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Purine Analogue 6-Methylmercaptopurine Riboside Inhibits Early and Late Phases of the Angiogenesis Process[1]

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ABSTRACT

Angiogenesis has been identified as an important target for antineoplastic therapy. The use of purine analogue antimetabolites in combination chemotherapy of solid tumors has been proposed. To assess the possibility that selected purine analogues may affect tumor neovascularization, 6-methylmercaptopurine riboside (6-MMPR), 6-methylmercaptopurine, 2-aminopurine, and adenosine were evaluated for the capacity to inhibit angiogenesis *in vitro* and *in vivo*. 6-MMPR inhibited fibroblast growth factor-2 (FGF2)-induced proliferation and delayed the repair of mechanically wounded monolayer in endothelial GM 7373 cell cultures. 6-MMPR also inhibited the formation of solid sprouts within fibrin gel by FGF2-treated murine brain microvascular endothelial cells and the formation of capillary-like structures on Matrigel by murine aortic endothelial cells transfected with FGF2 cDNA. 6-MMPR affected FGF2-induced intracellular signaling in murine aortic endothelial cells by inhibiting the phosphorylation of extracellular signal-regulated kinase-2. The other molecules were ineffective in all of the assays. *In vivo*, 6-MMPR inhibited vascularization in the chick embryo chorioallantoic membrane and prevented blood vessel formation induced by human endometrial adenocarcinoma specimens grafted onto the chorioallantoic membrane. Also, topical administration of 6-MMPR caused the regression of newly formed blood vessels in the rabbit cornea. Thus, 6-MMPR specifically inhibits both the early and the late phases of the angiogenesis process *in vitro* and exerts a potent anti-angiogenic activity *in vivo*. These results provide a new rationale for the use of selected purine analogues in combination therapy of solid cancer.

INTRODUCTION

In the adult, the proliferation rate of endothelial cells is very low compared with many other cell types in the body. Physiological exceptions in which angiogenesis occurs under tight regulation are found in the female reproductive system and during wound healing. Uncontrolled endothelial cell proliferation is observed in tumor neovascularization, angioproliferative diseases like Kaposi's sarcoma, and angiogenesis-dependent diseases like rheumatoid arthritis, psoriasis, and a number of eye diseases. Thus, it is conceivable that the availability of chemical agents that could prevent neovascularization would potentially have broad applicability as a therapy for a wide spectrum of diseases including cancer. Indeed, various angiogenesis inhibitors have been developed so far, and their efficacy has been evaluated in different *in vitro* and *in vivo* assays (1). Clinical evaluations of some of these inhibitors in cancer patients are in progress (2).

Purine analogues were developed in the early 50's as antineoplastic chemotherapeutic agents (3). These antimetabolites inhibit *de novo* purine synthesis and purine interconversion reactions, and their metabolites can be incorporated into nucleic acids (3). 6-TG[3] and 6-MMPR also alter membrane glycoprotein synthesis (4). Purine analogues can act as protein kinase inhibitors; 6-MMPR is a highly effective inhibitor of nerve growth factor-activated protein kinase N (5), and 2-AP inhibits proto-oncogene and IFN gene transcription (6). Moreover, purine analogues have found application in immunosuppressive and antiviral therapy (3). At present, 6-MMP and 6-TG continue to be used mainly in the management of acute leukemia.

Combination chemotherapy regimens for the management of solid tumors have been proposed in which purine analogues are administered in association with cytotoxic drugs (7–9). The inhibition of purine and pyrimidine synthesis by 6-MMPR in combination with *N*-phosphonacetyl-L-aspartate has been suggested to potentiate the activity of 5-fluorouracil (10). Furthermore, the two drugs in combination with 6-amino-nicotinamide seem to decrease the effective dose of adriamycin, possibly by decreasing tumor cell energy (11). These observations, together with the above-mentioned effects on cellular metabolism, gene expression, and signaling, indicate that purine analogues may potentiate the effect of cytotoxic drugs via various mechanisms of action.

Recently, the hypothesis that antiangiogenic compounds can be used in combination with cytotoxic drugs for the therapy of solid tumors has been advanced previously (12–14). Also, nucleoside antimetabolites have been demonstrated to inhibit the proliferation of endothelial cells in culture (15). These findings prompted us to investigate whether selected purine analogues may act as angiogenesis inhibitors. To this purpose, we evaluated the effects of 6-MMPR, 6-MMP, 2-AP, and AD on the early steps of the angiogenesis process (*i.e.*, cell proliferation, motility, and sprout formation) induced by the angiogenic FGF2 in cultured endothelial cells. Purine analogues were also tested for the capacity to affect the late differentiative phase of neovascularization (*i.e.*, formation of capillary-like structures by FGF2-transfected endothelial cells seeded on Matrigel). The *in vitro* observations were compared with the effect exerted *in vivo* by purine analogues on physiological and tumor-induced neovascularization of the chick embryo CAM and on the regression of newly formed blood vessels of the rabbit cornea. The results demonstrate that 6-MMPR, but not the other molecules tested, modulates the angiogenic activity of FGF2 *in vitro* and affect blood vessel formation and maintenance *in vivo*.

MATERIALS AND METHODS

Reagents. Purine analogues were purchased from Sigma Chemical Co. (St. Louis, MO). Human recombinant FGF2 was expressed and purified from *Escherichia coli* cell extract by heparin-Sepharose affinity chromatography as described previously (16).

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[3] The abbreviations used are: 6-TG, 6-thioguanine; AD, adenosine; 2-AP, 2-aminopurine; FGF, fibroblast growth factor; CAM, chorioallantoic membrane; ERK, extracellular signal-regulated kinase; MAEC, murine aortic endothelial cell; MBEC, murine brain microvascular endothelial cell, 6-MMP, 6-methylmercaptopurine; 6-MMPR, 6-MMP riboside; pZipbFGF2-MAEC, FGF2-transfected MAEC.

Cell Cultures. Fetal bovine aortic endothelial GM 7373 cells were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ) and grown in Eagle's MEM containing 10% FCS, vitamins, and essential and nonessential amino acids. Human umbilical vein endothelial cells, obtained from A. Mantovani (Mario Negri Institute, Milan, Italy), were grown in M199 medium supplemented with 20% FCS, 10 ng/ml FGF2, 100 μg/ml heparin, and 4 mM glutamine. Immortalized Balb/c MAECs (clone 22106) and MBECs (clone 10027) were obtained from R. Auerbach (University of Wisconsin, Madison, WI) and were grown in Dulbecco's modified MEM with 10% FCS added (17). MAECs were transfected with the expression vector pZipbFGF (provided by N. Quarto, New York University Medical Center, New York, NY), containing 1108-bp human FGF2 cDNA under the control of the Mo-MuLV LTR elements (18). The pZipbFGF2-MAEC clone, which expresses high levels of FGF2 (19), was used in the present study.

