Antiproliferative effect of trapidil, a platelet-derived growth factor antagonist, on a glioma cell line *in vitro*

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Platelet-derived growth factor (PDGF) is produced by glioma cells. However, there is heterogeneity among glioma cell lines in the production of PDGF. It has been demonstrated that U251MG cells produce a PDGF-like molecule while U105MG cells do not.

Trapidil, a specific antagonist of PDGF, competes for receptor binding with PDGF. Therefore, the inhibitory effect of trapidil on the proliferation of glioma cells was investigated *in vitro* using two glioma cell lines. At 100 μg/ml, trapidil significantly inhibited the proliferation of U251MG cells (which produce the PDGF-like molecule). At the same trapidil concentration, the proliferation of U105MG cells (which do not produce the PDGF-like molecule) was not inhibited. The inhibitory effect of trapidil was remarkable on Days 3 and 4 of culture. After 4 days of incubation, the proliferation of U251MG cells was 46% of the control preparation. Trapidil enhanced the antitumor effect of 3-((4-amino-2-methyl-5-pyrimidinyl)ethyl)-1-(2-chloroethyl)-1-nitrosourea (ACNU) against U251MG cells. The enhancing effect was highest on Days 4 and 6 of culture. After 6 days of incubation in the presence of 100 μg/ml trapidil and 1 μg/ml ACNU, the proliferation of U251MG cells was 18% of the control preparation. These findings suggest that trapidil interrupts the autocrine loop at the PDGF and PDGF-receptor level and that combination therapy with trapidil and ACNU may be useful in the treatment of glioma.

**KEY WORDS**

glioma • growth factor • trapidil

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**Materials and Methods**

**Drugs**

The drugs used in this study were trapidil (5-methyl-7-diethylamino-S-triazolo 1,5-a pyrimidine), recombinant monoclonal anti-PDGF antibody specific for PDGF B chain, and ACNU (3-((4-amino-2-methyl-5-pyrimidinyl)ethyl)-1-(2-chloroethyl)-1-nitrosourea).

**Tissue Culture Conditions and Drug Treatment**

Glioma cells in the exponential growth phase were harvested by a brief treatment with 0.25% trypsin, suspended in an RPMI 1640 medium that was free of serum, seeded into flat-bottomed 96-well microtiter plates in quadruplicate (5 x 10³ cells/well), and incubated at 37°C. After 24 hours of incubation, the cultures were washed and re-fed with 100 μl of supplemented medium. Trapidil or anti-PDGF at the indicated concentrations was added to each well in a volume of 10 μl. In some experiments, on Day 4 of incubation the culture was re-fed with fresh medium containing only 10% fetal bovine serum (FBS). At the selected times, the antiproliferative activity of trapidil or anti-PDGF antibody was determined by monitoring the number of metabolically active cells by means of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (see below).

In the same manner, trapidil, ACNU, or trapidil combined with ACNU at the indicated concentrations was added to each well. At selected times, the antiproliferative effect of trapidil and ACNU was determined by monitoring the number of metabolically active cells by means of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.
Antiproliferative effect of trapidil

Proliferative activity of trapidil, ACNU, or trapidil combined with ACNU was determined by MTT assay. Absorbance by drug-treated cultures was compared with that in the control cultures in supplemented medium. Growth inhibition was calculated and expressed as: % growth (± 1% standard deviation) = (A - background/B - background) × 100, where A represents absorbance of samples containing the agents and B represents absorbance of the medium control.

MTT Assay

To evaluate drug effects on cell growth, we used an automated microculture tetrazolium assay with MTT. Culture plates were incubated for various time periods. The MTT stock solution was prepared as follows: 4 mg MTT/ml phosphate-buffered saline was filtered in sterile manner through a 0.45-μm filter and stored at 4°C for a maximum of 1 month. The MTT working solution was prepared just prior to use by diluting the stock solution 1:1(v/v) in prewarmed standard culture medium, then was added in a 50-μl aliquot to each culture well. The cultures were incubated at 37°C for 4 hours, after which cell monolayers and formazan were inspected microscopically. Culture plates containing suspension lines or detached cells were centrifuged at low speed for 5 minutes and medium supernatants were removed by slow aspiration. Following thorough formazan solubilization by 1 N HCl-isopropanol, the absorbance of each well was measured using a microculture plate reader* at 570 nm.

