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Inhibitory Effect of Trapidil on Human Malignant Glial Cell Proliferation via Interruption of Autocrine Growth Stimulation

Otokrin Büyüme Stimülasyonunun Engellenmesi Yoluyla İnsan Malign Glial Hücreleri Üzerine Trapidilin İnhibitor Etkisi

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ABSTRACT

Two human malignant glial cell lines, U-251 and NMCG-I were used in this study. NMCG-I cell lines were been established from an astrocytoma, grade III by our clinic. Glial cells secrete platelet-derived growth factor (PDGF) - like molecules that stimulate their own growth in an autocrine manner. Based on this finding, a study was undertaken to examine the effect of trapidil, a drug known to have an antagonistic action against PDGF, on cell proliferation of human glial cell lines. Trapidil showed dose-dependent inhibition of glial cell proliferation in the absence of any exogenous mitogenic stimulation. The maximum effect was observed at a concentration of 100 mg/ml, with the decrease in cell growth compared to control cell lines (55% decrease for NMCG-I and 47% decrease for U-251). While the conditioned medium generated from U-251 and NMCG-I cell lines remarkably stimulated the proliferation of glial cells, with an increase of 67% for the control U-251 cell lines and 140% for the control NMCG-I cell lines, this effect was strikingly inhibited by the addition of trapidil. Epidermal growth factor remarkably stimulated the proliferation of glial cells, with an increase of 50% for the control U-251 cell lines and 73% for the control NMCG-I cell lines and this effect was also strikingly inhibited by the addition of trapidil. The maximum inhibitory effect, with a trapidil dose of 100 mg/ml, showed a 30% and 50% decrease in cell number, for U-251 and NMCG-1 respectively, compared to the EGF-stimulated cell growth. The overall results suggest that trapidil exhibits an inhibitory effect on glial cell proliferation by blocking the mitogenic stimulation induced by autocrine or exogenous growth factors, and may be considered as a possible new approach to the medical treatment of glial tumors.

KEY WORDS: Glial cells, trapidil, platelet derived growth factor, autocrine system.

ÖZ

Bu çalışmada, insan malign glial hücre tipleri olan U-251 ve NMCG-1 hücreleri kullanıldı. NMCG-1 hücre tipi kliniğimizde astrositom Grade III den elde edildi. Glial hücreler otokrin bir şekilde kendi büyümelerini stimüle eden bir molekül olan plateled-derived growth factor (PDGF) salgılarlar. Bu bulguya dayanarak, bu çalışma insan glial hücre türünün proliferasyonu üzerine PDGF'ye karşı bir antagonistik etkiye sahip olduğu bilinen bir ilaç olan trapidilin etkilerini incelemek için yapıldı. Trapidil herhangi birek sojen mitojenik stimulasyonun yokluğunda glial hücre proliferasyonuna doza bağımlı bir etki gösterdi. Maksimum etkiyi gösteren 100 ng/ml'lik konsantrasyonlarda, kontrol grup ile karşılaştırıldığında U-251 hücrelerinde %47, NMCG-1'de ise %55 azalma gözlendi. U-251 ve NMCG-1 hücre türlerinden elde edilen conditioned medium, kontrol grubu ile mukayese edildiğinde U-251 hücrelerinde %67 ve NMCG-1 hücrelerinde %140 artışa neden oldu. Bu etki trapidil ile belirgin olarak inhibe edildi. Epidermal growth factor (EGF), kontrol hücreleri ile mukayese edildiğinde U-251 hücrelerinde %50, NMCG-1 hücrelerinde %73'lük artış stimüle etti. Bu etki trapidilin ilavesi ile belirgin olarak inhibe edildi. EGF ile stimüle edilen hücre artışı ile kıyaslandığında 100 ng/ml lik trapidilin inhibitör etkisi, U-251 hücre sayısında %30, NMCG-1 hücre sayısında %50 düşüş olarak bulundu. Bu ortalama sonuçlar, trapidilin otokrin ve eksojen büyüme faktörleri tarafından sebep olunan mitojenik stimulasyonu bloke etme yoluyla glial hücre proliferasyonuna inhibitör etkisini göstermektedir ve glial tümörlerin medikal tedavisinde muhtemel yeni bir yaklaşım olarak düşünülebilinir.

