kamat AA, Landen CN, Han L, Thaker PH, et al. Stress hormone-mediated invasion of ovarian cancer cells. Clin Cancer Res 2006;12:369–75.
[48] Sokołowska P, Nowak JZ. Constitutive activity of beta-adrenergic receptors in C6 glioma cells. Pharmacol Rep 2005;57:659–63.
[49] Bai Y, Lu H, Machida CA. CRM 1-mediated degradation and agonist-induced down-regulation of beta-adrenergic receptor mRNAs. Biochim Biophys Acta 2006;1763:1076–89.
[50] Blaxall BC, Pellett AC, Wu SC, Pende A, Port JD. Purification and characterization of beta-adrenergic receptor mRNA-binding proteins. J Biol Chem 2000;275:4290–7.