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Dossier: Antioxidants in prevention of human diseases

Effect of resveratrol on matrix metalloproteinase-2 (MMP-2) and Secreted Protein Acidic and Rich in Cysteine (SPARC) on human cultured glioblastoma cells

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Abstract

***Introduction.* –** Glioblastoma is a highly malignant brain tumor with a high-invasive phenotype, so the prognosis is unfavorable, even in response to multidisciplinary treatment strategies. Obviously, therefore, a better therapeutic strategy is needed. Resveratrol has been reported to be one of the most potent chemopreventive agents inhibiting the cellular processes associated with tumor development, including initiation, promotion, and progression.

***Materials and methods.* –** In this study we used RT-PCR, western blot and SDS-zymography to investigate the effect of resveratrol on the expression of genes and proteins involved in the extracellular matrix remodeling associated with tumor invasion in human cultured glioblastoma cells treated for 24, 48 and 72 h. We analyzed the expression of matrix metalloproteinase-2 (MMP-2), the main mediator of glioblastoma invasiveness, and the Secreted Protein Acidic and Rich in Cysteine (SPARC), involved in the regulation of cell–matrix interactions.

***Results.* –** Our results show a dose-related decrease of MMP-2 mRNA and protein levels 72 h after resveratrol treatment, and lower SPARC gene and protein expression 72 h after resveratrol treatment. This indicates that resveratrol may influence the two major factors in the ECM remodeling occurring with tumor invasion, suggesting it may have uses as a therapeutic agent for brain tumors.

Keywords: MMP-2; SPARC; Resveratrol; Glioblastoma

1. Introduction

Brain tumors are one of the leading causes of death among young children and adults. Gliomas are the most common primary brain tumors, accounting for more than 40% of all central nervous system neoplasms [1]. Four major grades of gliomas are defined, glioblastoma being a highly malignant tumor typically affecting adults between 45 and 60 years of age [2]. Patients with malignant gliomas have a poor prognosis on account of the tumor's high-invasive capacity: they infiltrate diffusely into regions of the normal brain rendering total surgical removal impossible.

Tumor invasion partly depends on degradation of extracellular matrix (ECM) components mediated by tumor cell-secreted proteolytic enzymes such as matrix metalloproteinases (MMPs), a family of zinc-dependent proteases that break down ECM components [3,4]. The expression of MMPs in gliomas has been demonstrated, correlating glioma invasiveness with proteolytic activity of MMPs [5–7]. Gelatinases, particularly matrix metalloproteinase-2 (MMP-2), can be considered the prime factor in glioma invasiveness, since it can break down the basement membrane ECM components such as collagen type IV and laminin. MMP-2 expression also correlates with the progression and the degree of malignancy of gliomas [8,9].

Secreted Protein Acidic and Rich in Cysteine (SPARC) is a glycoprotein that influences a number of biological processes including cell differentiation, migration and proliferation. With its counter-adhesive properties, SPARC modulates cell–matrix interactions [10–12], and therefore, may have

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a functional role in tumor cell invasion of adjacent brain tissue. SPARC also takes part in proteolytic pathways by increasing the expression of collagenase and MMP-9, and activating MMP-2 [13]. This protein is frequently over-expressed in gliomas and its expression correlates with glioma invasion in vitro and in vivo [14–16]. SPARC may be a marker of invading cells.

The highly invasive phenotype of malignant gliomas means that patients have a poor prognosis, even using multidisciplinary treatment strategies including surgery, radiotherapy and chemotherapy [17,18]. Therefore, a better therapeutic strategy for malignant brain tumors is needed urgently.

In the search for new antitumoral agents over the past years, many plant extracts have been investigated. Resveratrol (*trans*-3,4′,5-trihydrostilbene) is a polyphenol found in various fruits and vegetables, and has many biological and pharmaceutical properties [19–21]. It is reported to be one of the most potent chemopreventive agents inhibiting cellular processes associated with tumor development, including initiation, promotion, and progression [22,23]. However, the precise mechanism of its anti-tumorigenic or chemopreventive activities is not understood, and only a few reports deal with the treatment of glioma with resveratrol [24,25].

Therefore, we designed this study to investigate the effect of resveratrol on highly malignant gliomas, evaluating the expression of genes and proteins involved in the mechanisms leading to tumor invasion. We analyzed MMP-2 and SPARC gene and protein expression in human cultured glioblastoma cells.

