

Dobesilate diminishes activation of the mitogen - activated protein kinase ERK1/2 in glioma cells

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Received: January 27, 2006; Accepted: February 24, 2006

Abstract

Fibroblast growth factors (FGFs) and their receptors, regularly expressed at high levels in gliomas, are further upregulated during the transition of the tumor from low- to high-grade malignancy, and are essential for glioma progression. FGFs induce upregulation of the mitogen-activated protein kinase (MAPK) signaling cascade in cultured glioma cells, which suggests that MAPK pathway participates in the FGF-dependent glioma development. Recently, it has been shown that dobesilate, an inhibitor of FGF mitogenic activity, shows antiproliferative and proapoptotic activities in glioma cell cultures. Accordingly, it should be expected this new synthetic FGF inhibitor to affect the activation levels of MAPK. Here we report that immunocytochemical and Western blot data unequivocally show that treatment of cell cultures with dobesilate causes a significant decrease of the intracellular levels of ERK1/2 activation, one of the components of the MAPK signalling cascade. This finding supports an important role for dobesilate in glioma growth, suggesting that dobesilate should be a treatment to be born in mind for glioma management.

Keywords: glioma • dobesilate • mitogen-activated protein kinase (MAPK)

Introduction

Significant improvement of outcome for patients with primary brain tumors remains elusive despite intense clinical and laboratory research. In particular, outcome for patients with glioblastoma multiforme

(GBM), the most common primary brain tumor in adults, remains unacceptable [1]. Thus, new innovative anti-glioma therapies are urgently needed.

We have recently reported that dihydroxy-2,5 benzenesulfonate (dobesilate), an oral agent for treatment of vascular complications of diabetic retinopathy [2], inhibits FGF-driven angiogenesis [3] and shows antiproliferative and proapoptotic activities in glioma cell cultures [4]. A detailed knowledge of the cell biology behind these effects may help to finely

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tune up a potential clinical application of this compound for the treatment of gliomas.

Regulatory mechanisms controlling proliferation, apoptosis and angiogenesis during tumorigenesis involve intracellular protein kinase cascades that transduce signals from the cells surface into changes in gene expression. Among the pathways often used to transduce these signals are the highly conserved mitogen-activated protein kinase (MAPK) or extracellular signal regulated protein kinase (ERK) cascades. These cascades consist of a three-kinase module that includes a MAPK (ERK) which is activated by a MAPK/ERK kinase (MEK), which in turn is activated by a MEK kinase (MEKK). The best characterized MAPK cascade consists of Raf, isoforms MEK1/2, and extracellular signal regulated protein kinase (ERK) 1/2. This cascade is under the control of Ras. [5, 6].

ERK1/2 have been implicated in mediating glioma cell proliferation [7, 8]. Furthermore, they constitute one of the most important cell signaling pathways activated by fibroblast growth factors (FGF) [8–14]. Prolonged activation and nuclear retention of ERKs is required for ERK-mediated enhancement of cell cycle entry. In addition, half of the activated ERKs remains bound to the cytoskeleton [6].

As it has been mentioned above, dobesilate inhibits FGF-promoted angiogenesis [3]. Given the strict requirement of FGF for glioma growth [15, 16], it seems reasonable to propose that the observed antiproliferative and proapoptotic activities of dobesilate in glioma cell cultures are also caused by an inhibition of FGF activity. If this is the case, alteration of the normal status of the FGF-triggered intracellular signaling system that drives cell division and survival should be expected. The research here reported was undertaken to test this hypothesis and, thus, to gain some insight in the cell biology of the dobesilate-induced inhibition of glioma proliferation.

Material and methods

Cell culture

Glioma C6 cells (2×10^4) were grown in Dulbecco's modified Eagle's medium (DMEM) [Gibco (Paisly, PA49RF,UK)] supplemented with 7.5% (v/v) fetal calf

serum. Antibiotics [penicillin (10 units/ml) and streptomycin (10 μ g/ml) (Gibco)] were added to the medium. Cultures were incubated at 37°C in a humidified chamber with 5% CO₂. Cells were set in coverslides and then treated with 100 μ M dobesilate potassium salt (Merck Farma y Quimica SA, Mollet del Vallés, Barcelona, Spain) for 1h, a concentration required to inhibit proliferation by 50% (IC₅₀) in glioma cultures [4]. We chose 1h for assessing the activated ERK1/2 levels after dobesilate treatment because its activation is detectable several minutes after FGF stimulation, and a subsequent increase in the levels of activated protein is detectable within 1h [14].