ANAHTAR SÖZCÜKLER: Glial hücreler, trapidil, plateled derived growth factor, otokrin sistem

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INTRODUCTION

Cell proliferation is regulated by growth factors that stimulate mitogenicity by binding to specific receptors on target cells. Platelet-derived growth factor (PDGF) is one of the growth factors produced by many kinds of cells. An autocrine loop through PDGF and PDGF-receptor is important for the proliferation of tumor cells. (4, 23). Appropriate concentration of an anti-PDGF neutralising antibody inhibited basal DNA synthesis and proliferation in the absence of added growth factors, suggesting a possible role for PDGF in autocrine stimulation of these cells (17). PDGF may be acting as a dominant mitogen to enhance DNA synthesis, and may function in autocrine stimulation (15).

The prognosis for patients with malignant gliomas treated with the currently available modalities of surgery, radiotherapy, and chemotherapy remains poor. Further improvements in patient survival may depend upon a deeper understanding of the growth factor pathways that regulate glioma proliferation. During the last several years, increasing interest has been focused on the role of various polypeptide growth factors such as epidermal growth factor EGF (3, 7, 20), platelet-derived growth factor (PDGF) (18), Fibroblast growth Factor (FGF's) (6) and the insulin-like growth factors (IGF's) (5) in the growth of malign gliomas. Many malignant glial lines have receptors for PDGF, and synthesize and secrete a PDGF-like mitogenic factor (2, 8, 11, 12, 16) suggesting the occurrence of autocrine stimulation (19).

Trapidil was originally developed as a coronary vasodilator, the pharmaceutical properties of which include a nitroglycerin-like vasodilating action, an inhibitory action on platelet aggregation through thromboxane A2 inhibition, enhancement of platelet aggregation protection by prostacyclin, facilitation of the biosynthesis of prostacyclin, and improvement of lipid metabolism (14, 15). Trapidil is a drug known to have an antagonistic effect on PDGF (14). In this study, we examined the effect of trapidil on the proliferation of low-passage human glial cells in culture and investigated the possibility of administering trapidil for the medical treatment of gliomas.

MATERIALS AND METHODS

This study was done in Tokyo International Medical Center Hospital.

Cell Culture

The human glioblastoma cell lines U-251 and NMCG-I (derived from spontaneously occurring human malignant gliomas)* were initially grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS)**. Cultures were established in 75-59 sq cm tissue-culture flasks***, maintained at 37oC in a humid atmosphere with 5% CO2 in air, and subcultured every 4 to 6 days by treating the monolayers with 0.25 % trypsin in Hanks' balanced salt solution.

Generation of Glial Cell - Derived Conditioned Medium

U-251 and NMCG-I that were passaged once were grown to confluence in 25-59 sq cm tissue culture flasks. After the growth medium was removed, the cells were washed twice with 6 ml PBS and then supplemented with 6 to 8 ml of either serum free growth medium or growth medium containing 5% vol/vol charcoal stripped FCS. After incubation at 37^{O}C for 24 hours. The resultant glioma-derived conditioned medium was collected. The conditioned medium was always used fresh for investigation, without a storage period.

Cell Growth Studies

Cells were plated into 25 - 59 sq cm tissue culture flaks (0.5 x 105 to 1 x 105 cells/flask, in 3 ml of growth medium containing 10% FBS. After incubation at 37 ^{O}C for 24 hours, the growth medium was completely removed, and the cells were incubated in Eagle's MEM containing 5% charcoal-stripped FCS with various treatments: Trapidil (5 - Methyl-7diethylamino-5-triazola 1.5-a pyrimidine)* in various concentrations (1 to 100 mg/ml, conditioned medium (50% vol/vol), and EGF (10 ng/ml). The medium was changed every 3rd or 4th day, and the cells were counted 8 to 14 days after treatment. The number of cells in each flask was determined by releasing cell nuclei in 2 ml HEPES buffer (10 mM) containing 1.5 mM MgCl2 and 0.2 ml Zapponin cell lysing agent and counting them with a cell counter.

* Glioma cell lines supplied by JAPAN Type Collection, International Medical Center, Tokyo, JAPAN.

** Fetal Calf serum supplied by GIBCO laboratories, Grand Island, New York.

*** Tissue-culture flasks manufactured by Greiner, Nürtingen, Germany.

* Trapidil provided by Shionogi Co Ltd., Tokyo, Japan.

** Zapponin cell lysing agent and cell counter manufactured by Coulter Electronics, Krefela, Germany.

Statistical Analysis

All studies were performed in triplicate. Statistical evaluation of all the data was by Student's t-test.