2. Materials and methods

2.1. Cell culture

Three human glioblastoma MI-cell lines (T60, T63, GBM) were obtained from biopsy specimens as described elsewhere [26]. Cell lines were maintained by serial passages in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere and were used within the first 20 passages. Glioblastoma (grade IV glioma) cells were cultured in RPMI supplemented with 10% FBS and antibiotics (10 U/ml penicillin, 10 mg/ml streptomycin) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Resveratrol treatment

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) was purchased from Sigma. A 100 mM solution of resveratrol was prepared in DMSO and stored at –20 °C. For treatment, this solution was diluted in RPMI 1640 and added to culture medium to the desired final concentrations (1 and 50 μM). Untreated cultures (VH) received the same amount of the solvent (DMSO 0.05%). VH and treated cells were incubated for 24, 48 and 72 h. At these intervals the supernatants were collected and cells were washed in PBS, trypsinized and harvested by centrifugation (100 × *g*, 5 min). Each cell line was cultured in duplicate. Glioblastoma cell viability was determined by Trypan blue staining.

2.3. RT-PCR analysis

Total RNA was extracted by a modification of the guanidine isothiocyanate/phenol/chloroform method (Tri-Reagent, Sigma). After DNase I digestion, 1 μg of total RNA was reverse-transcribed in 20 μl final volume of reaction mix (Promega). The following primers were used for RT-PCR: GAPDH 5′-ATTCCATGGCACCGTCAAGGCT, 3′-TCAGGTCCACCACGACACGTT (571 bp); MMP-2 5′-CCTCTCCACTGCCTTCGATACACC, 3′-AGCATCTATTCTTGGGCACCG (162 bp); SPARC 5′-ACCATGAGGGCCTGGATC, 3′-GGAGTGG ATTTAGATCACAAG (936 bp).

Amplification reactions were conducted in a final volume of 25 μl containing 2.5 μl of cDNA, 200 μM of the four dNTPs, 100 pmol of each primer, and 2.5 U of Taq DNA polymerase (EuroTaq, Euroclone). The RT-PCR protocols are listed in Table 1. The RT-PCR products were resolved by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and quantified by densitometric analysis (Image Pro-Plus).

2.4. SDS-zymography

ProMMP-2 protein levels were assessed in the supernatants of cultured glioblastoma cells by SDS-zymography. Supernatants were concentrated in an Amicon Y10 at 6500 × *g* for 15 min at 4 °C. The concentrated culture media were mixed 3:1 with sample buffer, containing 10% SDS. Four μg total proteins per sample were run under non-reducing/non-denaturing conditions onto 7.5% polyacrylamide gel (SDS-PAGE) co-polymerized with 1 mg/ml type I gelatin. The gels were run at 4 °C. After SDS-PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min each and incubated overnight in a substrate buffer at 37 °C (Tris–HCl 50 mM, $CaCl_2$ 5 mM, NaN_3 0.02%, pH 7.5). The MMP gelatinolytic activity was detected after staining the gels with Coomassie brilliant blue R250, as clear bands on a blue background [27]. To confirm the identity of MMP gelatinolytic activity, purified MMP-1 and MMP-2 (100 ng, Calbiochem) were run as controls.

Table 1
RT-PCR amplification conditions

Gene	Protocol	Cycles (*n*°)
MMP-2	Denaturation 94 °C 1 min	30
	Annealing 60 °C 2 min	
	Elongation 72 °C 3 min	
SPARC	Denaturation 94 °C 1 min	32
	Annealing 55 °C 1 min	
	Elongation 72 °C 1 min	
GAPDH	Denaturation 94 °C 30 s	25
	Annealing 62 °C 1 min	
	Elongation 72 °C 1 min	
	+72 °C 10 min to finalize extension	

2.5. Western blot

Concentrated culture media (20 μg of total proteins) were diluted in SDS-sample buffer, loaded on 10% SDS-polyacrylamide gel, separated under reducing and denaturing conditions at 80 V according to Laemmli [28], and transferred at 90 V to a nitrocellulose membrane in 0.025 M Tris, 192 mM glycine, 20% methanol, pH 8.3 [29]. After electroblotting, the membranes were air dried and blocked for 1 h After being washed in TBST, membranes were incubated for 1 h at room temperature in monoclonal antibody to SPARC (1:100 in TBST, Novocastra Laboratories) and, after washing, in HRP-conjugated rabbit anti-mouse serum (1:40,000 dilution, Sigma). Immunoreactive bands were revealed by the Opti-4CN substrate (Bio Rad).

2.6. Statistical analysis

All tests were run in duplicate. Data from the two runs are expressed as mean ± standard error (S.E.M.), and were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Neumann-Keuls test. P values less than 0.05 were considered significant.