Immunocytochemistry

For immunocytochemical studies rat C6 glioma cells were grown in coverslides and processed as previously reported [8]. Briefly, control and dobesilate-treated cultures were fixed in 4% paraformaldehyde, washed three times in PBS, permeabilized and then treated with a mouse anti-phospho ERK1/2 antibody (1:100; Sigma Chemical, St. Louis, MI. USA). Immunoreactivity was detected using Fast red as the chromogen for alkaline phosphatase conjugated to an anti-mouse secondary antibody. Negative control experiments used cultures that were incubated without primary antibody and subsequently processed with secondary antibody and reacted with chromogen. These cultures demonstrated a complete absence of reaction product. Immunocytochemical analysis was performed in three independent experiments. The percentage of positive-staining cells was estimated by randomly counting 500 tumor cells from three different high-power fields (x40) within one coverslip.

Western blot

C6 cells were seeded in six-well plates (Falcon), treated as described above, and harvested following the treatment. For whole cell extracts, cells were detached, harvested and lysed in a lysis buffer containing 10 mM Tris-HCl (pH 7.6), 10mM KCl, 1mM DTT, proteases and phosphatases inhibitors, 0.5% Triton and 0.1% SDS. The protein determination was done by BioRad protein assay dye reagent method. For Western blotting, 10 μ g of protein from each sample was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on a 9% polyacrylamide gel under reducing conditions. Proteins were then

transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech Europe, Cerdanyola, Barcelona, Spain). Membranes were blocked for 1 hour at room temperature in 3% powdered nonfat milk dissolved in a Tris/Tween solution (20mM Tris Ph 7.6, 0.1% Tween 20 in saline). Primary antibody [mouse anti-phospho ERK1/2 (1:4000). Sigma, Saint Louis, Missouri, USA] was diluted in blocking buffer and incubated with the membrane overnight at room temperature. After washing in TBS containing 0.5% Tween 20, the membranes were incubated with peroxidase-coupled goat anti-mouse antibody (Chemicon, Temecula, CA, USA) diluted 1:4000 for 1h at room temperature. The peroxidase activity was detected following the chemiluminescence method (ECL advance, Amersham). Membranes were incubated with Western blotting detection reagents (Amersham) and exposed to X-OMAT AR Films (Eastman Kodak, Rochester, NY, USA). Films were scanned on an Epson Perfection 2450 scanner (Japan) and optical density of each band was densitometrically determined using the Image Quant TL software (Amersham).

All experiments were repeated three times using independent cell preparation. A representative experiment from each series is presented. Data are the means \pm S.E.M. The significance of the results was determined by Student's t-test and nonparametric test. Differences are accepted as significant at $p < 0.05$.

Results

Activation of ERK1/2 in control cultures and in cultures treated with dobesilate.

In order to evaluate the activation levels of ERK1 and ERK2 isoforms in glioma cells, cultures were immunostained with an antibody elicited by an ERK-derived phosphopeptide that specifically recognizes the phosphorylated (activated) form of ERK (Material and methods). Indeed, pERK localization is mainly nuclear, but the cytoplasmic localization exists too, because the phosphorylation of such a protein occurs in the cytoplasm before its translocation to the nucleus [17]. Representative photomicrographs are shown in Fig. 1. As shown in the figure, the immunoreactivity predominantly accumulated in the perinuclear region of the cytoplasm. A weak immunoreactivity was also observed in the nuclei. The figure also shows that most of the

cells immunostain positively in the untreated sample. An important decrease in the number of stained cells was readily observed in the case of the cultures treated with dobesilate. A quantitative analysis of the immunostaining results was carried out as described in Material and methods. While a 93% of the untreated cells show positive immunostaining, the rate decreases down to a 30% in the dobesilate treated cultures. Furthermore, dobesilate treatment induced the formation of branch processes in glioma cells indicating differentiation into mature astrocytes (Fig. 1).

