# Mechanism of Dexamethasone Suppression of Brain Tumor–associated Vascular Permeability in Rats

## Involvement of the Glucocorticoid Receptor and Vascular Permeability Factor

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## Abstract

**Brain tumor–associated cerebral edema arises because tumor capillaries lack normal blood-brain barrier function; vascular permeability factor (VPF, also known as vascular endothelial growth factor, VEGF) is a likely mediator of this phenomenon. Clinically, dexamethasone reduces brain tumor-associated vascular permeability through poorly understood mechanisms. Our goals were to determine if suppression of permeability by dexamethasone might involve inhibition of VPF action or expression, and if dexamethasone effects in this setting are mediated by the glucocorticoid receptor (GR). In two rat models of permeability (peripheral vascular permeability induced by intradermal injection of 9L glioma cell–conditioned medium or purified VPF, and intracerebral vascular permeability induced by implanted 9L glioma), dexamethasone suppressed permeability in a dose-dependent manner. Since 80% of the permeability-inducing activity in 9L-conditioned medium was removed by anti–VPF antibodies, we examined dexamethasone effects of VPF expression in 9L cells. Dexamethasone inhibited FCS- and PDGF-dependent induction of VPF expression. At all levels (intradermal, intracranial, and cell culture), dexamethasone effects were reversed by the GR antagonist mifepristone (RU486). Dexamethasone may decrease brain tumor–associated vascular permeability by two GR-dependent mechanisms: reduction of the response of the vasculature to tumor-derived permeability factors (including VPF), and reduction of VPF expression by tumor cells. (*J. Clin. Invest.* 1996. 98:1400–1408.) Key words: cerebral edema • vascular endothelial growth factor • glioma • mifepristone • steroids**

## Introduction

Brain tumor–associated vascular permeability arises because tumor capillaries lack the usual blood-brain barrier function of normal brain capillaries (1). This phenomenon is thought to occur at least in part because permeability factors produced by tumor cells act upon the tumor-associated vasculature. As a result, plasma components leak from the blood stream into the extracellular space of the brain, creating brain tumor–associated cerebral edema (2–4). This cerebral edema compounds the neurologic dysfunction associated with the underlying tumor.

Glucocorticoids reduce brain tumor–associated vascular permeability and cerebral edema, reduce intracranial pressure, improve accompanying symptoms, and provide palliation of patients with malignant gliomas by prolonging useful neurologic function and life span (5–7). Although dexamethasone is a mainstay in the treatment of brain tumor patients, the mechanisms by which glucocorticoids reduce brain tumor–associated cerebral edema are poorly understood, and considerable morbidity is associated with their use. Iatrogenic Cushing's syndrome, the result of high dose glucocorticoid therapy, is characterized by truncal obesity, glucose intolerance, myopathy, immunosuppression, hypertension, and adrenal suppression.

Whereas most of the side effects of glucocorticoid treatment are presumed to be mediated by the glucocorticoid receptor (GR)[1] (8, 9), it is not known whether the dexamethasone effect on brain tumor–associated edema is dependent on interaction with the GR. Doses of dexamethasone that are used clinically for treating brain tumor–associated cerebral edema vary widely and far exceed those that saturate the pituitary glucocorticoid receptors and suppress the hypothalamic–pituitary–adrenal axis. It has often been suggested that these high doses are required because inhibition of cerebral edema occurs through a pharmacologic mechanism that does not require the GR and that steroids lacking glucocorticoid effects might be appropriate in this capacity (10–16).

Similarly, it is not fully understood at what level this dexamethasone effect on brain tumor–associated edema occurs: (*a*) inhibition of the effects of permeability factors on the capillary bed, and/or (*b*) inhibition of the production of permeability factors by tumor cells. Vascular permeability factor (VPF, also known as vascular endothelial growth factor, VEGF) has been shown to be a likely mediator of increased vascular permeability in some models of peripheral tumors (17, 18), and the VPF secreted by glioma cells has been proposed to play the same role in brain tumors (19, 20). Although VPF expression is clearly increased in response to hypoxia in rat C6 glioma–derived cells (21, 22), the regulation of VPF in glioma cells by

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1. *Abbreviations used in this paper:* 9LCM, 9L cell conditioned medium; $^{14}$C-AIB, α [1-$^{14}$C] aminoisobutyric acid; GR, glucocorticoid receptor; K, blood-to-brain transfer constant; KLH, keyhole limpet hemocyanin; recVPF, recombinant human VPF; RU486, mifepristone; VPF, vascular permeability factor.

other inducers or by dexamethasone has not been well studied, although evidence exists that such regulation may occur in human glioma cells (19, 23). In addition, dexamethasone has been shown to interfere with the early effects of VPF on endothelial cells (24).

These observations emphasize the need for experimental studies that define the mechanism of action and the optimal dosing regimens for glucocorticoid treatment of brain tumor–associated cerebral edema. Further, knowledge of the relative importance of GR- vs non–GR-mediated effects is essential to predict if compounds such as the 21-aminosteroids, which have a structure similar to dexamethasone but lack receptor-mediated side effects, will be effective in reducing brain tumor–associated cerebral edema (15, 25). Such knowledge should provide rational guidelines for optimizing the use of steroids in the treatment of brain tumor–associated cerebral edema while limiting side effects, and be helpful for the development of novel therapeutic approaches to this disease.

These studies were undertaken to elucidate the mechanisms by which dexamethasone suppresses brain tumor–associated cerebral edema, particularly with respect to the roles of the GR and VPF. To address these issues, we used the 9L gliosarcoma brain tumor model in the rat. This model was chosen because of several relevant features. 9L cells secrete VPF both in culture and in vivo (22, 26). The ability of the 9L tumors implanted in the rat brain to increase brain tumor–associated vascular permeability and brain edema is well documented (27–31). Dexamethasone reduces brain tumor–associated cerebral edema in the 9L model as well as in humans (31–36). We examined the effects of dexamethasone on three parameters: (*a*) the ability of 9L cell conditioned medium (9LCM) or purified VPF to induce vascular permeability in peripheral vessels (Miles assay); (*b*) the expression of VPF mRNA by 9L cells in culture; and (*c*) the degree of vascular permeability associated with 9L tumors implanted in the brain using α [1-$^{14}$C] aminoisobutyric acid ($^{14}$C-AIB) as a tracer. To determine to what extent the effects of dexamethasone involve the GR, we examined the reversibility of the dexamethasone effects by the GR antagonist mifepristone (RU486). We report that suppression of brain tumor–associated cerebral edema by dexamethasone may occur through actions both on the tumor cell and on the associated vasculature, and that these effects are GR-mediated and involve VPF.