RESULTS

Effect on Glioma Cell Proliferation

Cell growth studies were performed in U-251 and NMCG-I glial cell lines to investigate the effect of trapidil in the absence of any exogenous mitogenic stimulation. A concentration of trapidil ranging from 1 to 100 mg/ml caused significant inhibition of cell proliferation in a dose-dependent manner. The inhibitory effects of the concentrations on U-251 and NMCG-1 cells compared to control specimens were 10% and 4% for the 1mg/ml concentration, 16% and 8% for the 5mg/ml concentration, 23% and 16% for the 10ng/ml concentration, 32% and 28% for the 50ng/ml concentration, and 47% and 55% for the 100mg/ml concentration, respectively.

Effect on Conditioned Medium - Stimulated Cell Proliferation

To examine the effect of trapidil on the growth stimulation induced by conditioned medium derived from glioma cells that were passaged, cell growth studies were performed in the U-251 and NMCG-I cell lines. The increase in cell proliferation was 25% for 12.5% vol/vol, 47% for 25% vol/vol, and 67% for 50% vol/vol on U-251 cells. The increase in NMCG-1 cells was 50% for 12.5% vol/vol, 100% for 25% vol/vol, and 140% for 50% vol/vol (Figure 2). The addition of trapidil (10 and 100 g/ml) led to a dose-dependent inhibition of this conditioned medium-stimulated cell growth. The inhibitory effect, with a trapidil dose of 10 mg/ml, was a 15% and 25% decrease in cell number for U-251 and NMCG-1, respectively, compared to the conditioned medium-stimulated cell growth. The inhibitory effect, with a trapidil dose of 100 mg/ml, showed a 25% and 45% decrease in cell number for U-251 and NMCG-1, respectively, compared to the conditioned medium-stimulated cell growth. (Figure 3)

Effect on Epidermal Growth Factor Stimulated Cell Proliferation

To examine the effect of trapidil on the growth stimulation induced by EGF on cells that were passaged, cell growth studies were performed in U-251 and NMCG-I cell lines. Specimens showed 50% and 73% increase in cell proliferation in the presence of EGF 10 ng/ml for U-251 and NMCG-1 cells, respectively. The addition of trapidil (10 and 100 mg/ml) led to a dose-dependent inhibition of this EGF-stimulated cell growth. The maximum inhibitory effect, with a trapidil dose of 100 mg/ml, showed a 30% and 50% decrease in cell number for U-251 and NMCG-1 cells, respectively, compared to the EGF-stimulated cell growth. On the other hand, the inhibitory effect, with a trapidil dose of 10 mg/ml, showed a 20% and 27% decrease in cell number for U-251 and NMCG-1 cells, respectively (Figure 4.).

DISCUSSION

Malignant glial cells secrete platelet-derived growth factor-like molecules that stimulate their own growth in an autocrine manner.

Inhibitory Effect on Malignant Cell Proliferation

We found that the inhibitory effect of trapidil on cell proliferation in U-251 and NMCG-1 cells was 47% and 55%, respectively, in the absence of any exogenous mitogenic stimulation. These results are similar to some other studies (7, 12).

Trapidil was originally developed as a coronary vasodilator. Its unique characteristic as an antagonist to PDGF has been reported solely by Ohnishi et al. (14) who showed that trapidil (3 to 10 mg/ml) inhibited BALB/c 3T3 cell proliferation stimulated by PDGF, but did not inhibit that stimulated by fibroblast growth factor or Ca3(PO4)2. As the mechanism of growth regulation by trapidil remains unclear, Kuratsu et al. postulated that trapidil affects the intracellular signal transduction pathway, protein kinase C (PKC) activity and c fos expression in cells stimulated with serum containing growth factors (9). Trapidil and suramin block the PDGF-induced calcium response and inhibit the PDGF-initiated tyrosine phosphorylation of the PDGF receptor as detected by Western blot analysis using an antibody specific for phosphotyrosine (1).

In our study, we used that U-251 and NMCG-1 cells. We investigated the inhibitory effect of trapidil on these cells stimulated by Conditioned Medium. It was observed that there was a decrease of 25% and 45% on U-251 and NMCG-1 cells, respectively (with 100mg/ml dose of trapidil).