Cell Proliferation Assays. Cell proliferation assays were performed on GM 7373 cells as described previously (20). Briefly, cells were seeded at 70,000 cells/cm^2. After overnight incubation, cells were incubated for 24 h in fresh medium containing 0.4% FCS and 10 ng/ml of FGF2 in the presence of increasing concentrations of purine analogues. At the end of the incubation, cells were trypsinized and counted in a Burker chamber.

Wounding of Endothelial Cell Monolayer. GM 7373 cells were allowed to reach confluence. Then, wounds were created in the cell monolayer with a 1.0-mm-wide rubber policeman. Cultured medium and detached cells were removed and monolayers were incubated in fresh medium to which were added 10% FCS and the purine analogue under test. At different time points, the width of the wound was measured using an ocular grid. At 2 days, cells were fixed with acetic acid:methanol (1:3, vol:vol), incubated with 0.5 μg/ml Hoechst 33258 stain to visualize cell nuclei, and photographed using an inverted fluorescence photomicroscope.

Preparation of Three-Dimensional Gels. Fibrin gels were prepared as described previously (21), with minor modifications. Briefly, MBEC aggregates, prepared on agarose-coated plates exactly as described previously (22), were seeded onto fibrin-coated 24-well plates. Immediately after seeding, 500 μl of calcium-free medium containing fibrinogen (2.5 mg/ml) and thrombin (250 mU/ml) were added to each well and allowed to gel for 5 min at 37°C. Then, 500 μl of culture medium were added on top of the gel. Culture medium was renewed every 48 h. When present, FGF2 and purine analogues were added to both fibrin gel and cell culture medium at the indicated concentrations. In all of the experiments, the fibrinolytic inhibitor trasylol was added to the gel and to the culture medium at 200 KIU/ml to prevent the dissolution of the substrate (21). Formation of radially growing cell sprouts was observed during the next 5 days.

Matrigel, an extracellular matrix extract of the murine EHS tumor grown in C57/b16 mice, was provided by A. Albini (Instituto Nazionale per la Ricerca sul Cancro, Genova, Italy). Two hundred μl/well Matrigel (10 mg/ml) were used to coat 48-well plates at 4°C. After gelatinization at 37°C, pZipbFGF2-MAECs were seeded onto Matrigel-coated dishes at 75,000 cells/cm^2 in the absence or presence of purine analogues. Newly formed endothelial cell "cords" and "tubes" were photographed using an inverted phase contrast photomicroscope.

Western Blot Analysis of ERK-2. MAECs were grown at subconfluence in 60-mm dishes in DMEM containing 10% FCS. Then, cells were incubated for 1 h with the purine analogue (100 μM) under test. After incubation, FGF2 (10 ng/ml) was added to the culture medium. After 20 min, Western blot analysis of the cell extracts (60 μg of protein) was performed exactly as described previously (23) using anti-ERK-2 antibodies (kindly provided by Dr. Y. Nagamine, Friedrich Miescher Institute, Basel, Switzerland). Phosphorylation of ERK-2 was evidenced as a mobility shift on the gel (23).

CAM Assays. In a first series of experiments, 10-μl methylcellulose disks containing 5 nmol of 6-MMPR or 2-AP, 25 nmol of AD, or 400 ng of affinity-purified anti-FGF2 antibody (provided by D. B. Rifkin, New York University Medical Center, New York, NY), were implanted on the top of chick embryo CAMs at day 8 (24). CAMs were examined daily and photographed *in ovo* under a stereomicroscope. In some experiments, India ink was injected i.v. and the CAMs were fixed in Serra's fluid, dehydrated in a graded series of alcohols, and made transparent in methylbenzoate. At day 12, CAMs were processed for light microscopy, and the angiogenic response was assessed by a planimetric method of "point counting" (24). Briefly, every 3rd section within 60 serial slides from an individual specimen was analyzed by a 144-point mesh inserted in the eyepiece of a Leitz-Dialux 20 photomicroscope. Six randomly chosen microscopic fields of each section were evaluated at ×160 for the total number of the intersection points that were occupied by vessels transversally cut (diameter ranging from 3 to 10 μm). Mean values ±SD for vessel counts were determined for each analysis. The vascular density was indicated by the final mean number of the occupied intersection points as a percentage of the total number of intersection points. The statistical significance of differences among the mean values of the intersection points in the experimental series was determined by the Student *t* test for unpaired data. The planimetric method of point counting was also used on the same slides for the quantitation of fibroblast density within CAM mesoderm.

In a second set of experiments, fresh biopsies of the uterus were collected under sterile conditions from 10 patients with adenocarcinoma of the endometrium who had not received any previous treatment (*i.e.*, radiotherapy, chemotherapy, or hormonal therapy). The diagnosis was confirmed by histological examination in all of the cases. Specimens were minced in sterile RPMI 1640 to obtain 1–2 mm^3 fragments that were grafted onto the CAM of chick embryos at day 8 as described previously (25). Care was taken to select for the study those fragments free from necrosis and bleeding. Four h after grafting, embryos (20 animals per experimental group) were treated with vehicle or with 25 nmol of 6-MMPR pipetted directly onto the implant. At day 12, all of the CAMs were processed for light microscopy.

Rabbit Cornea Assay. Slow-release pellets containing 100 ng of FGF2 (1.0 × 1.0 × 0.5 mm) were prepared in sterile conditions incorporating the growth factor into a casting solution of an ethynil-vinyl copolymer (Elvax-40) in 10% methylene chloride (10 μl/droplet). In the lower half of New Zealand White rabbit eyes (Charles River, Calco, Como, Italy), anesthetized with sodium pentothal (30 mg/kg), a micropocket (1.5 × 3.0 mm) was surgically produced using a pliable iris spatula 1.5 mm wide. The pellets were implanted in the micropockets located in the transparent avascular corneal stroma. Subsequent daily observations of the implants were made with a slitlamp stereomicroscope without anesthesia. Induced by FGF2, angiogenesis started on day 4 and progressed to vascularize the corneal stroma in the next 7–10 days. At day 14, once angiogenesis was consistent, the animals were randomly divided into three groups (four animals per group) that underwent topical treatment with 6-MMP, 6-MMPR, or vehicle, given as ophthalmic ointment (100 μl twice a day). To this purpose, the test compounds were solubilized in DMSO and incorporated in sterile Vaseline (1:20, vol:vol) at 1 mM final concentration. Data were expressed as angiogenesis score, calculated as vessel density × distance from limbus in mm. Density values of 1, 2, 3, 4, and 5 corresponded to 0–25, 25–50, 50–75, 75–100, and more than 100 vessels per cornea, respectively (26).