## Methods

*Reagents.* Dexamethasone sodium phosphate was obtained from The Butler Co. (Columbus, OH) for use in animals, and from Sigma Chemical Co. (St. Louis, MO) for use in cell culture. RU486 (17β-hydroxy-11β-[4-dimethylamino phenyl]-17α-[1-propynyl]estra-4,9-dien-3-1) was graciously provided by Roussel, UCLAF (Paris, France). The 21-aminosteroids, tirilazad mesylate (U-74006F, 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16α-methyl-pregna-1,4,9(11)-triene-3,20 dione monomethanesulfonate), and U-74500A (21-{4-[5,6-*bis* (diethylamino)-2-pyridinyl]-1-piperazinyl}-16α-methyl-pregna-1,4,9(11)-triene-3,20 dione hydrochloride) were graciously provided by The Upjohn Co. (Kalamazoo, MI). DME, penicillin–streptomycin, and fetal calf serum were supplied by Gibco Laboratories (Grand Island, NY). Recombinant human VPF and PDGF-BB were purchased from R & D Systems, Inc. (Minneapolis, MN). Ketamine was obtained from Fort Dodge Laboratories (Fort Dodge, IA). Xylazine was supplied by the Mobay Corp. (Shawnee, KS). Serum corticosterone levels were measured by radioimmunoassay (Hazleton Laboratories, Vienna, VA).

*9L cell conditioned medium.* 9L gliosarcoma cells were grown in DME, 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin to near confluence, washed twice with PBS, and changed to serum-free DME. After 6 d, medium was exhaustively dialyzed against a solution of 0.25 M ammonium bicarbonate (adjusted to pH 7.4 using glacial acetic acid) using a Spectra/Por 6, 25-kD membrane (Spectrum Medical Industries, Inc., Laguna Hills, CA). The dialyzed solution was frozen, lyophilized, and redissolved in PBS. Pilot studies demonstrated that all of the permeability-inducing activity in 9LCM was retained by this procedure. The protein concentration in PBS solutions containing lyophilized conditioned medium was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA).

*Miles assay.* The Miles assay uses intradermal injection of test substances and intravascular injection of Evans blue dye (which binds to endogenous serum albumin) as a tracer to assay permeability in peripheral vessels. The assay was performed essentially as described (37), and all animal procedures were performed in accordance with the National Institutes of Health (NIH) Animal Care and Use Guidelines. Fisher 344 rats (250–300g; Taconic Farms Inc., Germantown, NY) were given intraperitoneal injections of steroid or carrier. 6 h later, the animals were anesthetized using 90 mg/kg intraperitoneal ketamine and 10 mg/kg Xylazine, and, after removing a blood sample for corticosterone level, given intracardiac injection of 30 mg/kg 2% Evans blue (Sigma Chemical Co.) in PBS. Intradermal 0.1-ml injections of 9LCM, recombinant human VPF (rec VPF), or PBS (background) were placed on the dorsal skin in rows of three. The animals were killed 30 min after injections and the skin removed. Wheals were resected using an 8-mm diameter trephine (Roboz Surgical Instrument Co., Inc., Rockville, MD) and incubated in 2 ml formamide (Fluka AG, Buchs, Switzerland) at 45°C for 48 h. OD of the extracts was read at 620 nM in a spectrophotometer (6/20; Coleman, Oak Brook, IL).

*Anti–VPF antibodies.* A peptide corresponding to the 25 $NH_2$-terminal amino acids of mature rat VPF (26) was synthesized and coupled to the carrier protein keyhole limpet hemocyanin (KLH) by 0.2% glutaraldehyde (38). Antibodies were raised in rabbits both to the VPF-KLH conjugate (anti–VPF) or to KLH alone (control antiserum). Adsorption of VPF from 9LCM was performed by incubating 9LCM with antibodies bound to protein A agarose (Pierce Chemical Co., Rockford, IL) as described previously (20).

*VPF expression in 9L cells.* 9L cells were plated in T–75-cm$^2$ flasks and grown to ~ 75% confluency, washed twice in PBS, and changed to serum-free DME. After 48 h, the culture medium was changed to fresh medium containing the additions indicated in the figure legends. For cells exposed to hypoxia, medium was first degassed under vacuum for several hours. A 5% $CO_2$/95% $N_2$ mixture was bubbled through this medium and the medium transferred to flasks that were also flushed with the gas mixture and immediately sealed. Analysis of the medium confirmed hypoxic conditions ($PO_2$ of ~ 20 vs ~ 150 mmHg for normoxia). After 6 h, cells were harvested and RNA isolated according to Chomczynski and Sacchi (39). RNA was subjected to electrophoresis and transfer as previously described (40). Blots were probed using the insert from a Bluescript vector containing rat $VPF_{164}$ cDNA (41) or a mouse actin probe (Ambion Inc., Austin, TX). Probes were labeled with $^{32}$P in a random primer extension reaction and hybridized using Quik Hyb (Stratagene Inc., La Jolla, CA) according to the manufacturer's instructions. Consistent results were obtained from two or three separate experiments.

