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Expression of hyaluronidase by tumor cells induces angiogenesis *in vivo*

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ABSTRACT Hyaluronic acid is a proteoglycan present in the extracellular matrix and is important for the maintenance of tissue architecture. Depolymerization of hyaluronic acid may facilitate tumor invasion. In addition, oligosaccharides of hyaluronic acid have been reported to induce angiogenesis. We report here that a hyaluronidase similar to the one on human sperm is expressed by metastatic human melanoma, colon carcinoma, and glioblastoma cell lines and by tumor biopsies from patients with colorectal carcinomas, but not by tissues from normal colon. Moreover, angiogenesis is induced by hyaluronidase$^+$ tumor cells but not hyaluronidase$^-$ tumor cells and can be blocked by an inhibitor of hyaluronidase. Tumor cells thus use hyaluronidase as one of the "molecular saboteurs" to depolymerize hyaluronic acid to facilitate invasion. As a consequence, breakdown products of hyaluronic acid can further promote tumor establishment by inducing angiogenesis. Hyaluronidase on tumor cells may provide a target for anti-neoplastic drugs.

Tumor metastasis is a complex phenomenon involving a sequence of events that remain poorly understood (1, 2). To metastasize, tumor cells must be able to avoid intercellular adhesion, detach from the tumor mass, and overcome physical barriers imposed by extracellular matrix. Furthermore, tumor cells must be able to induce the growth of new blood vessels, a process known as angiogenesis (3, 4). Influxes of new blood vessels further promote tumor growth and migration of tumor cells to distant sites.

The integrity of the extracellular matrix is maintained by a three-dimensional network of collagen fibers, which is reinforced by an amorphous ground substance composed of long chain polymers of mucopolysaccharides, glycosaminoglycans, and proteoglycans (5, 6). The most common glycosaminoglycans found in tissues are hyaluronic acid, chondroitin, and chondroitin sulfate. In normal healthy tissues, the extracellular matrix is maintained in a steady-state of equilibrium of very slow dynamic changes, with the formation of new macromolecules balanced by turn over and decay. The generalized stromal changes observed in the vicinity of invading tumor cells suggest that tumor cells can break down the extracellular matrix. Many enzymes capable of breaking down extracellular matrix proteins have been identified in malignant human tumor cells. Members of the matrix metalloproteinase family have been studied most extensively (7, 8). Enzymes capable of breaking down the ground substance and proteoglycans have received less attention (9, 10).

Hyaluronic acid functions as "cement" in holding the protein components in the extracellular matrix (11, 12). Therefore, hyaluronic acid plays an important role in the maintenance of an intact architecture of normal tissues. In 1928, a "spreading factor" that enhances the spreading of viral agents was found in the testicular extracts from animals and human (13). It was later characterized as a hyaluronan degrading enzyme and called hyaluronidase (14). In 1947, Fishman *et al.* (15) and his colleagues reported the presence of hyaluronidase activity in malignant tissues similar to the hyaluronidase activity present in the testis. Necrotic tumors have higher hyaluronidase activity, and hyaluronidase activities were elevated in the sera of cancer patients (16, 17). Hyaluronidase has also been identified in insect venom and snake venom. Presence of hyaluronidase in the venom enhances the spread of the toxin (18, 19). Many hyaluronidases have been described based on their enzymatic action (14). These hyaluronidases can be divided into two categories based on their pH requirement for enzymatic activity. An acidic hyaluronidase is a lysosomal enzyme with a pH optimum of approximately pH 3; this enzyme is inactive in neutral pH. The other hyaluronidase is the neutral sperm hyaluronidase.

Although hyaluronidase activities have been reported in animal cells for decades, the molecular identity of hyaluronidase was revealed only recently. A protein (PH-20) required for binding of guinea pig sperm to the zona pellucida was identified (20). The cDNA for PH-20 was cloned and found to have significant homology with the bee venom hyaluronidase gene (21, 22). Despite the significant homology between PH-20 and bee venom hyaluronidase, PH-20 is a membrane glycosylphosphatidylinositol (GPI)-anchored protein and bee venom hyaluronidase is an exoenzyme. Human PH-20 mRNA was detected in the testis but not in the spleen, ovary, or liver (22).