RESULTS

Effect of Purine Analogues on Cultured Endothelial Cells. FGF2 is able to induce an angiogenic phenotype in endothelial cells either when added exogenously to cell cultures or when produced endogenously. This phenotype includes endothelial cell proliferation, protease production, and cell migration (27, 28). To evaluate a possible angiosuppressive activity of purine analogues, 6-MMPR, 6-MMP, 2-AP, and AD (Fig. 1) were tested for the capacity to inhibit cell proliferation in FGF2-treated endothelial GM 7373 cells (20) and to affect cell migration induced by the mechanical wounding of the endothelial cell monolayer (a response that depends on the autocrine activity exerted by FGF2 produced endogenously by the injured monolayer; Ref. 28). As shown in Fig. 2*A*, all of the purine analogues inhibit the FGF2-induced proliferation of GM 7373 cells. However, 6-MMPR was approximately 1000 times more effective (ID_{50} = 0.5 μM) than the other compounds tested (ID_{50} = ~1 mM). Moreover, when GM 7373 cells were incubated for 3 days with 30 μM purine analogue and the culture medium was changed to fresh medium with no addition, the inhibitory effect induced by 6-MMPR on cell proliferation was promptly reversed. In contrast, no inhibition was exerted by the other molecules tested (Fig. 2*B*). It must be pointed out that cell viability was still higher than 95% in all of the experimental groups

after 8 days of treatment as evaluated by trypan-blue staining of trypsinized cells. Taken together, these data indicate that 6-MMPR exerts a reversible cytostatic effect on GM 7373 cells.

6-MMPR significantly affected also the capacity of GM 7373 cells to repair a mechanically wounded cell monolayer. Indeed, an ~50% reduction in cell migration was observed in cultures treated with 100 μM 6-MMPR for 2 days after wounding, whereas a complete repair occurred during the same time period in control cultures as well as in cultures treated with 100 μM AD or 6-MMP. Only a slight delay in wound repair was observed in 2-AP-treated cultures (Fig. 3).

The capacity of purine analogues to affect the first steps of the angiogenesis process was investigated further by the *in vitro* sprout formation assay (22). In this assay, endothelial cell aggregates were embedded into the fibrin gel in the presence of angiogenic stimuli, and the formation of radially growing endothelial sprouts followed. Accordingly, MBEC aggregates invaded the gel and formed solid sprouts after 2–3 days in culture when incubated in the presence of 30 ng/ml FGF2. Thirty μM 6-MMPR fully prevented sprout formation, whereas 6-MMP, 2-AP, and AD were ineffective (Fig. 4, *A* and *B*). Thus, 6-MMPR inhibits FGF2-induced endothelial cell proliferation, migration, and sprout formation in the μM range of concentrations. In contrast, no significant effect was exerted by 6-MMP, 2-AP, or AD on the early steps of the angiogenesis process.

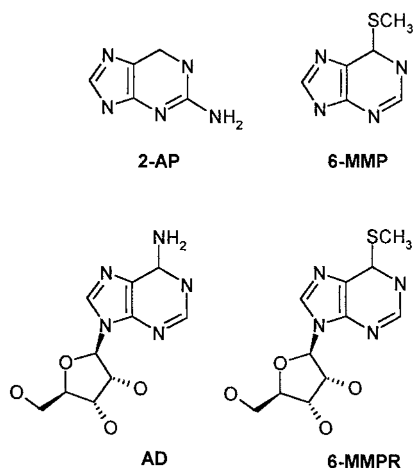

Fig. 1. The structure of the purine analogues used in the present study.

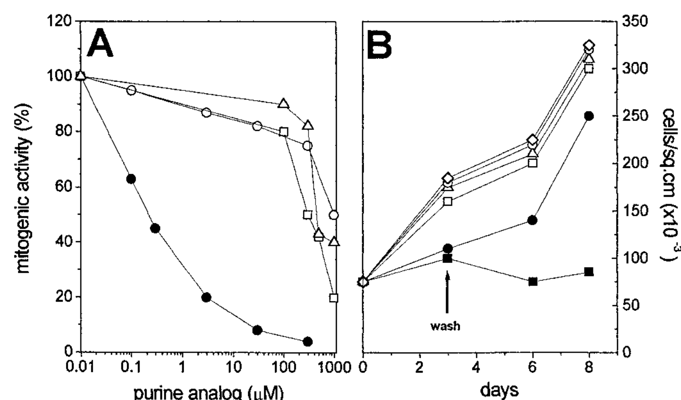

Fig. 2. The effect of purine analogues on the mitogenic activity of FGF2. GM 7373 cells were seeded at 70,000 cells/cm^2. After overnight incubation, cells were incubated for 24 h in fresh medium containing 0.4% FCS and 10 ng/ml FGF2 in the absence or presence of increasing concentrations of 6-MMPR (●), 6-MMP (□), 2-AP (△), or AD (○). At the end of the incubation, cells were trypsinized and counted in a Burker chamber (*A*). Under these experimental conditions, cultures incubated in 0.4% FCS or 0.4% FCS plus FGF2 undergo 0.1–0.2 and 0.7–0.8 cell population doublings, respectively (20). In *B*, cells were incubated with vehicle (◇) or with 30 mM 6-MMPR (●, ■), 6-MMP (□), 2-AP (△), or AD (○). After 3 days, one-half of the 6-MMPR-treated cell cultures were washed (*arrow*) and maintained in fresh medium with no addition (●), whereas all of the other cell cultures continued to receive the indicated purine analogue. Cells were counted at the indicated times. All of the experiments were repeated three times with similar results.