*Brain tumor model.* The 9L brain tumor model was established as previously described (42). Briefly, Fisher 344 rats were anesthetized using intraperitoneal ketamine and Xylazine and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). Syngeneic 9L gliosarcoma cells ($5 \times 10^4$) in 5 μl PBS were injected into the white matter (coordinates, 1 mm anterior and 3 mm lateral to the bregma; depth of inoculation, 3.5 mm) of the right cerebral hemisphere using a 10-μl Hamilton syringe connected to the manipulating arm of the stereotaxic apparatus. Animals were randomly divided into treatment groups on day 16, at which time animals were given in-

traperitoneal injections of steroids or control solution every 8 h for 48 h. On day 18, the right femoral artery and vein were cannulated with the animal under anesthesia. After obtaining a baseline blood sample, 100 μCi of $^{14}$C-AIB (American Radiolabeled Chemicals, St. Louis, MO) was injected into the femoral vein. Arterial blood samples were obtained at 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 10, and 15 min and centrifuged to obtain serum for scintillation counting. After the last blood sample, animals were killed, and the brain was removed, covered with an embedding matrix containing polyvinyl alcohol and polyvinyl glycol (O.C.T. compound; Miles Laboratories Inc., Elkhart, IN), and rapidly frozen in a beaker of 2-methylbutane (EM Science, Gibbstown, NJ) in dry ice (43).

*Quantitative autoradiography.* Frozen tissue sections were prepared on the cryotome (Microm, Heidelberg, Germany). 10-μm histologic sections were stained with hematoxylin and eosin. 20-μm autoradiographic sections were placed on Bainbridge Board (Charles T. Bainbridge's Sons, Cranbury, NJ) along with radioactive standards ($^{14}$C-plastic autoradiographic standards calibrated by the Laboratory of Cerebral Metabolism, National Institute of Mental Health [Bethesda, MD] to 20-μm brain sections containing known amounts of $^{14}$C) (44). The sections were placed in a cassette with SB-5 film (Eastman-Kodak Co., Rochester, NY) for 6 d and were evaluated using the method of Hiesiger et al. (43). The outlines of cortex and tumor, as determined from inspection of the histologic sections, were superimposed on the corresponding autoradiographic images to define the boundary of the tumor on the autoradiogram. After calibrating the equipment to a reference standard of 1,000 pixels per mm$^2$, the area within the tumor outline was measured and the mean tumor diameter calculated. The $^{14}$C-AIB radiographic standards were used to convert the optical density of the autoradiographic images to nCi/g. The radioactivity, nCi/g, of the tumor was measured and the unidirectional blood-to-brain transfer constant (K) for the tumor was calculated by dividing the tumor concentration of $^{14}$C-AIB by the integral of the plasma concentration of $^{14}$C-AIB from 0 to 15 min (43).

## Results

*Neutralization of 9L glioma permeability activity by anti–VPF antibodies.* 9L cells produce VPF in culture and when implanted in vivo (22, 26). However, the extent to which VPF is responsible for the permeability activity produced by 9L cells in not known. To address this question, we incubated 9LCM with anti–VPF antibodies and assayed for permeability activity using the Miles assay. Although the Miles assay is not completely representative of the brain tumor situation, it is a useful method for analyzing the nature, effects, and regulation of tumor-derived permeability factors. Approximately 80% of permeability activity was removed by this antibody (Table I). The inability of this antibody to inhibit all of the permeability activity is probably due either to the presence of other permeability factors in the 9LCM or to the inability of the antibody to recognize all of the potential VPF conformations.

*Table I. Adsorption of Permeability Activity from 9LCM by Anti–VPF Antibodies*

| Antiserum | Permeability | Percent, relative to control |
|---|---|---|
| None | 26.5±6.8 | 100 |
| Anti–VPF | 5.6±1.4 | 21* |
| Anti–KLH | 28.2±8.8 | 106‡ |

9LCM was incubated with immobilized IgG from either control (anti–KLH carrier) or anti–VPF (VPF peptide+KLH) antiserum. Control 9LCM or 9LCM incubated with the indicated antibody was assayed for activity in the Miles assay. Permeability is expressed as $OD_{620} \times 100$. Data are expressed as the mean±SD ($n = 3$). Background values (PBS) have been subtracted. *Significantly different from control ($P = 0.006$); ‡not significantly different from control ($P = 0.8$).

*Dexamethasone suppression of permeability induced by 9L conditioned medium and VPF in peripheral vessels.* Initial experiments were designed to determine the concentrations of 9LCM required for induction of vascular permeability in the

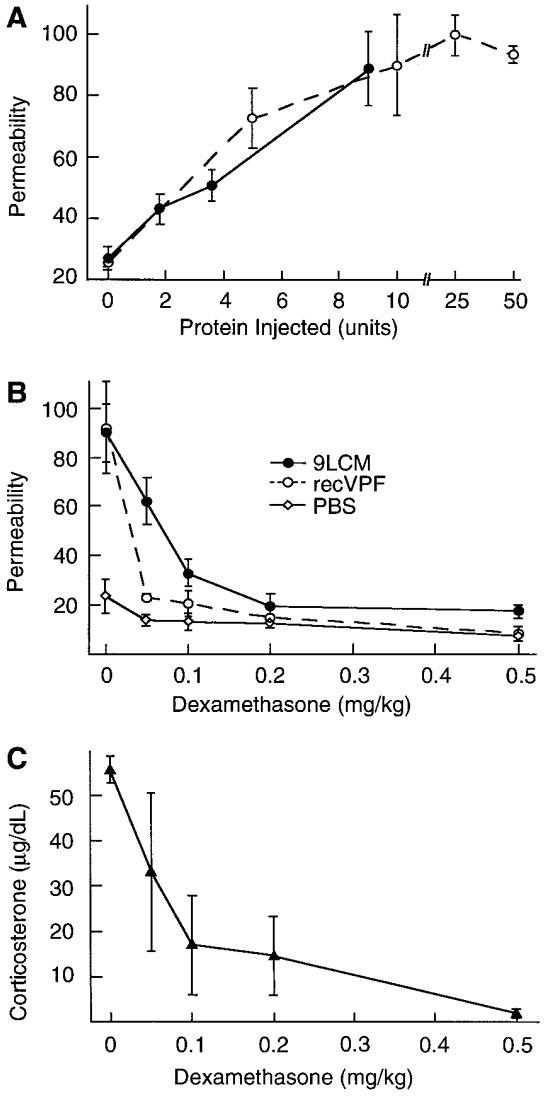


*Figure 1.* Effect of dexamethasone on induction of cutaneous vascular permeability and on corticosterone levels. (*A*) Animals were given 0.1-ml intradermal injections of 9LCM (-●-) or recVPF (-○-). 1 U protein injected equaled 5 μg for 9LCM and 1 ng for recVPF. Permeability was determined by the Miles assay and expressed as $OD_{620} \times 100$. Each point represents mean±SEM of nine 9LCM or four recVPF animals. (*B*) Rats were given a single intraperitoneal injection of dexamethasone (controls received PBS) at the indicated dose. 6 h later, animals were given intradermal injections of 45 μg 9LCM, 10 ng recVPF, or PBS as indicated. Permeability was determined by the Miles assay. Each point represents mean±SEM of 4–10 animals. (*C*) Rats were given a single dose of dexamethasone as above. 6 h later, blood for corticosterone determination was obtained by cardiac puncture immediately before performance of the Miles assay. Bars represent the mean±SD of seven to eight animals.

