Inhibition of Angiogenesis by the Matrix Metalloprotease Inhibitor N-[2R-2-(Hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan Methylamide

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ABSTRACT

The inhibitor N-[2R-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide specifically blocks several matrix metalloproteases, enzymes which are thought to be involved in angiogenesis. An extract of Walker 256 carcinoma in Hydron pellets implanted in the corneas of Sprague-Dawley rats was used to stimulate angiogenesis from the vessels of the limbus. Angiogenesis was graded visually as the distance penetrated into the cornea and the number of vessels generated. The vessel area was also measured by image analysis using Image 1 software. Continuous i.v. administration of N-[2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide at 32 mg/kg/day (n = 17) via syringe pump reduced vessel number [25.06 ± 5.9 (SEM) compared to 65.33 ± 9.0] and vessel area (26.14 ± 3.2 mm² compared with 40.96 ± 4.6 mm²), but not distance penetrated, compared to vehicle-treated control rats over 6 days. These results confirm the suspected role for matrix metalloproteases in angiogenesis and suggest that inhibitors of these enzymes may be angiostatic agents.

INTRODUCTION

Angiogenesis, the formation of new blood vessels, occurs in both normal and pathological processes (1–4). New blood vessel growth occurs normally in ovulation, corpus luteum formation, placental development, wound healing, and exercised muscle. Angiogenesis is also associated with pathological conditions such as benign and malignant tumors, psoriasis, arthritis, inflammation, and some ocular diseases (1, 4, 5). In cases of retinoproliferative diseases such as retrolental fibroplasia and diabetic retinopathy, new capillary formation interferes with visual function (2, 6).

Solid tumor growth requires development of capillaries from the host to the tumor. Tumors will not grow larger than 2 mm without angiogenesis (7). This observation has stimulated great interest in the study of angiogenesis over the last 2 decades. In 1976 Folkman and Cotran proposed that “once a tumor take has occurred, every increase in the tumor cell population must be preceded by an increase in new capillaries that converge on the tumor” (7, 8). Tumor-induced angiogenesis is stimulated by factors secreted by the tumor itself (9). Cotran proposed that “once a tumor take has occurred, every increase in the tumor cell population must be preceded by an increase in new capillaries that converge on the tumor” (7, 8). Tumor-induced angiogenesis is stimulated by factors secreted by the tumor itself (9).

Remodeling and penetration of basement membrane by sprouting endothelial cells are thought to require proteolysis of extracellular matrix components by tissue plasminogen activator and/or MMPs (10). Inhibition of angiogenesis in the cornea by collagenase inhibitors from cartilage suggests a critical role for the MMPs (11). A recent report showed that minocycline, a weak inhibitor of collagenase with a 50% inhibitory concentration of 190 μM (12), decreases angiogenesis and growth of a tumor implanted in rabbit corneas (13). GM 6001 is a potent inhibitor of human skin fibroblast interstitial collagenase with a Kᵢ of 0.4 nM (14). It also inhibits human neutrophil interstitial collagenase, the Mₑ, 72,000 gelatinase (gelatinase A), Mᵦ, 92,000 gelatinase (gelatinase B), and stromelysin (Kᵢ, 0.1, 0.5, 0.2, and 30 nM, respectively) (15). It weakly inhibits the metalloprotease angiotensin-converting enzyme and the serine protease plasmin with Kᵢ values in the μM range. We report here that GM 6001, an inhibitor with specificity for the MMPs, significantly reduces the number and area of new vessels in rat corneas implanted with Hydrogel pellets containing an extract of the malignant tumor Walker 256 carcinosarcoma.

MATERIALS AND METHODS

All chemicals were reagent or USP grade. GM 6001 was prepared as described previously (14).

Preparation of the Hydrogel Pellets. Walker 256 solid carcinosarcoma from Dr. Arthur E. Bogden of the Biomeasure Tumor Bank (Hopkinton, MA) was grown in Sprague-Dawley rats. A tumor was harvested from a donor rat and viable portions were placed in a Petri dish containing cold PBS. The tumor was removed from the PBS, its volume was estimated, and it was homogenized by hand in an equal volume of PBS with a Dounce grinder. After centrifugation, the protein concentration of the supernatant was 30–35 mg/ml using the Bradford method. Hydrogel polymer type NCC (Hydro Med, New Brunswick, NJ) was dissolved in 95% ethanol to a final concentration of 12% (w/w). A 1:1 mixture of 12% Hydrogel and tumor extract was mixed by vortexing for 2 min. Then 1.5 μl of this mixture was pipetted twice onto the ends of 1.5-mm-diameter Teflon rods and allowed to dry overnight in the refrigerator. The dry 1.5-mm disks were easily removed from the rods with forceps and placed into surgically created pockets in rat corneas.