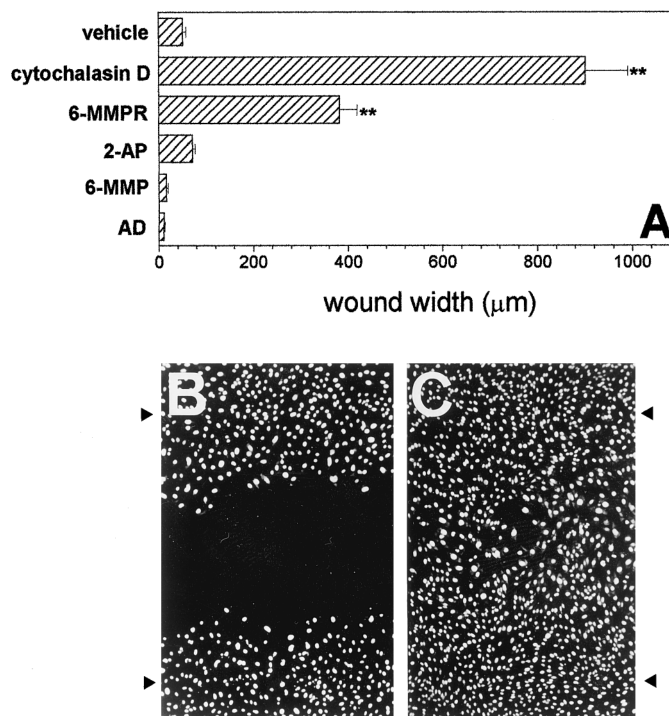

Fig. 3. The effect of purine analogues on the repair of wounded endothelial cell monolayer. Confluent GM 7373 cell monolayers were wounded with a 1.0-mm-wide rubber policeman. Then cultures were incubated in fresh medium with or without 100 μM purine analogue. Cytochalasin D at 0.5 μg/ml was used as a control. After 2 days, cells were fixed, nuclei were stained with Hoechst 33258 stain, and the width of the wound was measured using an ocular grid (*A*). Each point is the mean ± SD of three observations. Student's *t* test, treated *versus* vehicle: **, $P < 0.01$. Representative microscopic fields of 6-MMPR-treated (*B*) and AD-treated (*C*) cultures were photographed using an inverted fluorescence photomicroscope. *Arrowheads*, the width of the wound at T_0.

The late phase of neovascularization is characterized by the evolution of solid endothelial cell sprouts into capillary tubes. *In vitro*, the culture of different endothelial cell types on Matrigel, a laminin-rich, gelled basement membrane matrix, results in the formation of vascular tubes, a phenomenon known as "spontaneous angiogenesis" (29). Previous observations in our laboratory had shown that MAECs acquire the capacity to undergo morphogenesis on Matrigel after FGF2 transfection and that this process depends on an autocrine mechanism of action of the overexpressed growth factor (8). Thus, to evaluate whether purine analogues can also affect the late, differentiative steps of the angiogenesis process, 6-MMPR, 6-MMP, 2-AP, and AD were tested for the capacity to prevent vascular tube formation on Matrigel by pZipbFGF2-MAECs. As shown in Fig. 4, *C* and *D*, only 6-MMPR exerted a significant inhibitory effect on the morphogenesis of pZipbFGF2-MAECs when tested at 100 μM.

To elucidate the mechanism of action of purine analogues as FGF2 antagonists, they were evaluated for the capacity to affect the activation of the FGF receptor-dependent intracellular signaling pathway in endothelial cells. To this purpose, FGF2-treated MAECs were assessed for the phosphorylation of ERK-2, a key molecule in the signal transduction Ras/Raf-1/mitogen-activated protein kinase kinase/ERK-2 pathway switched on by the growth factor in responsive cells (23). As shown in Fig. 5, FGF2 induces the rapid phosphorylation of ERK-2, detected by Western blot analysis as a mobility shift of the protein in SDS-PAGE (23). Preincubation of MAECs with 100 μM 6-MMPR fully prevented FGF2-induced phosphorylation of ERK-2, whereas all of the other purine analogues were ineffective.

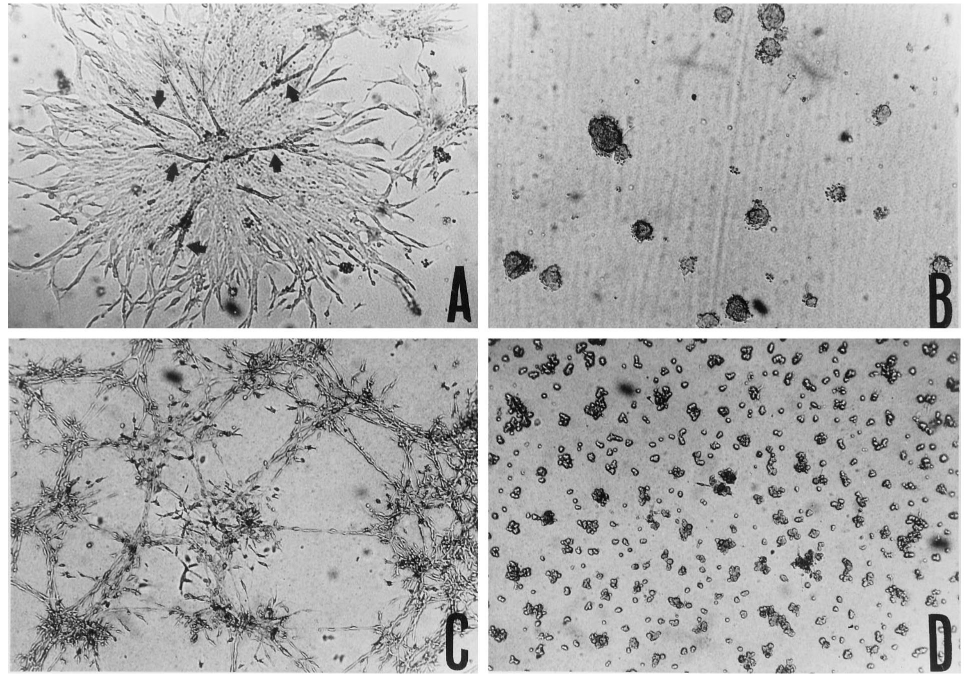

Fig. 4. The effect of purine analogues on *in vitro* endothelial cell sprouting and morphogenesis. MBEC aggregates were embedded in fibrin gel to which was added 30 ng/ml FGF2 in the absence (*A*) or in the presence (*B*) of 30 μM 6-MMPR and photographed after 5 days. Endothelial cell sprouts are marked by *arrowheads*. pZipbFGF2-MAECs were seeded on Matrigel in the absence (*C*) or in the presence (*D*) of 100 μM 6-MMPR and photographed after 2 days. Sprouting (*A*) and morphogenesis (*C*) were observed in control cultures but were fully prevented in 6-MMPR-treated cultures (*B*, *D*). No inhibition of sprouting and morphogenesis was observed in cultures treated with 6-MMP, 2-AP, or AD (not shown).