The present report demonstrates that trapidil inhibits in vitro cell proliferation of human gliomas. The studies using glioma-derived CM revealed that trapidil inhibits conditioned medium-stimulated cell proliferation. Remarkable inhibition of conditioned medium-stimulated DNA synthesis by trapidil was

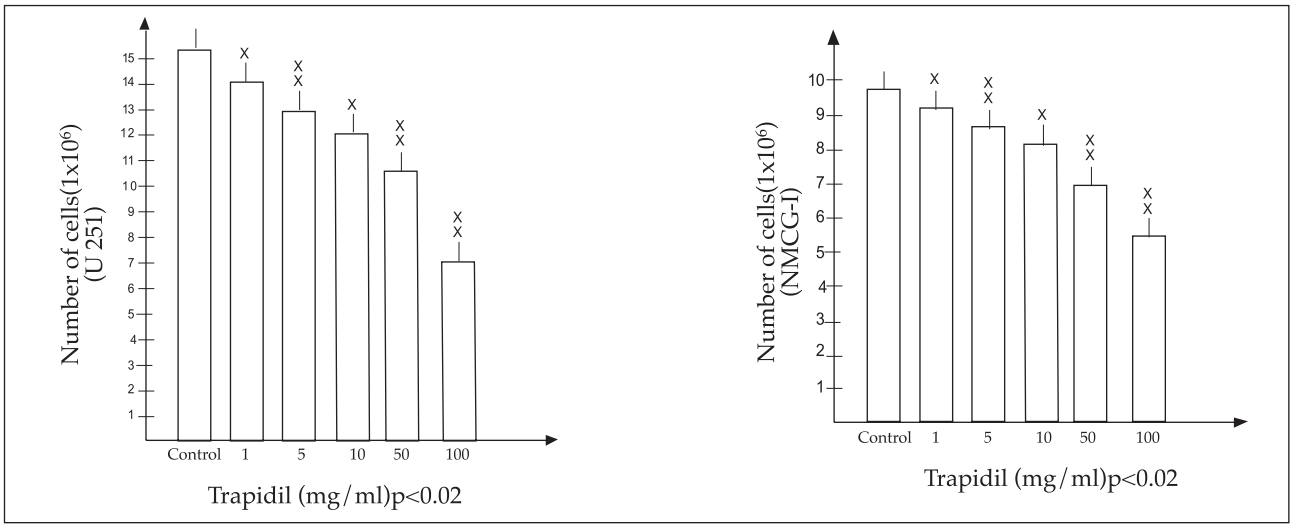

Figure 1. Graphs showing the effect of trapidil on glial cell line proliferation. Vertical bars represent the Standard deviation. Left and Right: A dose-dependent inhibitory effect was observed on proliferation in the U-251 cell line and NMCG-I cell line. U-251 glial cells were seeded at 5x105 cells/flask and NMCG-I glial cells were seeded at 1 $x10^5$ cells/flask. The number of cells was determined 8 days after treatment with various doses of trapidil. Statistical significance: $^{x}p < 0.02$, $^{xx}p < 0.01$, compared to control.

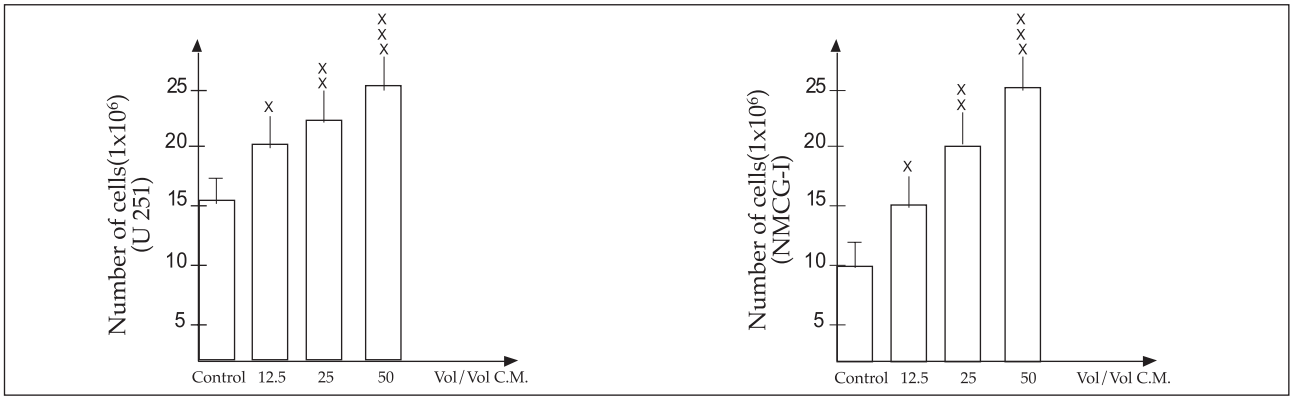

Figure 2. Graphs showing the effect of conditioned medium (C.M.) on glial cell lines. Both U-251 cells and NMCG-I cells showed a maximum increase in cell proliferation in the presence of conditioned medium (50% vol/vol growth medium containing 5 % charcoal - stripped fetal calf serum). Statistical significance: , $^{x}p < 0.02$, $^{xx}p < 0.01$, , $^{xxx}p<0.001$