Surgical Procedures. Sprague-Dawley rats (250–300 g each) were kept in rooms with the temperature controlled to 24°C on a 12-h light/dark cycle with rat chow and tap water ad libitum. Under sodium pentobarbital anesthesia an indwelling catheter was placed in the inferior vena cava through the right femoral vein, passed under the skin, exteriorized from the interscapular area, and protected with an helicoid spring sutured to the skin at the point of exteriorization (16). A drop of tetracaine hydrochloride was applied to the cornea, each eye was propitized, and a small superficial incision was made at the apex of the cornea. A corneal pocket was then created by separating the lamella of the stroma with an iris spatula adapted for the rat as described by Gimbrone et al. for rabbits (17). The pocket was enlarged toward the limbus with its base 0.5–0.7 mm from the corneal-limbal junction. A 1.5-mm Hydrogel pellet containing the tumor extract was placed in each pocket and the animals were housed individually in cylindrical cages and allowed to recuperate.

The venous lines were passed through the center top openings of the cages and were primed with drug or vehicle. The lines were connected through liquid-tight swivels to a Harvard infusion pump delivering 0.8 ml/day for 6 days. Vessels continued to grow toward the pellets throughout the 6-day period with no evidence of regression. Animals were then reanesthetized with sodium pentobarbital through the catheter, removed from the cages, and placed in the supine position. The upper abdomen and thorax were opened rapidly, a polyethylene cannula was placed cephalad into the thoracic aorta, and 5 ml of India ink were quickly injected. The eyes were enucleated, fixed in 5% formalin, evaluated under a dissecting microscope, and photographed.

Experimental Groups: Drug and Vehicle. Two sets of experiments were performed, high and low dose. In the high dose experiment, 10 mg/ml (25.7
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mm) GM 6001 were dissolved in 55% dimethyl sulfoxide in water due to its low solubility in aqueous solutions. Eighteen eyes receiving the high dose drug (32 mg/kg/day) were evaluated and 16 eyes receiving 55% dimethyl sulfoxide vehicle were evaluated. The final number of eyes evaluated in each group was 17 and 15, respectively, due to perforation of the corneas. In the low dose experiment, GM 6001 was dissolved in enough dimethyl sulfoxide to make a final dilution of 0.1% dimethyl sulfoxide and 0.4 mg/ml drug (about 1.03 mM) in 50 mM N,N-(2-hydroxyethylpiperazine)-N'-2-ethanesulfonic acid buffer (pH 7.5), near its maximum solubility in aqueous solutions. Six eyes were treated with the low dose drug (1.28 mg/kg/day) and six were treated with the 0.1% dimethyl sulfoxide vehicle. Only 5 eyes were evaluated in the drug-treated group because one eye was dehydrated inadvertently after fixation.

Evaluation of Neovascularization. The degree of neovascularization was evaluated in three ways. The number of vessels (excluding secondary branches) was counted (or estimated for massive neovascularization). The distance of penetration from the limbus into the cornea was graded using an arbitrary scale from 0 to 5. Finally, the new vessel area in one 2-mm² fixed-field encompassing the region between the limbus and the pellet was measured by video image analysis.

The distance of penetration was graded using the following scale: grade 0–1, vessels at the limbus with no or minimal penetration into the cornea; grade 2, vessels penetrate into the cornea up to one-half of the distance to the pellet; grade 3, vessels penetrate to the border of the pellet; grade 4, vessels penetrate up to one-fourth of the diameter of the pellet; and grade 5, vessels penetrate up to or greater than one-half of the diameter of the pellet.

For measurement of vessel area by image analysis, still-mode video pictures were recorded using an Olympus dissection microscope (×64) and a Hamatsu video camera. To reduce reflections from the cornea and to prevent drying, specimens were mounted on an O-ring in a closely fitting plastic chamber filled with 5% formalin and covered with a glass slide. A standardization slide (Grapicules, Tonbridge, England) provided the correct linear length measurements at any magnification. The observation field was a 1- x 2-mm rectangle covering the area between the pellet and the limbus where neovascularization occurred. Data acquisition and storage were achieved with Image 1 software.

Fig. 1. A, vehicle-treated eye from the high dose group; B, a GM 6001-treated eye (32 mg/kg/day) from the same group. The Hydron pellet in A and B and the new vessels in focus in the foreground in A are clearly visualized by the India ink injection; in B vessels appear to be fewer and of smaller diameter than in A.
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RESULTS

Tumor extract-induced angiogenesis was significantly reduced by GM 6001 in the high dose (32 mg/kg/day) group when vessel number (25.06 ± 5.9 compared to 65.33 ± 9.0 in controls) or vessel area (26.14 ± 3.2 mm² compared to 40.96 ± 4.6 mm² in controls) was measured. Differences were not significant between the low dose group (34.17 ± 5.8 vessels and 28.57 ± 5.7 mm²) and its control (62.00 ± 16.8 vessels and 51.02 ± 16.2 mm², respectively). There was no significant reduction in vessel penetration at any dose.