Effect of Purine Analogues on the Vascularization of Chick Embryo CAM. On the basis of the *in vitro* observations, purine analogues were evaluated for their capacity to affect the basal growth of new blood vessels *in vivo* in the CAM (12). After the i.v. injection of India ink, the macroscopic vascular pattern of CAMs—exposed to 5 nmol of 2-AP or 25 nmol of AD from day 8 to day 12 of development—was indistinguishable from that of control embryos (Fig. 6*A*). In contrast, a zone free of blood vessels was evidenced beneath the implants containing 5 nmol of 6-MMPR (Fig. 6*B*) and was similar to that observed in embryos treated with neutralizing anti-FGF2 antibody (not shown; see Ref. 24).

Histological observation of CAM sections at day 12 showed that the CAM of control and AD-treated embryos was formed by flat chorion and allantoid epithelia with capillary blood vessels located at the base of the chorion and by the mesoderm containing large arteries and veins, fibroblasts, and a few leukocytes (Fig. 6, *C* and *D*). Normally developed blood vessels were detectable also in the CAM of 2-AP-treated embryos (Fig. 6*E*). In contrast, no blood vessels were recognizable beneath the chorion or in the mesoderm of CAMs treated with 6-MMPR (Fig. 6, *F* and *G*) or anti-FGF2 antibody (Fig. 6*H*). Furthermore, the chorion was thickened and transformed into a three-layered cuboidal epithelium in most of the embryos treated with 2-AP and 6-MMPR (Fig. 6, *E* and *F*). Quantitation of blood vessel density by a planimetric method of point counting confirmed the morphological observations and pointed to an anti-angiogenic effect of 6-MMPR (Table 1). Interestingly, at variance with anti-FGF2 antibody, none of the purine analogues affected fibroblast density in CAM mesoderm (Table 1), ruling out a possible nonspecific cytotoxic effect of these compounds at the dose tested.

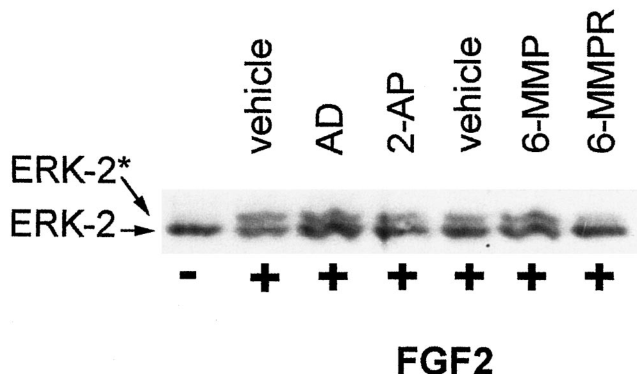

Fig. 5. The effect of purine analogues on FGF2-induced ERK-2 activation in endothelial cells. MAECs were incubated for 1 h at 37°C with vehicle or with 100 μM AD, 6-MMP, 2-AP, or 6-MMPR. After incubation, FGF2 (10 ng/ml) was added to the culture medium (+). After 20 min at 37°C, Western blot analysis of the cell extracts was performed using anti-ERK-2 antibodies. Activation of ERK-2 is evidenced as a mobility shift on the gel (*ERK-2**).

To assess whether purine analogues are also able to inhibit tumor-induced angiogenesis, 6-MMPR was evaluated for the capacity to affect neovascularization induced by human tumor specimens when grafted onto the chick embryo CAM at day 8 (30). To this purpose, human endometrial adenocarcinoma biopsies were used. In agreement with previous observations (25), tumor specimens grafted onto the CAM were surrounded after 96 h by numerous allantoic vessels that invaded the implants (Fig. 7*A*). In contrast, the allantoic blood vessels did not penetrate the grafts treated with 25 nmol of 6-MMPR (Fig. 7*B*). At microscopic examination, the untreated pathological implants (Fig. 8, *A* and *B*) showed numerous blood vessels within their stroma and at the boundary between the implants and the CAM mesenchyme in which numerous host vessels invaded the graft. In contrast, no blood vessels were detectable inside the pathological specimens treated with 6-MMPR (Fig. 8*C*).

Effect of 6-MMPR on Newly Formed Blood Vessel Maintenance in the Rabbit Cornea. Recent observations have shown that angiogenic stimuli are essential for the survival of newly formed blood vessels (31–33). On this basis, 6-MMPR was evaluated for the capacity to affect the maintenance of capillaries evoked in the rabbit cornea by an angiogenic stimulus. To this purpose, animals were implanted with a pellet containing 100 ng of FGF2. After 14 days, once a consistent vascular network had been elicited by the angiogenic stimulus, animals received 6-MMPR as a topical treatment (100 nmol given every 12 h from day 14 to day 23) or the vehicle. The extent of the blood vessel network was scored until day 28. As shown in Fig. 9, 6-MMPR caused a rapid and signifi-