3. Results

3.1. Cell viability and proliferation

Phase contrast microscopy of cultured human glioblastoma cells confirmed the heterogeneity of shape and of the nucleus (Fig. 1a). There was a dose-related decrease of proliferation in glioblastoma cells treated with either 1 or 50 μM resveratrol (Fig. 1b).

3.2. Gene expression

The gene expression analysis is presented in Fig. 2. MMP-2 mRNA levels tended to be lower 48 h after resveratrol, and the reduction was significant, and dose-related, after 72 h (15%, N.S. after 1 μM; 29%, $P < 0.05$ after 50 μM) (Fig. 2a). SPARC gene expression tended to be dose-dependently down-regulated with both doses at all three intervals (Fig. 2b).

3.3. SDS-zymography

SDS-zymography analysis was done on the supernatants of glioblastoma cells cultured for 72 h. The zymogram contained two lysis bands corresponding to proMMP-2 and proMMP-9. Densitometric analysis of the proMMP-2 band indicated a dose-related decrease of the inactive gelatinase A 72 h after resveratrol treatment (10%, N.S. after 1 μM; 18%, $P < 0.05$ after 50 μM) (Fig. 3 a, b). ProMMP-9 levels were unaffected by resveratrol treatment.

3.4. Western blot

Densitometric analysis of immunoreactive bands corresponding to SPARC revealed that SPARC protein levels tended to be decreased by resveratrol 72 h after treatment of glioblastoma cells (Fig. 4 a, b).

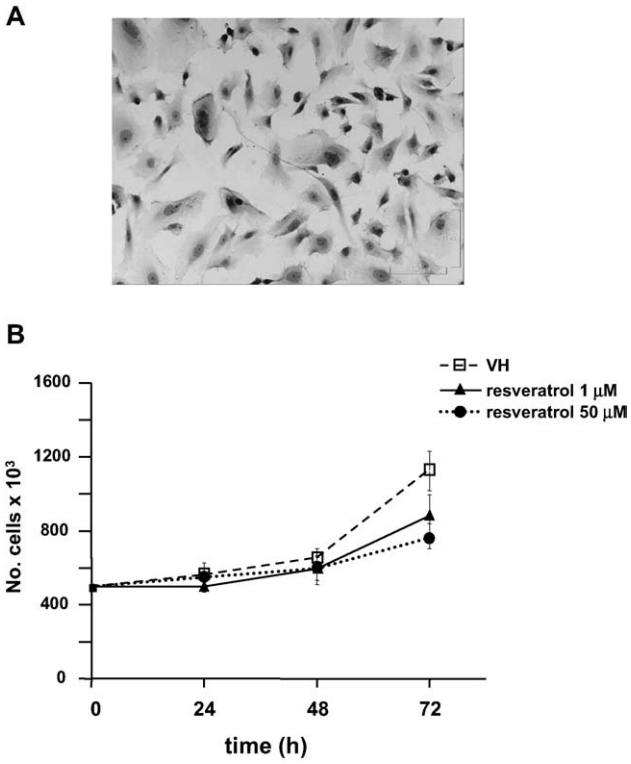

Fig. 1. a) Microphotographs of cultured glioblastoma cells after hematoxylin–eosin staining showing the heterogeneity of cellular shape and of the nucleus. Bar: 100 μm. b) Time-dependent effect of resveratrol on cultured glioblastoma cell proliferation. Cells were plated in T-75 flasks (500,000 cells/flask) and allowed to attach. Fresh medium containing 1 and 50 μM resveratrol was added, and cells were counted at the times indicated. Glioblastoma cells incubated with vehicle (VH) were used as controls. Each time point represents the mean ± S.E.M. of duplicate samples.

4. Discussion

Resveratrol can cross the blood–brain barrier and is taken up by brain tissue [30]. This is a further pointer to resveratrol's potential in the therapy of brain tumors. The precise mechanisms of the anti-carcinogenesis effect of resveratrol remain largely unknown. It is partly attributable to its antioxidant activity, but experimental evidence indicates that these properties are related to the compound's ability to cause cell-cycle arrest in the G1 phase [31] or in the S-G2 phase transition, or to trigger apoptosis in a variety of cancer cell lines [32–34].

This is consistent with our findings of a dose-related decrease of glioblastoma cell proliferation, particularly evident 72 h after resveratrol treatment.