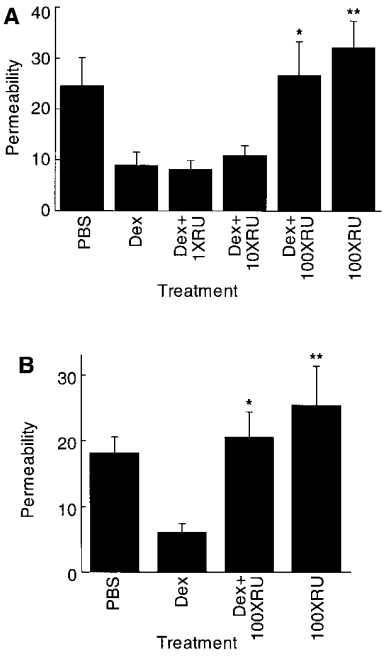


*Figure 2.* Effect of RU486 on dexamethasone inhibition of cutaneous permeability. Rats received a single intraperitoneal injection of control (*PBS*), dexamethasone (*Dex*), RU486 (*RU*), or Dex+RU as indicated. The dexamethasone dose was 0.075 mg/kg in all cases. (*A*) RU486 was given at 1×, 10×, or 100× dose (0.075, 0.75, or 7.5 mg/kg, respectively) as indicated. 6 h later, cutaneous permeability was induced by intradermal injection of 9LCM (18 μg/injection) and permeability was determined by the Miles assay and expressed as $OD_{620} \times 100$. Background values (intradermal injection of PBS) have been subtracted. Bars represent mean±SEM of five to nine animals. *Significantly different from Dex alone ($P = 0.02$), not significantly different from controls; **not significantly different from control or Dex + 100×RU. (*B*) As above except that permeability was induced by injection of 5 ng recVPF. Bars represent mean±SEM of four animals. *Significantly different from Dex alone ($P = 0.015$), not significantly different from controls; **not significantly different from control or Dex+100×RU.

Miles assay. Since ~20% of the permeability activity in 9LCM was not removed by the anti–VPF antibody, recombinant VPF alone was also included for comparison with the 9LCM. The dose-response curve was linear at least to 45 μg of 9LCM protein, up to 10 ng of recVPF (Fig. 1 *A*). Further experiments were done within the linear range to allow easy detection of suppression of permeability by steroids. To establish whether dexamethasone inhibits the permeability induced by intradermal injection of 9LCM or recVPF, rats were treated with various doses of dexamethasone 6 h before the Miles assay. Previous work in our laboratory demonstrated effective suppression of permeability with dexamethasone given 6 h before the Miles assay in guinea pigs (19). The vascular permeability induced by either 9LCM or recVPF was reduced in a dose-dependent manner by dexamethasone (Fig. 1 *B*). A dexamethasone dose of 0.1 mg/kg produced ~ 70% reduction in the permeability induced by 9LCM. A dexamethasone dose of 0.075 mg/kg was established as the $ED_{50}$ for suppression of 9LCM-induced permeability. Vascular permeability induced by recVPF was at least as easily suppressed as that induced by 9LCM and is consistent with the hypothesis that VPF is the predominant permeability-enhancing agent in 9LCM. Dexamethasone suppression of the permeability response to conditioned medium is also quantitatively similar to suppression of permeability induced by histamine (data not shown), indicating that dexamethasone suppression of permeability is not necessarily specific to the permeability-enhancing agent.

*Suppression of serum corticosterone by dexamethasone.* If dexamethasone suppression of vascular permeability is mediated by the GR, then the dose-response curve should mimic the curve for a known GR-mediated effect of dexamethasone, namely suppression of serum corticosterone levels. Therefore, we examined dexamethasone suppression of serum corticosterone levels in these rats (Fig. 1 *C*). The dose-response curve for suppression of Evans blue extravasation in the Miles assay correlated with suppression of corticosterone levels ($r = 0.77$, $P < 0.001$), suggesting that both processes are receptor mediated.

*Reversal of dexamethasone effect by RU486.* With the use of RU486, a GR antagonist, it is possible to block the GR-mediated effects of dexamethasone in experimental animals. To examine