We hypothesized that tumor cells may express a hyaluronidase protein similar to the one present on sperm. Expression of hyaluronidase would facilitate the metastasis of tumor cells. Based on the published human PH-20 sequence, we investigated whether PH-20 mRNA is present in human tumor cell lines. We report here that a hyaluronidase similar to the one on human sperm is expressed by metastatic human melanoma, colon carcinoma, and glioblastoma cell lines and by tumor biopsies from patients with colorectal carcinomas, but not by tissues from normal colon. Moreover, angiogenesis is induced by hyaluronidase$^+$ tumor cells but not hyaluronidase$^-$ tumor cells. Angiogenesis induced by hyaluronidase$^+$ tumor cells was blocked by an inhibitor of hyaluronidase. The significance of these findings with regard to the potential of using hyaluronidase as a diagnostic marker and a target for therapy will be discussed.

MATERIALS AND METHODS

Cell Lines. SMMU-1 and SMMU-2 are human melanoma cell lines (23). The characterization of the VACO colon carcinoma cell lines have been described in detail (24). STT and CRT are glioblastoma cell lines kindly provided by R.

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Abbreviations: RT–PCR, reverse transcription–PCR; GPI, glycosylphosphatidylinositol.

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Ransohoff (Cleveland Clinic Research Foundation, Cleveland). HG7 and HG2 are glioblastoma cell lines kindly provided by R. Johnson (Case Western Reserve University). All other cell lines were obtained from the American Tissue Culture Collection.

Reverse Transcription–PCR (RT–PCR). RNAs were extracted from cells or tissues using the TRIZOL reagent kit (GIBCO/BRL). Two pairs of primers were used for each cell line: primers 2 and 3 and primers 1 and 3. The expected RT–PCR product from primers 2 and 3 is 504 bp. The expected RT–PCR product from primers 1 and 3 is 759 bp. For RT reactions, 2.5 μg of total RNA and 1 μl of oligo(dT) primer (100 ng) were added to a microfuge tube and the volume was brought to 12 μl by adding diethyl-pyrocarbonate (DEPC)-treated H_20. The mixture was heated at 60°C for 5 min and then cooled on ice. Four microliters of 5× RT buffer [2 μl of 0.1 M dithiothreitol/1 μl 10 mM dNTP/1 μl reverse transcriptase (Superscript BRL)] was added, and the mixture was incubated at 42°C for 1 hr. Reverse transcriptase was inactivated by heating and the cDNA mixture was diluted by adding 30 μl of TE buffer. For PCRs, 1 μl 3′ primer (50 ng), 1 μl 5′ primer (50 ng), 5 μl 10× *Taq* buffer, 1.5 μl 50 mM $MgCl_2$, and 0.5 μl *Taq* polymerase (Perkin–Elmer/Cetus) were added to 5 μl of diluted cDNA in a total volume of 50 μl. Tubes were covered with 50 μl of mineral oil. PCRs were carried out for 35 cycles in a PTC-100 Thermal controller (MJ Research, Cambridge, MA) at 94°C for 5 min followed by 35 cycles at 94°C for 1 min, 63°C for 1 min, and 72°C for 10 min. PCR products were run on 1% agarose gel and visualized by ethidium bromide staining.

Northern Blot Analysis. Northern blot analysis was performed with 10 μg of total RNA prepared by using RNA^{ZOL} (Biotecx Laboratories, Edmonton, Canada). After transfer to Hybond-N membrane (Amersham) and UV cross-linking, the blot was hybridized with PH-20 or β actin probes generated by random primer labeling (Boehringer Mannhein). After hybridization, the PH-20 blots were washed three times for 20 min at 60°C in 0.5× standard saline citrate (SSC) (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) and 0.1% SDS and exposed to x-ray film for 10 days. β actin blots were washed three times for 20 min at 60°C in 0.5× SSC and 0.1% SDS, once for 20 min at 60°C in 0.1× SSC and 0.1% SDS, and exposed to x-ray film over night.

Induction of Angiogenesis in the Cornea of Mice. Tumor cells were loaded into a syringe with a 33-gauge needle and injected into the corneas of mice with the aid of an ophthalmologic microscope. Each normal female BALB/c mouse (8–10 weeks old) received 1×10^5 tumor cells in 10 μl. All the mice were coded, and the degree of neovascularization was determined by slit lamp examination. A color photograph of each cornea was taken at each time point. The degree of angiogenesis was determined by two independent individuals based on the neovascularization revealed in the photographs in a blind fashion. Maximum neovascularization received a numerical score of 3, indicating the formation of new blood vessels all the way from the limbus of the cornea to the center of the pupil. If the maximal migration of blood vessels was between the limbus and the center of the pupil, the animal received a score of 1.5. If no blood vessel outgrowth occurred, the score was 0. For the inhibition of angiogenesis by apigenin, one $\times 10^5$ SMMU-2 tumor cells were mixed with either phosphate-buffered saline (PBS) or different concentrations of apigenin (Sigma) in PBS. Tumor cells were then injected into the corneas of normal mice. Each group consisted of five mice. The degree of angiogenesis was determined as described.