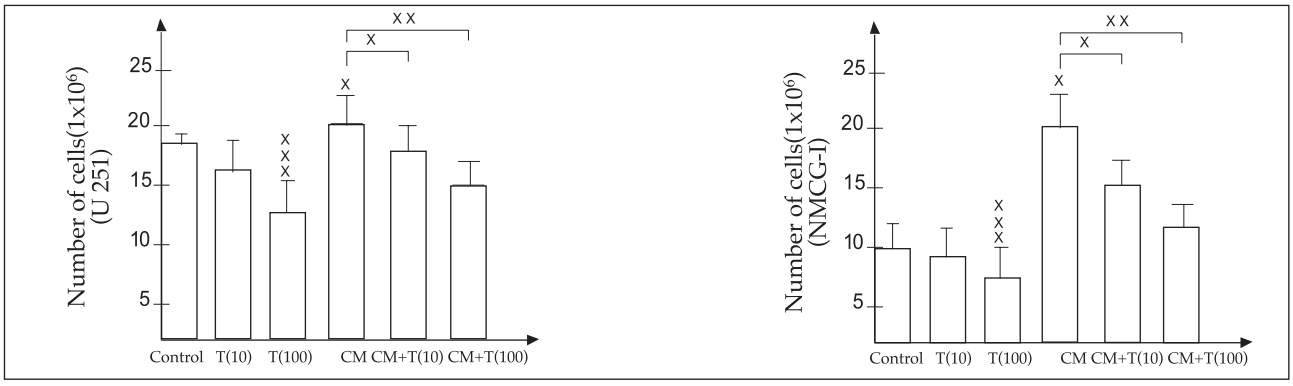

Figure 3. Graphs showing the effect of trapidil on conditioned medium (CM)-stimulated cell proliferation. T(10) = trapidil at a dose of 10 mg/ml, T(100) = trapidil at a dose of 100 mg/ml. Vertical bars denote the standard deviation: $^{x}= p < 0.05$, $^{xx}= p < 0.01$, $^{xxx}= p < 0.01$, compared to control, or CM with trapidil compared to CM alone. An inhibitory effect was observed on CM-stimulated cell proliferation in human glial cell lines (U-251 and NMCG-I). U-251 cells were seeded at 5 x 10^4 cells/flask and NMCG-I cells were seeded at 1 x 105 cells/flask, and the number of cells was determined 13 days after treatment with glioma-derived CM (50 % vol/vol in growth medium containing 5% charcoal-stripped fetal calf serum) in the presence of trapidil (10 to 100 mg/ml)