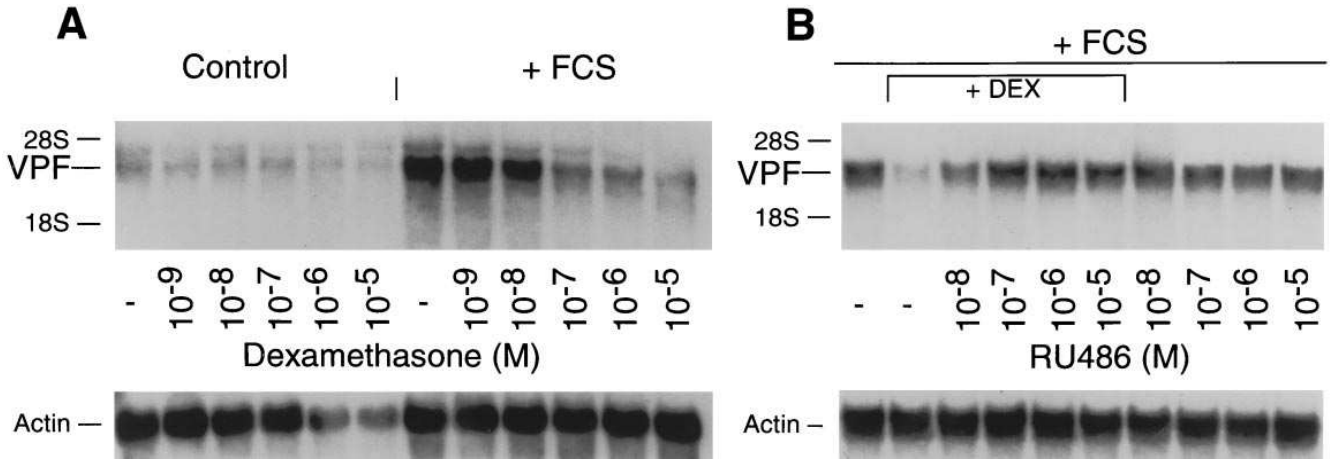


*Figure 3.* Effects of FCS, dexamethasone, and RU486 on VPF expression in 9L cells. 9L cells were grown to ~ 75% confluency and changed to serum-free medium. After 48 h, additions were made simultaneously as indicated. FCS was added to a final concentration of 10%. Cells were harvested after 6 h and total RNA isolated. Northern blots (20 μg RNA) were analyzed using a probe for rat VPF and mouse actin. (*A*) Cells were cultured in the presence of increasing concentrations of dexamethasone in either the absence (*Control*) or presence of FCS. Dexamethasone concentration is indicated below. (−) Indicates the absence of dexamethasone. (*B*) FCS was added to all cells along with steroids as indicated. In this experiment, where added, dexamethasone (*DEX*) was present at a final concentration of $10^{-7}$ M. Increasing concentrations of RU486 were added as indicated. (−) Indicates the absence of RU486.

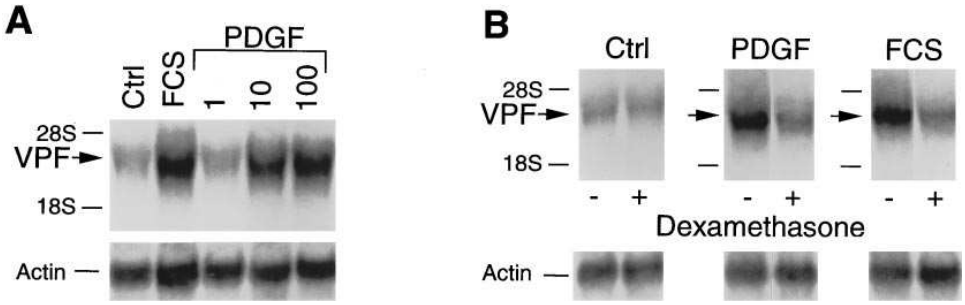


*Figure 4.* Effect of PDGF of VPF expression in 9L cells. Cells were treated as described in Fig. 3. Cells were harvested 6 h after the indicated additions. Northern blots (20 μg RNA) were analyzed using a probe for rat VPF and mouse actin. (*A*) Cells received no additions (*Ctrl*), 10% FCS, or 1, 10, or 100 ng/ml PDGF. (*B*) Cells received no inducer (*Ctrl*), 10 ng/ml PDGF, or 10% FCS. Additions were made simultaneously with (+) or without (−) $10^{-7}$ M dexamethasone.

directly whether dexamethasone effects were GR mediated, we used RU486 to determine if dexamethasone suppression of 9LCM-induced permeability depended on activation of the GR. Rats were treated with dexamethasone $ED_{50}$ (0.075 mg/kg), either alone or with RU486 at doses 1, 10, or 100 × greater than dexamethasone. Dexamethasone $ED_{50}$ was chosen to enhance sensitivity to the effects of GR antagonism by RU486. RU486 reversed the antipermeability effect of dexamethasone when given in doses 100 × greater than dexamethasone, but not in doses 1 or 10 × greater (Fig. 2 *A*). RU486 alone did not affect permeability. The antagonism of the dexamethasone effects by RU486 at 100× concentration is consistent with previous results demonstrating RU486 inhibition of known receptor-mediated effects (45, 46). The RU486 experiments were also performed using recVPF as the permeability-inducing agent. The dexamethasone suppression of recVPF activity was also reversed by RU486 (Fig. 2 *B*), which is consistent with the hypothesis that dexamethasone inhibition of VPF-induced permeability requires the GR.

*Effects of dexamethasone on VPF expression in 9L cells.* Dexamethasone has been shown in some normal cell types to inhibit the induction of VPF mRNA that occurs in response to tumor promoters (47, 48). Since VPF is responsible for at least 80% of the permeability activity produced by 9L cells, a dexamethasone-dependent inhibition of VPF expression in glioma cells provides another possible mechanism by which steroids might reduce vascular permeability in brain tumors. Other than hypoxia (22), the influence of other possible regulators of VPF expression in 9L cells has not been examined. Therefore, we examined VPF mRNA levels in the presence of FCS and dexamethasone. VPF mRNA was markedly upregulated in response to FCS (Fig. 3 *A*). In the presence of dexamethasone, basal levels of VPF were not affected, but the ability of FCS to induce VPF mRNA was inhibited in a dose-dependent manner (Fig. 3 *A*). Inhibition was nearly complete by $10^{-7}$ M dexamethasone. To determine whether or not this effect of dexamethasone also occurs through a GR-mediated mechanism, we attempted to reverse the dexamethasone-dependent inhibition of VPF induction with RU486. RU486 blocked the dexamethasone inhibition of FCS-mediated VPF induction (Fig. 3 *B*), indicating that the dexamethasone-dependent inhibition of FCS-mediated VPF induction occurs via a mechanism requiring the GR. RU486 alone did not affect the FCS-dependent induction of VPF expression (Fig. 3 *B*) or the basal level of VPF expression (data not shown).

Since PDGF is a major mitogen in serum and has been proposed as an important growth factor in brain tumors (49, 50), it was of interest to determine directly the effect of PDGF on VPF expression in these cells. PDGF induced expression of VPF mRNA in 9L cells in a dose-dependent manner (Fig. 4 *A*). As with the FCS-dependent induction of VPF mRNA, VPF induction by PDGF was also inhibited by dexamethasone (Fig. 4 *B*).