Effect of dobesilate in the activation of ERK1/2 in glioma cells

Cell immunostaining does not allow the analysis of the activation of ERK at the molecular level. In order to examine the extent at which the activation affects at each of the two ERK isoforms, a Western blot analysis of a crude homogenate of the cultures was carried out, as described in Material and methods. Results appear in Fig. 1e. A summary of the quantitative analyses of these results is represented in Fig. 1f. As shown in both figures, there are significant differences between dobesilate-treated and untreated samples in the activation levels of both ERK1 and ERK2. The levels of activated ERK1 are clearly higher than those of ERK2 in the untreated sample (Fig. 1e and 1f).

Discussion

It is well documented that MAPK is a key signal-transducing protein in cell proliferation [6]. FGF has been found to elicit mitogenic responses through the activation of MEKs pathways in several cell types [8–14]. More precisely, binding of FGF to its affinity receptors induces rapid phosphorylation of ERK *in vivo* [13]. In addition, several endogenous angiogenesis inhibitors like the 16-KDa fragment of prolactin or like angiostatin can block FGF-induced phosphorylation of ERK isoforms p42/p44 *in vitro*, which further stresses the relevance of these pathways in the transduction of FGF intracellular signaling [18, 19]. It has been also reported that FGF and their receptors

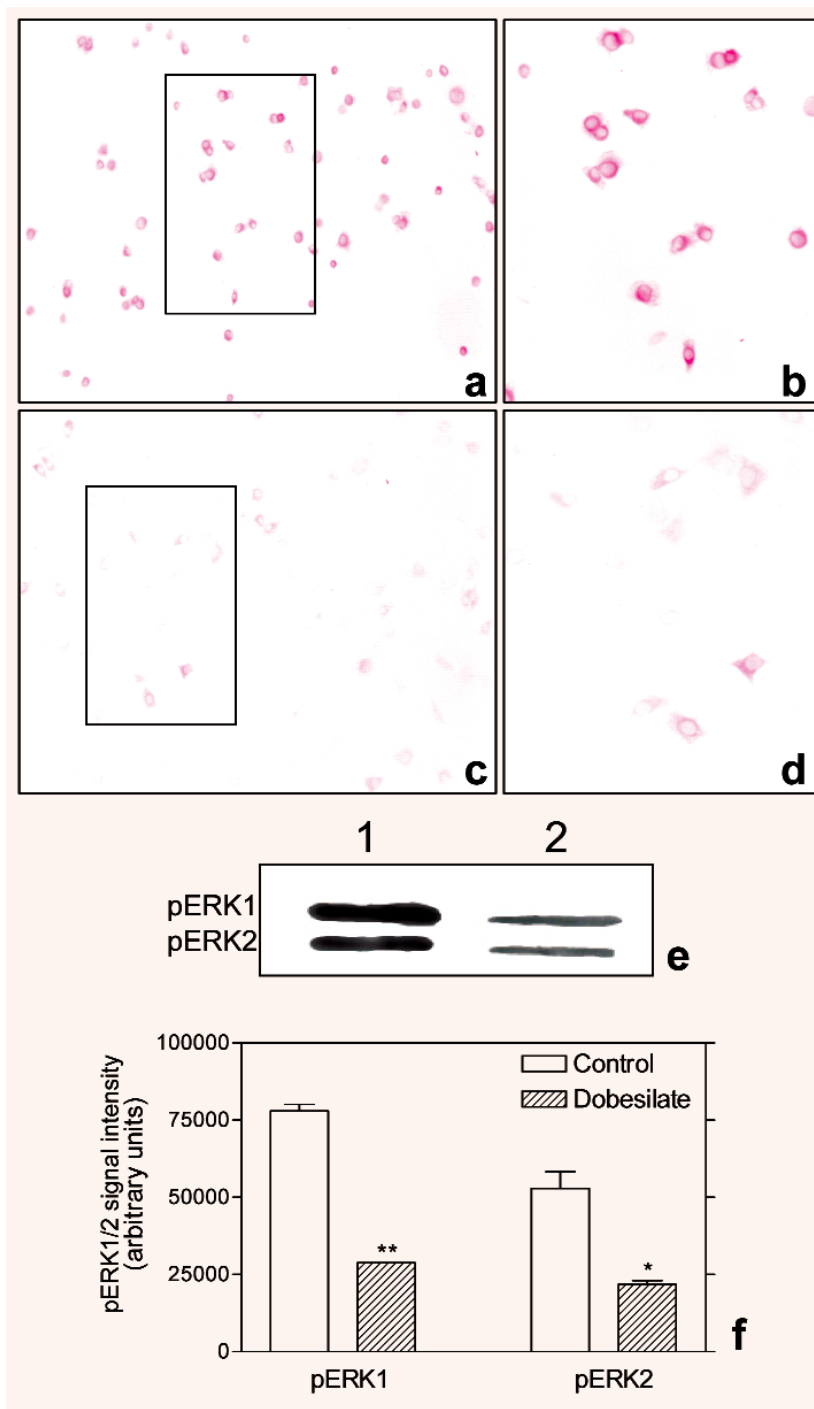


Fig. 1 Immunocytochemical and Western blot detection of ERK1/2 in glioma cells. Panels **a** and **b**, untreated glioma cells. Panels **c** and **d**, glioma cells treated with dobesilate (100 μM for 1 h). Panels **b** and **d** are high-powered views of the boxed areas shown in the two respective panels of the left; **e**, Western blot analysis shows that ERK1/2 protein is less detectable after dobesilate treatment of glioma cells; lane 1, control cells; lane 2, dobesilate-treated cells; **f**, quantification levels of ERK1/2 were determined by densitometric analysis (* $p < 0.05$; ** $p < 0.01$) (**a** and **b**, original magnification $\times 40$; **c** and **d**, $\times 80$) Results are representative of data obtained in three independent experiments.

are expressed at high rates in gliomas, and that their synthesis is upregulated during the transition from low-to-high-grade malignancy [15, 16]. We therefore investigated whether this important signaling event in glioma cell proliferation was affected by dobesilate, as an assessment of whether FGF signaling was involved in the observed inhibitory activities of dobesilate [4],