Since resveratrol exerts a blocking effect on all tumor development phases, we analyzed this effect on the mechanisms involved in tumor invasion in highly malignant gliomas. The physical processes of invasion involve disengagement of the cells from their microenvironment, followed by breakdown of the surrounding matrix, cell movement, and

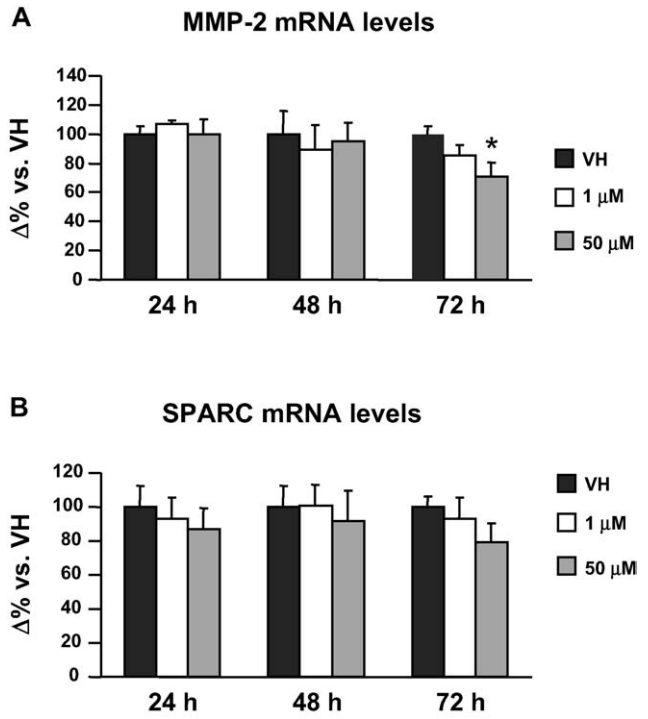

Fig. 2. Bar graphs showing steady-state mRNA levels of MMP-2 (a) and SPARC (b) in cultured human glioblastoma cells at the times indicated. Changes in mRNA levels are expressed as normalized optical densities relative to GAPDH mRNA. Values are mean ± S.E.M. * $P < 0.05$ vs. VH.

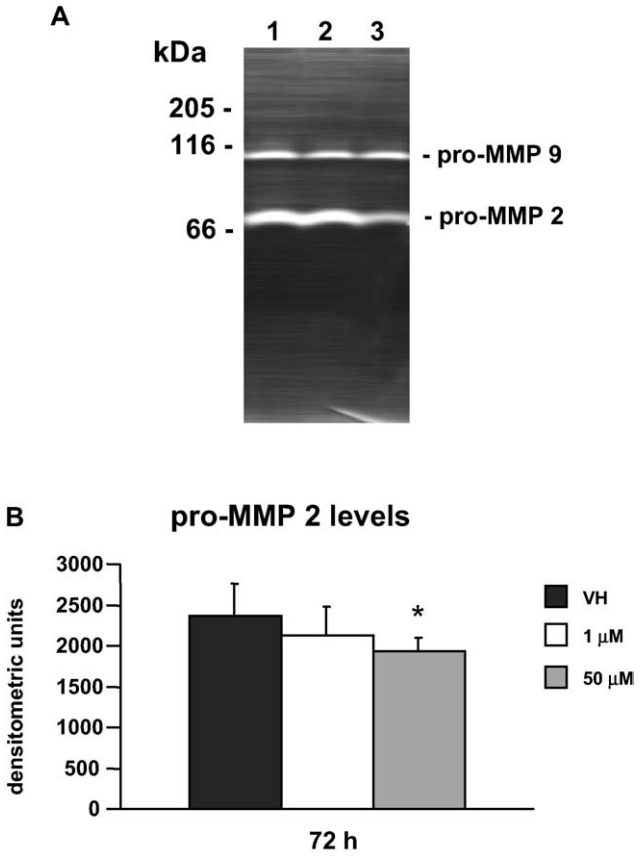

Fig. 3. a) Representative SDS-zymography showing two lysis bands consistent with proMMP-2 and proMMP-9. Glioblastoma supernatants were electrophoresed on 7.5% gels. 1: VH; 2: resveratrol 1 μM; 3: resveratrol 50 μM. b) Bar graphs showing proMMP-2 levels after densitometric scanning of lysis bands. Values are mean ± S.E.M. * $P < 0.05$ vs. VH.

re-establishment of the local environment at a new location. This allows glioma cells at the tumor-invasive front to overcome the ECM barrier, and to penetrate adjacent brain structures. Since this is accomplished by an ECM remodeling process involving MMP-2 and SPARC [35–37], we investigated MMP-2 and SPARC gene and protein expression in human cultured glioblastoma cells treated with two different doses of resveratrol. We used 1 μM resveratrol since a similar resveratrol concentration is reached in the plasma of rats after oral administration of red wine [38]. The 50 μM concentration was not cytotoxic in a neuroblastoma cell line [39] and almost completely inhibited lymphocyte cell proliferation [40].