Hypoxia has also been reported to increase VPF in 9L cells (22) and is thought to be relevant to induction of VPF in human tumors (21, 51). However, unlike the FCS-dependent induction of VPF, dexamethasone failed to inhibit the VPF induction occurring in 9L cells under hypoxic conditions (Fig. 5 *A*), suggesting that the increase in VPF by FCS and hypoxia may be occurring through different mechanisms in these cells. In addition, the level of VPF observed in the presence of both hypoxia and FCS is greater than the level observed in the presence of either inducer alone (Fig. 5 *B*). Although dexamethasone was unable to suppress the increase in VPF expression that occurs in the presence of hypoxia alone, dexamethasone was able to partially suppress the induction that occurred in the presence of both hypoxia and FCS (Fig. 5 *C*), presumably by inhibiting the FCS-mediated component of the VPF increase as observed above.

*Suppression of vascular permeability in the 9L brain tumor by dexamethasone.* If the observed effects of dexamethasone on peripheral vessel permeability and on 9L cells in culture are

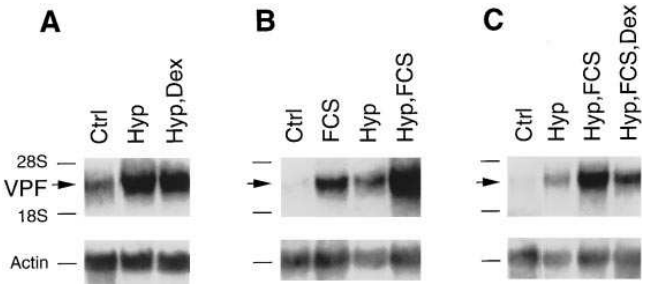


*Figure 5.* Effect of dexamethasone and FCS on induction of VPF by hypoxia. Cells were treated as described in Fig. 3. Northern Blots (20 μg RNA) were analyzed using a probe for rat VPF and mouse actin. Hypoxic induction was carried out as described in Methods. Control cells (*Ctrl*) were maintained in a normoxic environment and received no additions. (*A*) Cells were maintained in a hypoxic environment in the absence (*Hyp*) or presence (*Hyp,Dex*) of dexamethasone ($10^{-7}$ M) for 6 h. (*B*) Cells were exposed to either hypoxia (*Hyp*) or FCS alone, or to both inducers simultaneously (*Hyp,FCS*) for 6 h. (*C*) As in *B* except that some cells received dexamethasone simultaneously with exposure to both hypoxia and FCS (*Hyp, FCS, Dex*).

relevant to brain tumors in vivo, then dexamethasone suppression of vascular permeability in the tumor-associated vasculature should also be reversible by RU486. The 9L homograft brain tumor model using the amino acid analogue $^{14}$C-AIB as a tracer quantifies vascular permeability in the tumor-bearing brain by determining the blood-to-brain transfer constant for AIB (52). Despite the widespread use of the 9L brain tumor model for the study of brain tumor–associated permeability and brain tumor–associated cerebral edema (27–30, 53) and its inhibition by dexamethasone (31, 54), a dose–response curve for this dexamethasone effect had not been established previously. To establish this curve, tumor-bearing rats received the indicated dose of dexamethasone for 48 h before the measurement of capillary permeability. Treatment of rats with increasing doses of dexamethasone produced a dose-dependent decrease in $^{14}$C-AIB blood-to-brain tumor transfer (Fig. 6). Dexamethasone at 1 mg/kg per d produced a 47% reduction in the transfer constant compared to control ($P = 0.003$). Increasing the dose to 10 mg/kg per d had an additional effect, producing a 60% reduction in K. Dexamethasone treatment did not affect tumor size. Control tumor mean diameter was 5.4±1.6 mm and that of dexamethasone-treated animals was 5.4±1.9 mm (0.1 mg/kg per d), $P = 0.9$; 5.9±1.7 mm (1.0 mg/kg per d), $P = 0.6$; and 6.0±0.8 mm (10 mg/kg per d), $P = 0.5$. The histologic appearance of the 9L tumor, a solid core of tumor cells with finger-like projections reaching into the surrounding brain, was identical in the control and dexamethasone-treated groups.

*Effect of RU486 on dexamethasone effects in brain tumors.* The higher doses of dexamethasone required to inhibit permeability in the brain tumor model compared with the peripheral model suggests that non–GR-mediated mechanisms may be involved in the dexamethasone suppression of brain tumor–associated vascular permeability. To examine more directly the importance of the receptor-mediated effects of dexamethasone on brain tumor–associated vascular permeability, RU486 was given with dexamethasone to tumor-bearing animals and permeability was assessed, as described above. This experiment was performed with animals implanted at the same time as those used to establish the dose–response relationship with dexamethasone. Animals were treated with 100 mg/kg per d RU486 or 1 mg/kg per d dexamethasone alone, or with those doses of RU486 and dexamethasone together (Fig. 6). RU486 reversed the effect of dexamethasone on permeability ($P = 0.008$), but had no effect on permeability when given alone.

*Effect of 21-aminosteroids on brain tumor–associated vascular permeability.* The 21-aminosteroids are structurally similar to glucocorticoids, but lack their GR effects. To assess the effect of the 21-aminosteroids on brain tumor–associated permeability, animals were treated with either U-74006F (Tirilazad) or U-74500A instead of dexamethasone, and permeability was assessed by the $^{14}$C-AIB method as above. To improve detection of any effect on permeability, 10 mg/kg per d 21-aminosteroid, equal by mass to the highest dose of dexamethasone used in our 9L brain tumor trials, was chosen. Neither 21-aminosteroid suppressed permeability (Fig. 6), suggesting a requirement for the GR in dexamethasone suppression of permeability. In fact, U-74500A treatment resulted in an increased transfer constant (K = 5.86±1.88) compared to control (K = 3.59±0.92, $P = 0.01$). Treatment with 21-aminosteroids did not affect tumor size; control tumor mean diameter was 5.4±1.6 mm and that of U-74006F–treated animals was 6.4± 0.6 mm, $P = 0.2$, and that of U-74500A–treated animals was 5.5±0.4 mm, $P = 0.6$.