RESULTS

Demonstration of PH-20 mRNA in Metastatic But Not in Nonmetastatic Human Melanoma Cell Lines by RT–PCR and Northern Blot Analysis. RT–PCR was used to investigate whether PH-20 mRNA was present in human melanoma cell lines derived from a patient with metastatic melanoma. The SMMU-1 cell line was established from the primary melanoma, whereas the SMMU-2 line was derived from a lymph node metastasis from the same patient (23). The primer sequences used for RT–PCR and their locations within the PH-20 gene are illustrated in Fig. 1*a*. Correct RT–PCR products were detected in metastatic SMMU-2 melanoma cells but not in primary SMMU-1 melanoma cells or in normal melanocyte mRNA (Fig. 1*b*).

The 759-bp RT–PCR product from SMMU-2 was cloned and sequenced. The sequences derived from SMMU-2 were identical to the human PH-20 sequence. Additional RT–PCR using other primers revealed that tumor hyaluronidase mRNA also contains the GPI anchor sequence (results not shown), which indicates that tumor hyaluronidase is also a GPI-anchored protein.

Northern blotting was used to characterize the mRNA in SMMU-2 tumor cells. A 2.4-kb mRNA band was present in SMMU-2 tumor cells but not in SMMU-1 tumor cells (Fig. 2). This 2.4-kb mRNA corresponds to the PH-20 mRNA present in human sperm. β actin mRNA was present in both SMMU-1 and SMMU-2 tumor cells.

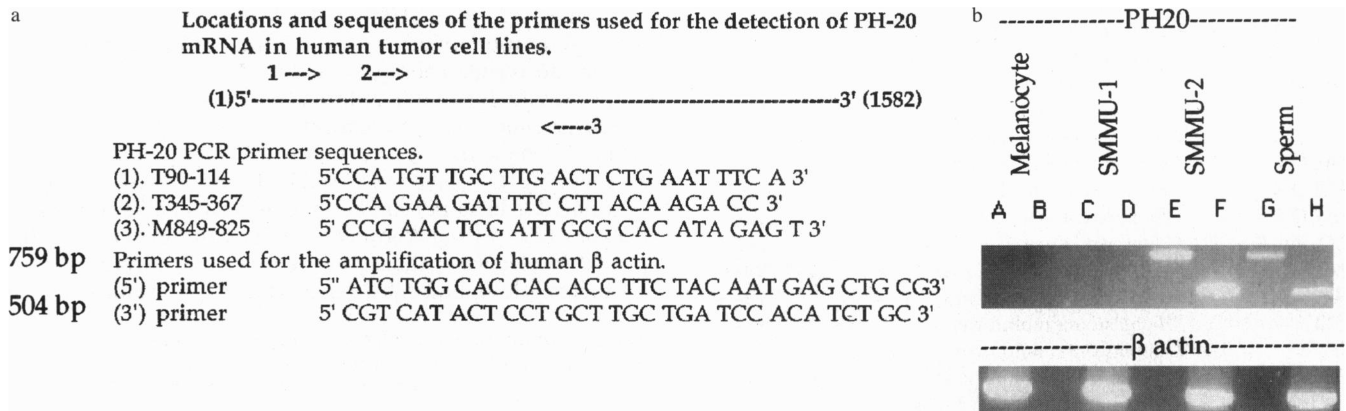

FIG. 1. PH-20 mRNA is present in human tumor cell lines. (*a*) Locations and sequences of the primers used for the detection of PH-20 mRNA in human tumor cell lines. (*b*) PH-20 mRNA is present in sperm and SMMU-2, but not SMMU-1 or normal melanocytes. Lanes A and B contain RNA from normal melanocytes. Lane A contains the RT–PCR product from primers 1 and 3 and lane B contains product from primers 2 and 3. Lanes C and D contain RNA from SMMU-1. Lane C contains the RT–PCR product from primers 1 and 3 and lane D contains product from primers 2 and 3. Lanes E and F contain RNA from SMMU-2. Lane E contains the RT–PCR from primers 1 and 3 and lane F contains product from primers 2 and 3. Lanes G and H contain RNA from normal human sperm. Lane G contains the RT–PCR product from primers 1 and 3 and lane H contains the product from primers 2 and 3. Human β actin RT–PCR products were generated using the 5′ and 3′ primers described.