In our conditions resveratrol significantly lowered MMP-2 mRNA levels in glioblastoma cells after 72 h. This pattern is consistent with the results of the protein analysis, showing significantly reduced proMMP-2 levels at the same interval. Interestingly, the effects on both MMP-2 mRNA and protein levels were dose-related 72 h after resveratrol.

Resveratrol was found to inhibit MMP-2 in human liver fibroblasts [41], but the mechanism remains unknown. MMP-2 gene expression requires translocation of the transcription factor NF-kB to the nucleus [42,43] and since resveratrol has been reported to prevent the activation of NF-kB [44], its effect on MMP-2 gene expression is quite likely achieved through this inhibition.

SPARC is a matricellular protein that mediates interactions between cell and their extracellular environment. This protein has a major role in the promotion of glioma cell invasion, as confirmed by evidence that human glioma cells engineered to over-express SPARC adopt an invasive phenotype [16]. A further interesting role for SPARC in the promotion of tumor progression has also recently been suggested [45]: it may enable tumor cells to survive under the stressful conditions that surround the tumor, such as nutrient restriction, hypoxia and genomic instability. The expression of SPARC by gliomas induces cellular survival in serum-free conditions and the apoptotic rate of SPARC-expressing glioma cell lines is reduced relative to control line, representing a mechanism through which gliomas resist cell death [36]. SPARC, therefore, may be an important target for cancer therapy, as it is involved in tumor invasion and resistance to apoptosis. Our results show a tendency to dose-dependent down-regulation of SPARC gene expression 24, 48 and 72 h after resveratrol treatment. Moreover, 72 h after resveratrol treatment SPARC protein levels tended to be lower in glioblastoma treated cells, compared to VH.

Considered as a whole, our results show that resveratrol influenced MMP-2 and SPARC expression in glioblastoma cells. This suggests that the two major determinants in ECM remodeling associated with tumor invasion may be a target for resveratrol, suggesting it could serve in the therapy of brain tumors. The results also contribute to our knowledge of

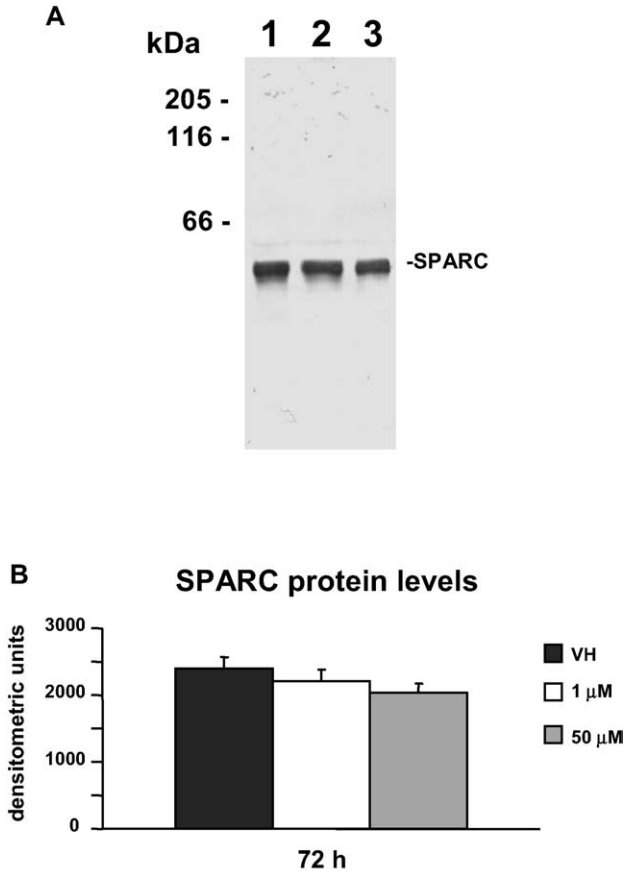

Fig. 4. a) Immunoblot analysis for SPARC protein in glioblastoma supernatants. The monoclonal antibody identifies a positive immunoreactive band in the 43 kDa region corresponding to SPARC. Reference weight markers are reported on the left. 1: VH; 2: resveratrol 1 μM; 3: resveratrol 50 μM. b) Bar graphs showing SPARC protein levels after densitometric scanning of immunoreactive bands. Values are mean ± S.E.M.

the mechanisms of the antitumor and chemopreventive potential of resveratrol, suggesting it may have some potential in therapy.

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