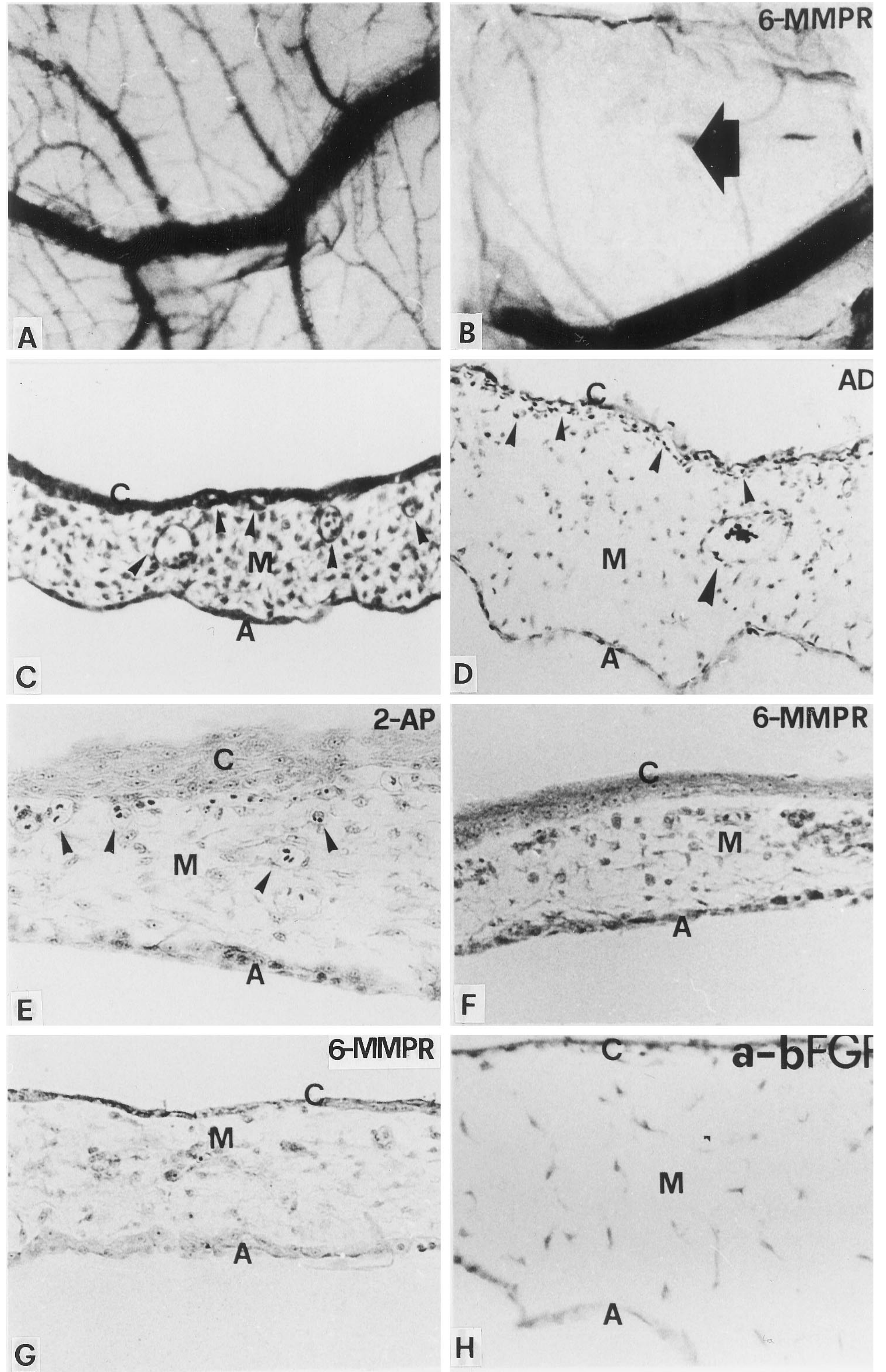

Fig. 6. The effect of purine analogues on physiological CAM vascularization. *A* and *B*, macroscopic observations. Five nmol of 6-MMPR absorbed on methylcellulose disc were implanted onto the CAM of an 8-day embryo, and the anti-angiogenic response was evaluated at day 12. After i.v. injection of India ink, compare the avascular zone corresponding to the site of implant of 6-MMPR (*arrow* in *B*) with the normal morphology of the vasculature in CAM treated with control vehicle (*A*). *C–H*, semithin sections. In *C*, control CAM at day 12: the CAM is made up of an ectodermal epithelium (*letter C*), a capillary meshwork (*arrowheads*) running under the ectoderm, an intermediate mesenchyme (*letter M*) containing large vessels (*arrowheads*) and fibroblasts, and a deep endodermal epithelium (*letter A*). A similar vascularization is observed in AD-treated CAM (*D*). In contrast, no blood vessels are recognizable beneath the chorion and in the mesoderm of CAMs treated with 6-MMPR (*F*, *G*). Note also the thickening of the chorion in embryos treated with 6-MMPR (*F*) or with 2-AP (*E*). In *H*, CAM treated with anti-FGF2 antibody (400 ng/implant) shows no blood vessels beneath the ectoderm and in the intermediate mesenchyme, and the presence of loosely arranged fibroblasts. *A* and *B*, ×30; *C–G*, ×250; *H*, ×300.

cant regression of newly formed capillaries (resulting in 50 and 75% decrease of the angiogenesis score at 3 and 5 days, respectively, after the beginning of the treatment), whereas no significant modifications of the newly formed capillary network was observed in the animals treated with vehicle. It must be pointed out that the control analogue 6-MMP did not cause any significant effect on blood vessel maintenance when administered under the same experimental conditions (data not shown).

DISCUSSION

New blood vessel formation is a multi-step process that begins with the degradation of the basement membrane by activated endothelial cells that will migrate and proliferate, which leads to the formation of solid endothelial cell sprouts into the stromal space. Then, vascular loops are formed and capillary tubes develop with the formation of tight junctions and deposition of new basement membrane (34). Each

Table 1 *Effect of purine analogues on CAM vascularization*

Methylcellulose discs were implanted onto the CAM of 8-day embryos (20 animals per group). Vascularization and fibroblast density were quantiated at day 12 by a planimetric method of "point counting."

Treatment	Microvessel density		Fibroblast density	
	Intersection points (mean ± SD)	Area (% of total)	Intersection points (mean ± SD)	Area (% of total)
Control (vehicle)	8.0 ± 1.8	5.5	22 ± 4	15.2
AD (25 nmol/implant)	7.0 ± 1.5	4.9	20 ± 2	13.9
2-AP (5 nmol/implant)	7.0 ± 0.8	4.9	24 ± 3	16.7
6-MMP (5 nmol/implant)	7.5 ± 1.2	5.2	21 ± 3	14.5
6-MMPR (5 nmol/implant)	1.0 ± 0.3[a]	0.7	19 ± 2	13.2
Anti-FGF2 Ab[b] (400 ng/implant)	3.0 ± 0.7[a]	2.0	5 ± 1[c]	3.6

[a] $P < 0.001$ *versus* control, AD, 2-AP, and 6-MMP.
[b] Ab, antibody.
[c] $P < 0.001$ *versus* all of the other groups.

step of this process represents a potential target for the inhibitory action of angiosuppressive molecules (1). In the present paper, we describe for the first time the antiangiogenic activity of the purine analogue 6-MMPR. Our results indicate that 6-MMPR affects various cell proliferation-independent aspects of the angiogenesis process induced *in vitro* by FGF2 (*i.e.*, cell motility, sprout formation, and morphogenesis) at concentrations that are cytostatic but not cytotoxic for endothelial cells. This suggests that selected purine analogues may act as angiosuppressive molecules on multiple targets of the angiogenic process. This hypothesis is supported by the observation that the anti-angiogenic activity exerted *in vivo* by 6-MMPR on physiological blood vessel development of chicken CAM is not the mere consequence of a generic inhibitory action on cell proliferation, as demonstrated by the lack of effect of 6-MMPR administration on fibroblast density of the CAM mesoderm.