since dobesilate has been already reported to inhibit FGF activity in an angiogenesis model [3]. Furthermore, dobesilate inhibits FGF-induced proliferation of human fibroblasts [20]. As reported here, exposure to dobesilate resulted in an immediate relative short-term inactivation of ERK1/2 in glioma cells, as assessed by immunocytochemistry and Western blot. Although FGF is

poorly released in normal conditions, experimental evidence supports that extracellular FGF is responsible for the ERK activation [21], and autonomous proliferation of C6 rat glioma cells [22]. These findings suggest that the antiproliferative effect of dobesilate may be mediated *via* extracellular FGF/ERK1/2 pathway. Although the present results are obtained in FCS-supplemented culture conditions, the known ability of dobesilate to specifically inhibit FGF-driven actions [3, 20] suggests that dobesilate reduces ERK activation by antagonizing FGF-induced effects. However, the experimental conditions do not allow to discard the possible inhibitory effect of dobesilate on an ERK activating pathway independent of FGF. This inactivation appears parallel to the shift of the cell shape towards a more differentiate phenotype. Differentiation agents for treatment of malignancies constitute an attractive therapeutic strategy, particularly for gliomas [23–25]. Such agents have been used to inhibit glioma cell growth by changing their anaplastic phenotype in another more normal phenotype. Since differentiation status of glial cells can be assessed by glial fibrillary acidic protein (GFAP) [26], and the intensity of GFAP staining of glial tumors is inversely related to their degree of anaplasia [27], the effect of dobesilate in GFAP expression in those cells deserves future evaluation in longer exposure of culture treatment.

Low-molecular-weight compounds targeting growth factors seem attractive approaches for developing new drugs, because they normally show excellent pharmacological properties in term of stability and bioavailability [28]. A considerable effort has been directed toward the discovery of FGF inhibitor agents with these features and the evaluation of their therapeutic applications [22, 29–31]. Using structure-based screening procedures, we have previously designed several compounds with these characteristics that show antitumoral activities both *in vitro* and *in vivo* [32, 33]. The results reported here show that dobesilate is a new compound to be added to that list, at least, as a lead compound for the development of new therapeutic approaches to treat gliomas. Furthermore, dobesilate is unique in that it is currently in clinical use to treat vascular complications of diabetic retinopathy [2] and, hence, its pharmacokinetics is already known.

Acknowledgements

The present work was founded in part by Investread Europa SL. The authors thank C. Bourdier for editorial assistance and C. Muela for photographic work.

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