Fig. 1A shows an eye treated with 55% dimethyl sulfoxide vehicle alone, and Fig. 1B shows an eye treated with 32 mg/kg/day drug in this vehicle. The dramatic differences in neovascularization in this latter eye were not seen in all eyes. Note that the new vessels are in focus in the foreground while the vessels in the background are located in the iris. Fig. 2A shows a digitized still-video image of the same vehicle-treated eye as in Fig. 1A. A vessel area of 0.32 mm² (16% of the area of the field) was measured from the vessels highlighted in white. Fig. 2B shows the same drug-treated eye as in Fig. 1B with a vessel area of 0.045 mm² (2% of the area of the field).

Fig. 3 shows the effect of the drug compared to the vehicle on the number of vessels in the low and high dose experiments. The reduction in vessel number by drug is significant in the high dose group (P < 0.0005) but not in the low dose experiment. Fig. 4 shows the effect of both drug doses on the vessel area. The reduction in area is significant in the high dose group (P < 0.01). Neither dose of the drug had a significant effect on the distance of vessel penetration from the limbus toward the pellet implant. In the low dose group, the penetration averaged 3.8 ± 0.16 in vehicle-treated animals and 3.8 ± 0.16 in drug-treated animals. In the high dose group, penetration averaged 4.1 ± 0.07 in the vehicle group and 3.4 ± 0.2 in the drug group.

DISCUSSION

The reduction in vessel number and area by GM 6001 at 32/mg/kg/day supports the important role of matrix metalloproteases in angiogenesis. The reduction at 1.2 mg/kg/day appears to be similar to that of the higher dose but was not significant, possibly due to the smaller number of eyes (5 and 6 in the control and low dose groups, respectively, compared to 15 and 17 in the control and high dose groups, respectively). The image analysis measurement of vessel area should be more reproducible than counting overlapping and branching vessels. The sensitivity of the area method could be increased by reducing the standard 2-mm² area to the central 1-mm² area of angiogenesis. The size and distance from the limbus of the corneal

Fig. 2. A, digitized still-video image from the same vehicle- treated eye as in Fig. 1A. B, the same GM 6001-treated eye (32 mg/kg/day) as in Fig. 1B. The rectangle outlined in black in A and in white in B is the standard 2-mm² field analyzed. The vessels highlighted in white were used to calculate total new vessel area, 16.03% of the total image area of 2 mm² shown in A and 2.27% in B.

Fig. 3. Effect of GM 6001 on the number of new vessels at the low dose (1.28 mg/kg/day; n = 5 eyes) and the high dose (32 mg/kg/day; n = 15) compared to their vehicle controls (n = 6 and n = 17, respectively). Bars, SEM. The reduction in number by the high dose is significant (P < 0.0005).

Fig. 4. Effect of GM 6001 on the area of new vessels in the low (1.28 mg/kg/day) and high dose (32 mg/kg/day) groups. Bars, SEM. The high dose significantly reduced vessel area (P < 0.01).
implant strongly influence new vessel growth (7, 17–20). In the present experiments, the method of pellet formation assured a constant pellet diameter. However, variability in the small volumes of Hydron used to make the pellets (1.5 μl pipetted twice) could be a source of variability in the angiogenic stimulus. The distances of the pellets from the limbus were not significantly different between the vehicle- and drug-treated groups at each dose as measured on the photographs of each eye.

The inhibitor failed to reduce the distance of vessel penetration. This suggests that the number of endothelial cell sprouts (the number of vessels) was reduced, but that once sprouting occurred, further growth was not inhibited. If the inhibitor is acting by preventing penetration of an extracellular matrix barrier by cells, then the primary barrier, such as the endothelial basement membrane, must be located early in the sprouting process.

The smaller diameter of the vessels in the GM 6001-treated eye, shown in Fig. 1B, was apparent in most eyes of both drug-treated groups. This was not quantitated either visually or by the area measured by image analysis. The observed reduction in vessel diameter, number, and area would suggest the involvement of matrix metalloproteases in the initiation of a sprout and its transverse enlargement but not in its ability to penetrate parallel to the plane of the lamellae of the corneal stroma.

Inhibition of corneal neovascularization by GM 6001 is in agreement with the recent report that the tetracycline minocycline, a weak inhibitor of collagenase, dramatically reduces both angiogenesis and growth of tumors implanted in the rabbit cornea (13). Certain tetracyclines, some without antibacterial activity, inhibit these enzymes (although not strongly) and reduce collagenase activity in gingival crevicular fluid in patients with periodontal disease and in animals with pathologically excessive activity of these enzymes (21). However, others have shown that although another tetracycline, doxycycline, inhibits collagenase in vivo and reduces the recurrence of disease in patients, it does not affect the levels of collagenase in crevicular fluid (22). The mechanism of action of tetracyclines in MMP-related pathology is therefore not known. Their weak inhibition of these enzymes makes it unlikely that all of their in vivo effects are due to blocking MMPs.

In summary, GM 6001, a potent and specific inhibitor of matrix metalloproteases, significantly reduces vessel number and area but not the distance of vessel penetration into corneas implanted with a pellet containing an extract of Walker 256 carcinosarcoma.

REFERENCES

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