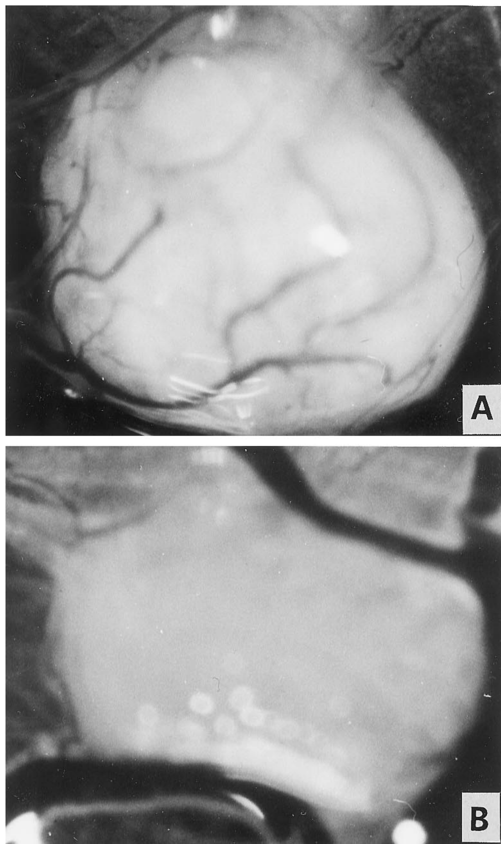

Fig. 7. The effect of 6-MMPR on tumor-induced CAM vascularization: macroscopic observations. Two fragments of the same biopsy from a human endometrial adenocarcinoma were grafted onto the CAM of chick embryos at day 8. After 4 h, vehicle (*A*) or 25 nmol of 6-MMPR (*B*) were pipetted directly onto the implant. CAMs were photographed at day 12. Note the absence of allantoic vessels invading the graft in 6-MMPR-treated biopsy (*B*) in respect to the control counterpart (*A*).

Previous observations (30) had shown that Walker 256 carcinosarcoma specimens, grafted onto the CAM, remain avascular without discernible host capillaries invading the pathological tissue for the first 72 h after implantation. During this period, vessels of the graft disappear. Then, numerous blood vessels originating from the CAM infiltrate the tumor. Recently, a similar sequence of events was observed for human endometrial adenocarcinoma grafts (25). In this study, we have found that 6-MMPR inhibits new blood vessel formation induced by grafted neoplastic uterine biopsies. These data indicate that 6-MMPR is able to prevent tumor angiogenesis *in vivo*.

The capacity of 6-MMPR to induce the regression of newly formed blood vessels in the rabbit cornea is also of interest. Tumor vascularization is a dynamic process characterized by the continuous formation of new vessels and the remodeling of existing ones, which indicates that angiogenesis, blood vessel maintenance, and regression represent interlaced phenomena (35). Previous observations (31) had demonstrated that the presence of an angiogenic stimulus (*e.g.*, vascular endothelial growth factor) is required for the maintenance of newly formed blood vessels during retinal neovascularization and tumor growth. Also, FGF-2 is essential for the *in vitro* survival of endothelial cells when they differentiate in capillary-like structures in three-dimensional collagen gel (32). Moreover, systemic administration of nitric oxide synthase inhibitor causes the regression of the vascular network evoked in the rabbit cornea by implants of human tumor biopsies (33). Our data are in keeping with these observations and further support the notion for 6-MMPR as an anti-angiogenic molecule.

Regulation of angiogenesis, vascular morphogenesis, maintenance, and remodeling seems to be due to a fine tuning of the balance among various tyrosine kinase receptors and their ligands, including different members of FGF, vascular endothelial growth factor, and angiopoietin families and their cognate receptors (35). Here we have shown that 6-MMPR prevents FGF2-induced ERK-2 phosphorylation in endothelial cells, a key step in the signal transduction pathway transduced by tyrosine kinase receptors for different angiogenic growth factors (23, 36). Interestingly, 6-MMPR does not affect tyrosine autophosphorylation of FGF receptor in L6 transfectants,[4] which indicates that the inhibition of ERK-2 activation by 6-MMPR is not the mere consequence of a general impairment of the tyrosine phosphorylating activity of the cell and that ERK-2 itself and/or other tyrosine kinases upstream of ERK-2 are the target of this purine analogue. Previous observations (5) had demonstrated that purine analogues can act as protein kinase inhibitors, 6-MMPR showing a high potency and relative selectivity for nerve growth factor-activated protein kinase N.

[4] M. Presta, unpublished observations.