Results

Antiproliferative Effect of Anti-PDGF

Plates containing U251MG or U105MG glioma cells were cultured with 2 μg/ml of anti-PDGF antibody for 3 days and then assayed. The U251MG cells were sensitive to anti-PDGF, the growth of these cells was 44.9% ± 6.6% of the control level. The growth of U105MG cells was not inhibited (Fig. 1).

Antiproliferative Effect of Trapidil

In the next set of experiments, we examined the antiproliferative effects of trapidil on U251MG and U105MG cells. The U251MG cells were sensitive to the antiproliferative effects of trapidil. As shown in Fig. 2, the addition of trapidil at a dose of 10 μg/ml decreased cell growth to 87% of the control level, and at a dose of 100 μg/ml caused inhibition of cell growth to 46% of the control monolayer. On the other hand, U105MG cells were only slightly sensitive to trapidil at a dose of 100 μg/ml.

After 2 days of exposure to trapidil (100 μg/ml), the growth of U251MG cells was inhibited to 82%. After 4 days of exposure, cell growth inhibition reached a plateau of 46% (Fig. 3).

Antiproliferative Effects of Trapidil with ACNU

In the next set of experiments, we examined whether the combination of trapidil and ACNU produced additive or synergistic cytostatic effects. As shown in Fig. 4, when the medium contained ACNU alone (1 μg/ml), the proliferation of U251MG cells was 63.2% of the control cultures. In the presence of combined ACNU (1 μg/ml) plus trapidil, cell proliferation was further inhibited. Cell growth was 56.3% and 31.4% of the control in the additional presence of 10 and 100 μg/ml trapidil, respectively, suggesting that the combination treatment produced additive effects.

While the number of U251MG cells gradually increased in serum-free medium containing 100 μg/ml trapidil until the 4th day of culture, there was a rapid increase after the change on Day 4 to fresh medium.
FIG. 3. The antitumor effects of trapidil (100 μg/ml) on U251MG cells over time. The percent growth was calculated on Days 2, 4, and 6 using the MTT assay (see text).

FIG. 4. The antitumor effects of combined trapidil and ACNU on U251MG cells. The cells were incubated with ACNU alone (0.1 to 5.0 μg/ml, open circles), or ACNU plus trapidil (10 μg/ml, squares), or ACNU plus trapidil (100 μg/ml, solid circles).

FIG. 5. At 4 days after initiation of the cultures (arrow), the media containing only trapidil (100 μg/ml) or trapidil (100 μg/ml) plus ACNU (1 μg/ml) were removed and the cultures re-fed with medium containing only 10% fetal bovine serum (FBS). The number of cells treated with trapidil alone increased rapidly after the change of culture medium (open circles). In contrast, the number of cells treated with trapidil plus ACNU decreased after the change to medium containing 10% FBS (solid circles).

containing only 10% FBS (Fig. 5). This indicates that the antiproliferative effect of trapidil is cytostatic for U251MG cells. The number of U251MG cells treated with combined trapidil plus ACNU did not rapidly increase after re-feeding with fresh medium containing 10% FBS.

These experiments suggest that combined treatment with trapidil and ACNU exerts cytostatic effects on U251MG cells. The antiproliferative effects over time of combined 100 μg/ml trapidil and 1 μg/ml ACNU were similar to those observed in the presence of either agent alone. The percent cell growth was markedly suppressed 6 days after the initiation of culture (Fig. 6).

Discussion

Growth Factor

Growth factors such as PDGF, epidermal growth factor, and fibroblast growth factor have been reported to stimulate the proliferation of astrocytes in vitro. Platelet-derived growth factor, which is released from platelets, is one of the growth factors in serum and has been shown in vitro to stimulate the proliferation of fibroblasts, smooth-muscle cells, and human glial cells.