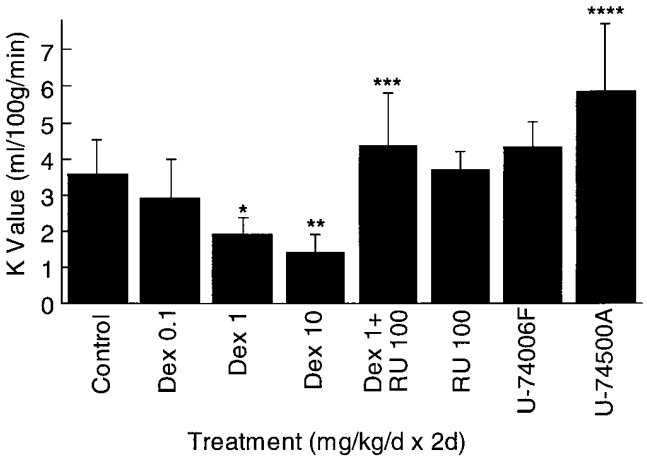


*Figure 6.* Effect of dexamethasone, RU486, and 21-aminosteroids on brain tumor vascular permeability. Rats were implanted with 9L tumor cells as described. On day 16, intraperitoneal administration of dexamethasone (*Dex*) and/or RU486 (*RU*) was begun at the indicated dose (*mg/kg/d*) and was given in divided doses every 8 h for 2 d. The 21-aminosteroids, U-74006F and U-74500A, were given at 10 mg/kg per d as described above. $^{14}$C-AIB infusions were done on day 18 and the transfer constant was determined as described in Methods. Values represent mean±SD of four to nine animals. Values significantly different from control: *$P = 0.003$, **$P = 0.001$, ****$P = 0.012$; ***value significantly different ($P = 0.008$) from 1 mg/kg per d dexamethasone alone.

## Discussion

That dexamethasone reduces brain tumor–associated edema in animal models and clinical settings is well known. However, the mechanisms by which this occurs are poorly understood. Our results suggest that dexamethasone suppression of vascular permeability in the rat 9L brain tumor model may occur through two mechanisms: inhibition of the effects of tumor-derived permeability factors on the vascular bed, and inhibition of the production of VPF by tumor cells. Reversibility of these dexamethasone effects by RU486 demonstrates the requirement at both levels for the GR.

Evidence is mounting that VPF secretion by glioma cells may underlie the increased vascular permeability associated with these tumors. VPF and its receptors are upregulated in both rodent and human brain tumors (20–22, 55). The major inducer of permeability contained in 9L conditioned medium is VPF (this report). Previous work from our laboratory demonstrated that 75-99% of the cutaneous permeability response to human glioma cell–conditioned medium and to glioma cyst fluids is also blocked by an antibody to VPF (20). Therefore, elucidation of the mechanisms by which VPF is regulated in these tumors is an important task. The VPF gene contains an AP-1 binding site (56), and VPF increases in response to substances that interact with that site such as serum, growth factors (including PDGF), and tumor promoters (47, 48, 57). VPF also increases in response to serum in some human glioma cell lines (23) and in rat 9L cells (this report). We demonstrate here that induction of VPF expression by this mechanism is in-

hibited by dexamethasone, and that the inhibition is reversed by the GR antagonist RU486. Dexamethasone inhibition of VPF induction most likely results from binding of the dexamethasone–GR complex to the AP-1 transactivator complex, which in turn prevents the AP-1 transactivator complex from stimulating transcription of genes containing an AP-1 site (58, 59). Although this effect of dexamethasone does not require GR-dependent gene transcription, it does require the binding of dexamethasone to the GR.

VPF is also induced by hypoxia in these cells, but this induction was not inhibited by dexamethasone. This is in agreement with previous results from NIH 3T3 cells (48), and suggests that induction by hypoxia in these two cell lines may result from an increase in VPF mRNA stability. This process is independent of the AP-1 site and therefore not affected by dexamethasone (48). A hypoxia-mediated increase in VPF mRNA stability also occurs in retinal epithelial cells (60). That VPF induction by FCS and hypoxia are occurring through different mechanisms in 9L cells would explain why the two inducers increase VPF more than either one alone and why dexamethasone only partially inhibits the increase observed in the presence of both inducers. However, in another glioma cell line, hypoxia had been shown to increase VPF mRNA through transcriptional activation (61). In addition, expression of Fos and Jun (components of the AP-1 transactivator complex) and subsequently VPF are upregulated by hypoxia in a hepatoma cell line (62), in which case dexamethasone suppression of hypoxia-mediated VPF induction is certainly possible. Since the regulation of VPF by hypoxia, growth factors, and dexamethasone has not yet been studied in human glioma cells, the extent to which dexamethasone decreases vascular permeability in brain tumor patients through an inhibition of VPF production remains to be determined. It is of interest that PDGF induces VPF mRNA in 9L cells and that this induction is inhibited by dexamethasone. Since PDGF expression is often elevated in human gliomas (50, 63), it is possible that PDGF also induces VPF expression in the human setting, and that this expression may be inhibited by dexamethasone administration.

We have demonstrated that the GR is the common mediator of dexamethasone inhibition of FCS-dependent VPF induction in 9L cells, and of dexamethasone suppression of vascular permeability in the periphery and the brain. Despite this, certain differences regarding dexamethasone effects were observed between the two models used for studying vascular permeability. Suppression of permeability in the brain tumor model is not as complete as in the periphery, and higher doses of dexamethasone are needed to be effective in the brain than in the periphery. Although a dose of 0.2 mg/kg of dexamethasone suppressed permeability by $\sim$ 90% in the skin, only 60% inhibition of permeability in the brain tumor was observed at a dose of 10 mg/kg. Possible explanations for these differences follow. (*a*) Permeability to large molecules (as measured by Evans blue extravasation in the skin) is more easily suppressed than permeability to small molecules (as measured by AIB in the brain). However, dexamethasone inhibition of permeability is not complete in 9L brain tumor models even when Evans blue is the tracer (54). (*b*) Dexamethasone is unable to inhibit hypoxic induction of VPF in 9L cells that may occur in vivo. However, 9L tumors implanted intracranially do not exhibit extensive necrosis (reference 22 and J.D. Heiss, unpublished observations), which suggests that hypoxia may not be the primary inducer of VPF in this model. (*c*) There may be permeability factors other than VPF, the effects of which are not suppressed by dexamethasone. Interestingly, in the Miles assay, suppression at 0.5 mg/kg, dexamethasone was more complete (100% vs 88%, $P = 0.10$) for recVPF- than for 9LCM-induced permeability, raising the possibility that the small percentage of permeability-enhancing activity of 9LCM that is insensitive to dexamethasone suppression may not be due to VPF. (*d*) At a given dose of systemically administered dexamethasone, the brain level of dexamethasone is lower than that in the periphery.