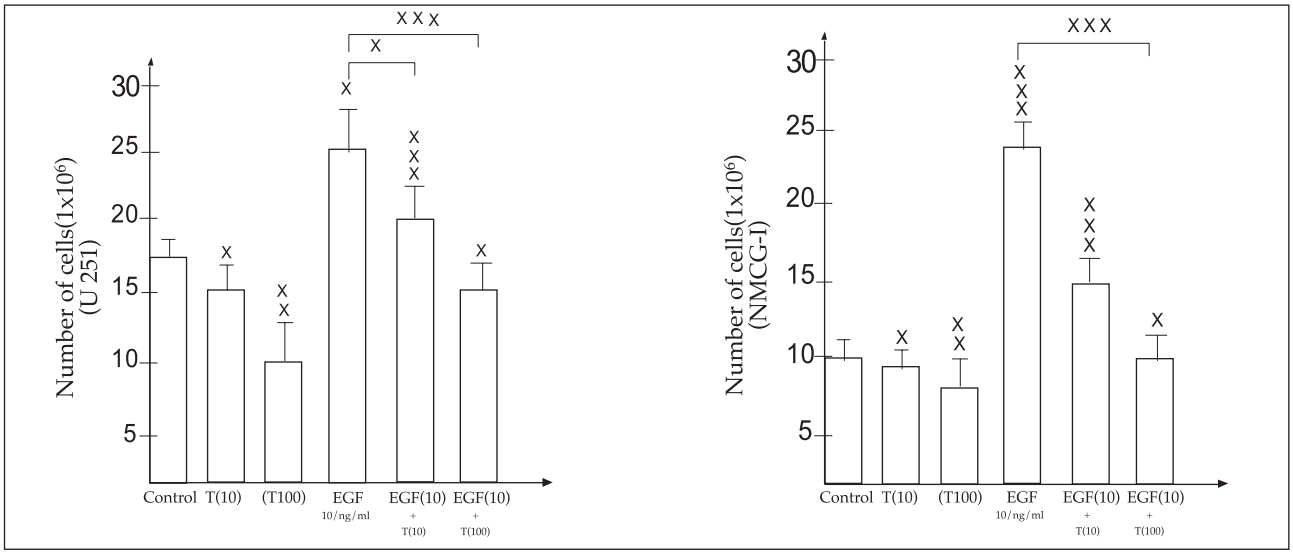

Figure 4. Graphs showing the effect of trapidil on epidermal growth factor (EGF)-stimulated cell proliferation. EGF (10) = Epidermal growth factor at a dose of 10 ng/ml. T(10) = trapidil at a dose of 10 mg/ml, T(100) = trapidil at a dose of 100 mg/ml. Vertical bars denote the standard deviation: ^{x}= $p < 0.02$, ^{xx}= $p < 0.01$, ^{xxx}= $p < 0.01$ compared to control, or EGF (10 ng/ml) with trapidil compared to EGF alone. An inhibitory effect was observed on EGF-stimulated cell proliferation in human glial cell lines (U-251 and NMCG-I). U-251 cells were seeded at $5x10^4$ cells/flask and NMCG-I cells were seeded at 1 x 105 cells/flask, and the number of cells were determined 13 days after treatment with EGF (10 ng/ml), containing 5% charcoal-stripped fetal calf serum in the presence of trapidil (10 to 100 mg/ml).

observed even when there was no effect on the basal DNA synthesis. A similar antiproliferative effect of trapidil has been reported on glioma cells (21): trapidil inhibited the proliferation of U-251 MG cells which produce PDGF-like molecules (8), but did not inhibit that of U-105 MG cells which do not produce PDGF-like molecules (18). Our report demonstrated that trapidil inhibited the proliferation of both U-251 MG cells and NMCG-I MG cells which produce PDGF-like molecules. Trapidil has also been shown to inhibit the baseline and PDGF-induced cell proliferation in cultured glioblastoma cells (17).

Effect as an Antagonist to Epidermal Growth Factor

In the end of study, U-251 and NMCG-1 groups stimulated by EGF were found to have on increase of 50% and 70% respectively. 100 mg/ml Trapidil was given to these groups. It was observed that there was 30% decrease in U-251 and 50% decrease in NMCG-1 cells.

Although the anti-PDGF action of trapidil seems to be certain and several studies have been reported using trapidil as an anti-PDGF drug (8), its precise mechanism is still unknown, and no report indicates whether the antagonistic effect of trapidil is specific to PDGF or not. Trapidil and suramin also inhibit the EGF-initiated calcium response in T98G cells (1), but only partially inhibit EGF- initiated tyrosine phosphorylation at the same concentration (1). Trapidil and suramin inhibit PDGF- and EGF-initiated early biochemical events, and thus suppress growth factor-induced cell proliferation (1). Our results further show that trapidil inhibits EGF-stimulated cell proliferation.

Trapidil as a Therapeutic Drug for Malignant Gliomas

Our data on the inhibitory effect of trapidil on in vitro malignant glioma growth introduce the possibility of a new method for medical adjuvant therapy for malign gliomas. Trapidil is different from any other known therapeutic agents, such as gender-specific steroids and dopamine agonists, because of its novel mechanism of action. It has also been reported that trapidil not only antagonizes the effect of PDGF, but also inhibits the proliferation induced by EGF, emphasizing its broad spectrum of action (1, 22). Furthermore, because of this unique mechanism, an enhanced effect can be expected if trapidil is used in combination with another agent with a different mechanism of action.

Trapidil is already in clinical use as a drug treatment for angina pectoris or as maintenance therapy after cerebral apoplexy, and thus a large amount of clinical data is available. A standard oral

trapidil dose of 100 mg in a human results in a peak plasma level of 3 to 4 mg/ml. Since our data showed that the inhibitory effect of trapidil is already observed at a dose of 10 mg/ml, there may be a good chance that trapidil would have an inhibitory effect on the malignant glioma growth in patients if used in clinical trials with or without other drugs in combination.

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