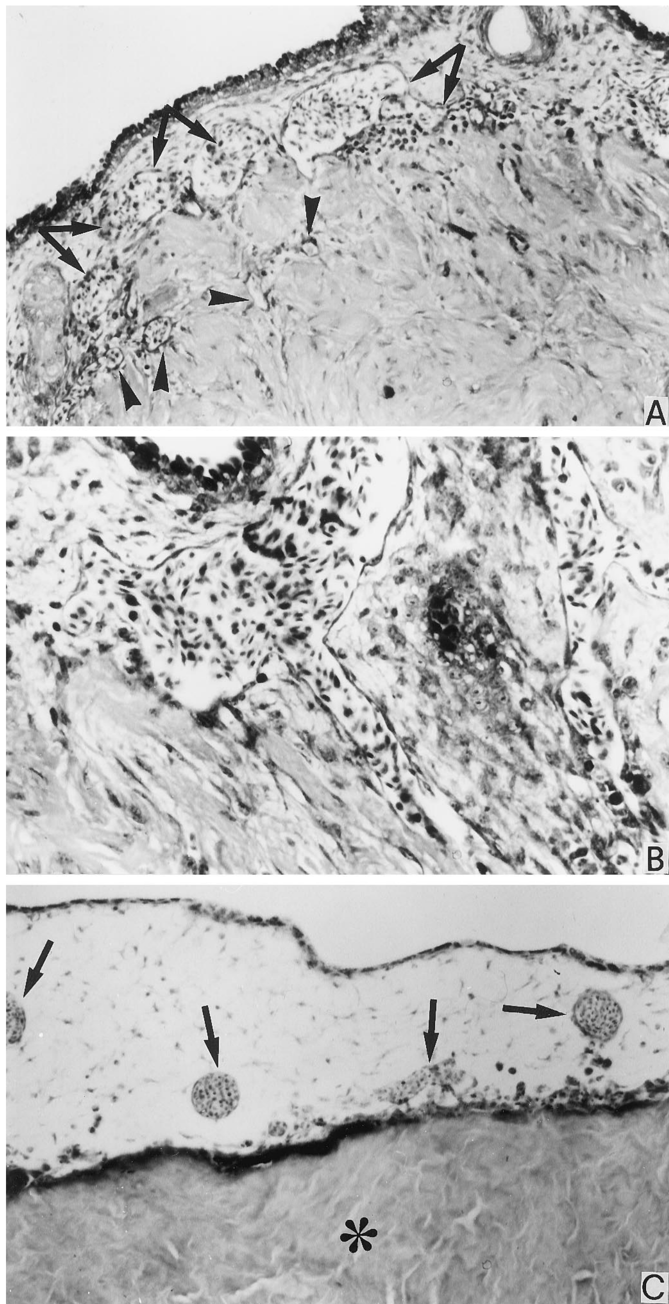

Fig. 8. The effect of 6-MMPR on tumor-induced CAM vascularization: histological observations. Fragments of human endometrial adenocarcinoma biopsies were grafted onto the CAM and treated with vehicle (*A* and *B*) or 25 nmol of 6-MMPR (*C*) as in Fig. 7. At day 12, CAMs were processed for light microscopy. In *A*, vehicle-treated implants show numerous blood vessels within their stroma (*arrowheads*) and at the boundary between the implant and the CAM mesenchyme (*arrows*). In *B*, numerous host vessels invade the vehicle-treated graft. In *C*, blood vessels of the CAM (*arrows*) do not infiltrate the grafted tumor treated with 6-MMPR (*), which remains avascular (*C*). *A* and *C*, ×250; *B*, ×400.

These data, together with the capacity of purine analogues to modulate gene expression in different experimental systems, indicate that selected members of this class of compounds can interfere with intracellular signaling and growth factor activities (6, 37, 38). Moreover, 6-MMPR alters membrane glycoprotein synthesis (4). Thus, besides the well-characterized effects of purine analogues as antimetabolic drugs able to inhibit *de novo* purine synthesis and purine interconversion reactions (3), different mechanisms of action may be responsible for the anti-angiogenic activity of 6-MMPR. Additional experiments will be required to clarify this point.

The chemical structure of the 6-MMPR molecule is of importance for its capacity to act as an angiogenesis inhibitor in that structurally-related compounds were ineffective. In particular, the riboside moiety and the 6-methylmercapto group of 6-MMPR seems to be of importance for its angiostatic activity. Indeed, inactive 6-MMP and 2-AP lack the riboside moiety, and AD differs from 6-MMPR only for the substitution of the 6-methylmercapto group with a 6-amino group (see Fig. 1). A detailed analysis on a larger number of compounds will be required to better define the structure-function relationship for angiostatic purine analogues.

Compelling experimental evidence indicates that tumor growth and metastasis are influenced by neovascularization (34, 39). Angiogenesis permits tumor growth by: (*a*) supplying oxygen and nutrients (the "perfusion effect"); (*b*) stimulating tumor cells via cytokines and growth factors produced by endothelial cells (the "paracrine effect"); and (*c*) facilitating tumor cell spreading and the growth of metastatic foci (the "metastatic effect"; Ref. 40). This makes newly formed intratumoral blood vessels attractive for therapeutic purposes. Thus, several angiogenesis inhibitors have been developed in recent years and have been shown to induce inhibition of the growth of primary tumors and to control metastasis in *in vivo* experimental models (1). Interestingly, recent studies (12–14) have suggested that the combined therapeutic approach targeting both tumor cells (by conventional cytotoxic agents) and endothelial cells (by angiogenesis inhibitors) may lead to synergistic antitumor effects.

At present, purine analogues are used mainly in the management of acute leukemia. However, combination chemotherapy regimens for the management of solid tumors have been proposed in which purine analogues may potentiate the effect of cytotoxic drugs, including 5-fluorouracil and adriamycin (7–11). Also, Phase I and II clinical trials of cytotoxic drug modulation by purine analogues have been performed (7, 9, 10). The rational for this therapeutic approach was based on the capacity of purine analogs to deplete the intracellular pool of natural nucleotides that compete with cytotoxic fluorinated nucleotides and to decrease tumor cell energy, thus facilitating apoptosis induced by DNA-damaging anticancer agents. Our findings pro-

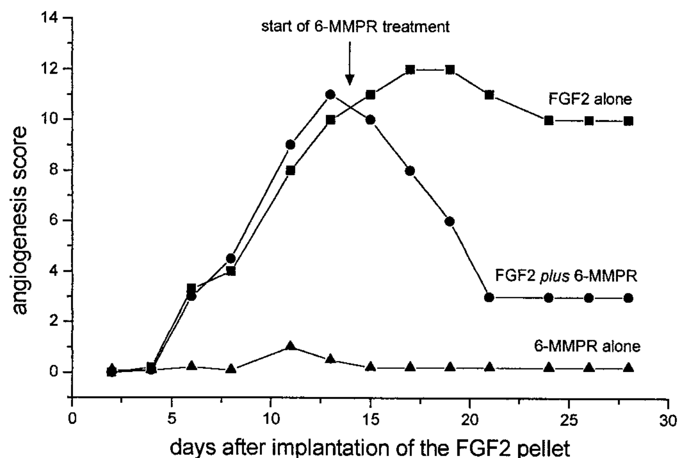

Fig. 9. The effect of 6-MMPR on newly formed blood vessel maintenance in the rabbit cornea. Slow-release pellets containing 100 ng of FGF2 (■, ●) or vehicle (▲) were implanted in the cornea of New Zealand White rabbits (4 animals per group). At day 14, animals underwent topical treatment with 6-MMPR (●, ▲) or vehicle (■), given as ophthalmic ointment (100 nmol of test compound in 100 μl of Vaseline twice a day from day 14 to day 28). Daily observations of the implants were made with a slitlamp stereomicroscope without anesthesia, and data were expressed as angiogenesis score, calculated as vessel density × distance from limbus in mm. Density values of 1, 2, 3, 4, and 5 corresponded to 0–25, 25–50, 50–75, 75–100, and more than 100 vessels per cornea, respectively (26). No effect on FGF2-induced neovascularization was observed when animals were given 6-MMP under the same experimental conditions (data not shown).

vide a new rationale for the use of selected purine analogues in combination chemotherapy in which 6-MMPR may act also as inhibitor of tumor vascularization.

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