Several findings support the explanation that the relative insensitivity to dexamethasone of the brain tumor model, compared to the Miles assay, may be a result of the relative impermeability of the brain to dexamethasone (64, 65). Tamargo, et al. (31) demonstrated that suppression of cerebral edema in the 9L tumor model depends more on brain dexamethasone levels than on plasma levels, since dexamethasone must penetrate the blood-brain barrier to suppress permeability in that model. They reported that even after 8 d of systemic administration of dexamethasone, levels of the steroid in the cerebral hemisphere containing a 9L tumor were only 4% of those in the plasma (31). Our finding that suppression of pituitary ACTH secretion and 9L-conditioned, medium-induced dermal vascular permeability occurs with dexamethasone doses that have little effect on the brain is consistent with the finding of Miller et al. (66) that dexamethasone activates rat pituitary GRs (type-II adrenal steroid receptors) at blood concentrations that do not activate GRs in the brain, even though the inherent affinity of the brain and pituitary GR for dexamethasone in vitro is the same. Our finding that the dexamethasone effect in both models is reversible by RU486 also suggests that the higher doses of dexamethasone required to activate the brain GR, and subsequently reduce brain tumor–associated vascular permeability, is at least partially explained by inadequate dexamethasone entry into the brain at lower doses.

The essential role of the GR in mediating the inhibition of vascular permeability by dexamethasone is further supported by the finding that the 21-aminosteroids, compounds with a glucocorticoid structure but without GR-mediated effects, were ineffective in suppressing permeability. The 21-aminosteroids, or lazaroids, possess the non–GR-mediated effects of methylprednisolone, which include inhibition of lipid peroxidation and stabilization of cell membranes. By reducing lipid peroxidation, the lazaroids reduce secondary tissue injury from ischemia and trauma. In addition, arachidonic acid–induced vasogenic brain edema has been inhibited in rats with the 21-aminosteroid U74006F (14). Our results show that these non–GR-dependent effects are of marginal, if any, importance in tumor-associated vascular permeability. The inability of non–GR-mediated actions of glucocorticoids to suppress brain tumor–associated cerebral edema is supported by the observations of Arbit et al., who found that the aminosteroid U-78517 actually increased cerebral edema in rats with 9L gliosarcomas (29). In addition, the aminosteroids lacked antipermeability effects in other rat brain tumor models, the C6 astrocytoma (67) and the Walker 256 carcinosarcoma (25), in which dexamethasone is effective. It is unknown why brain tumor–associated vascular permeability is unaffected by non–GR-mediated steroids. Although the 21-aminosteroids depend on free radical scavenging to reduce secondary brain injury after subarachnoid hemorrhage and trauma, oxygen free radicals are unlikely to be major causative factors of peritumoral brain edema because superoxide dismutase activity is similar in 9L brain tumors and normal brain

(68). The inability of non–GR-mediated steroids to affect brain tumor–associated permeability may also be related to the importance of VPF in this process. Since this report demonstrates that the dexamethasone effects on VPF actions occur through the GR, non–receptor-mediated steroid actions cannot be expected to reduce VPF-mediated increases in vascular permeability.

To isolate the permeability suppressant effects from the growth inhibitory effects of dexamethasone, our dexamethasone treatment began 16 d after implantation, when the 9L brain tumors were large and well established. If given earlier, such as 2 d after cell implantation, dexamethasone decreases 9L tumor size, vascular density, and permeability (54). Since the transfer constant is the product of permeability times the capillary surface area, reduction of capillary surface area alone can cause a reduction in the transfer constant. For the transfer constant to reflect permeability, vascular density in all treatment groups must be equal. Because glucocorticoids inhibit tumor angiogenesis during the exponential vascular growth phase, larger tumors are less susceptible to reductions in vascular density than recently implanted small tumors (69). Dexamethasone treatment for 48 h did not effect tumor diameter or tumor histology in our study. Glucocorticoid treatment of established rat tumors, rather than recently implanted tumor cells, better simulates glucocorticoid treatment of human brain tumors since glucocorticoids have been ineffective in suppressing growth of human brain tumors (70).

Since the suppression of brain tumor permeability by dexamethasone is mediated by the GR, the principle way to reduce the peripheral effects of exogenous glucocorticoid administration is by using the lowest effective dose of GR agonists, such as dexamethasone. Our study shows that the efficacy of an agent to suppress brain tumor–associated cerebral edema depends on its glucocorticoid potency, supporting the clinical use of dexamethasone, a potent glucocorticoid with minimal mineralocorticoid effects. Our study also shows that higher doses of dexamethasone are required to suppress vascular permeability in the intracerebral tumor setting than in the intradermal setting, in part because the blood-brain barrier, although more permeable than normal, still impairs drug delivery in the tumor model. Improving drug delivery across the blood-brain barrier would reduce the dexamethasone dose necessary to control cerebral edema. Implanting polymers containing dexamethasone directly into the brain and tumor is one experimental method used to treat brain tumor–associated cerebral edema that achieves high brain/plasma dexamethasone levels and avoids systemic glucocorticoid side effects (31). Other innovative, less invasive, approaches to avoiding glucocorticoid side effects are needed. Additional approaches for minimizing VPF production and subsequent interaction with the VPF receptors may also be useful in controlling this troublesome clinical problem.

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