We recently reported that a PDGF-like substance is secreted by U251MG cells but not by U105MG cells and that there is heterogeneity among glioma cells in the production of growth factor. In the present experiments, the growth of U251MG cells was inhibited by anti-PDGF antibody while that of U105MG cells was not (Fig. 1). This observation led us to hypothesize that the proliferation of U251MG cells is PDGF-dependent and that it might be inhibited by pharmacological agents capable of interfering with PDGF.

Receptor-Competitive Inhibition by Trapidil

Trapidil is thought to be a potent coronary vasodilator and it is an agent capable of interfering with PDGF. The PDGF-induced proliferation of BALB/c 3T3 fibroblasts was markedly suppressed when these cultures were pretreated with trapidil. However, fibroblast proliferation was not suppressed when PDGF was introduced into the cultures prior to the addition of trapidil, or when trapidil was introduced into the cultures less than 4 hours before the addition of PDGF, or
Antiproliferative effect of trapidil

![Graph showing percent growth over time](image)

**Fig. 6.** The antitumor effect of combined trapidil plus ACNU on U251MG cells over time. The cells were incubated with ACNU alone (1 μg/ml, open circles) or with a combination of trapidil (100 μg/ml) and ACNU (1 μg/ml, solid circles).

when mitogens other than PDGF were used to stimulate proliferation.\(^{1,2,10}\) Ohnishi, *et al.*,\(^{12}\) postulated that trapidil functioned as a competitive inhibitor, binding preferentially to PDGF-receptor sites on target cells, thus preventing PDGF from exerting its stimulating effect. The observation that 4 hours of preincubation with trapidil is necessary for the drug to exert its inhibitory effect confirms such a mechanism. Furthermore, Rutherford and Ross\(^{14}\) have shown that smooth-muscle cells can be stimulated to synthesize deoxyribonucleic acid (DNA) after 1 to 2 hours of exposure to PDGF. The anti-PDGF activity of trapidil was markedly weakened when the drug was added to the medium 2 hours after the induction of proliferation by PDGF, suggesting that trapidil does not directly reduce DNA synthesis.\(^{12}\)

A possible alternative to the above mechanism might be that trapidil exerts a direct cytotoxic effect on glioma cells. Because our experiments showed that U105MG cells, which were not dependent on PDGF, were not affected by trapidil (Fig. 2), a nonspecific cytotoxic effect of trapidil at a dose range up to 100 μg/ml can be ruled out.

Takamiya, *et al.*,\(^{19}\) who studied the association of PDGF with the appearance of reactive astrocytes following brain injury, found that the appearance of reactive astrocytes in the wound was dramatically suppressed by the administration of trapidil. In our experiments, trapidil inhibited the proliferation of PDGF-dependent glioma cells.

**Antiproliferative Effect of Trapidil and Suramin**

The antiproliferative effect of trapidil seems to be cytostatic, because trapidil-treated U251MG cells exhibited regrowth after re-feeding of the cultures with fresh medium containing 10% FBS. Combined treatment with trapidil plus ACNU more effectively inhibited the growth of U251MG cells. The observed effect seems to be cytotoxic because U251MG cells treated with trapidil plus ACNU did not show regrowth after re-feeding of the cultures with fresh medium containing 10% FBS (Fig. 5).

Suramin has attracted attention as an anticancer drug with a unique mechanism of action.\(^{2,5,17,18}\) Like trapidil, this compound inhibits the binding of PDGF to PDGF receptors, although its action is not PDGF-specific. Unlike trapidil, suramin can inhibit the binding of other growth factors (such as epidermal growth factor and tumor growth factor-beta) to their receptors.

The study reported here is preliminary. Further cell lines and techniques must be used for confirmation of our findings, and animal studies of the PDGF-producing glioma cell line are indicated.

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**References**


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