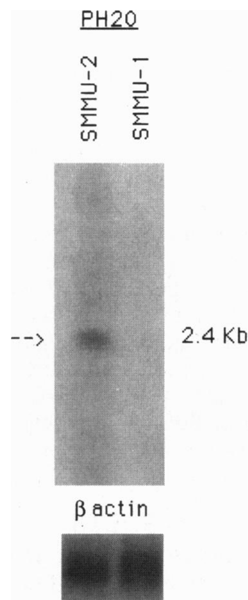

FIG. 2. Northern blot analysis of mRNA from SMMU-2 and SMMU-1 melanomas. Ten micrograms of RNA isolated from SMMU-2 or SMMU-1 was loaded into each lane and hybridized with PH-20 or β actin probes as described.

Demonstration of Hyaluronidase mRNA in Human Tumor Cell Lines and Biopsies from Patients with Colorectal Cancer But Not in Biopsies from Normal Colon Tissue. We next determined whether hyaluronidase mRNA is present in a panel of human tumor cell lines (Table 1). Not all tumor cell lines expressed hyaluronidase mRNA. Hyaluronidase mRNA was consistently present in metastatic colon carcinomas and in metastatic melanomas. All four tumor cell lines established from patients with aggressive glioblastoma multiform expressed hyaluronidase mRNA. Normal brain tissue did not express hyaluronidase mRNA. Hyaluronidase mRNA was also present in mRNA from four biopsies obtained from patients with colorectal cancers. Normal colonic mucosal tissues obtained from the same patients did not express hyaluronidase mRNA (Table 2).

Table 1. Expression of PH-20 mRNA in human tumor cell lines as determined by RT–PCR

Name	Tumor origin	PH-20 mRNA*
SMMU-2	Melanoma metastasis	Yes
SMMU-1	Primary melanoma	No
SK-Mel-1	Melanoma metastasis	Yes
SK-Mel-2	Melanoma metastasis	Yes
SK-Mel-3	Melanoma metastasis	Yes
Hs294T	Melanoma metastasis	Yes
	Normal melanocyte (n = 4)	No
VACO 444	Colon carcinoma (Dukes' B2)	No
VACO 481	Colon carcinoma (Dukes' B2)	No
VACO 531	Colon carcinoma (Dukes' B2)	Yes
VACO 432	Colon carcinoma (Dukes' B2)	Yes
VACO 5	Colon carcinoma (Dukes' C)	Yes
VACO 6	Colon carcinoma (Dukes' C)	No
VACO 8	Colon carcinoma (Dukes' D)	Yes
VACO 9	Colon carcinoma Metastasis	Yes
VACO 10	Colon carcinoma Metastasis	Yes
VACO 457	Colon carcinoma Metastasis	Yes
STT	Glioblastoma multiforme	Yes
CRT	Glioblastoma multiforme	Yes
HG7	Glioblastoma multiforme	Yes
HG2	Glioblastoma multiforme	Yes
PL/1	Glioma	No
U251	Glioma	Yes
Hs683	Glioma	Yes
H4	Glioma	No
	mRNA from normal white matter	No
	mRNA from normal gray matter	No

*PH-20 mRNA was determined using the RT-PCR protocols described in Fig. 1.

Hyaluronidase Bearing Tumor Cells Induce Angiogenesis *in Vivo*. Oligosaccharide fragments of hyaluronic acid induce angiogenesis (25, 26), which is an important process in tumor progression (3, 4). Tumor cells may therefore use hyaluronidase to digest hyaluronic acid, whose degradation products may induce angiogenesis. We investigated whether hyaluronidase$^+$ SMMU-2 cells induce angiogenesis in the corneas of mice. Blood vessel formation developed in all mice injected with SMMU-2 cells and persisted for at least 2 weeks (Table 3 and Fig. 3). Multiple vessels formed at the limbus and grew toward the site of injection. In mice injected with hyaluronidase$^-$ SMMU-1 cells, blood vessel outgrowth was limited and never reached the distance achieved by hyaluronidase$^+$ SMMU-2 cells.

This experiment was confirmed with another pair of colon carcinoma cell lines. Similarly, strong angiogenesis involving many hyperemic blood vessels developed in four of five animals injected with hyaluronidase$^+$ colon carcinoma VACO5 cells. However, only one of five animals injected with hyaluronidase$^-$ VACO6 cells developed significant angiogenesis; the blood vessels developed in VACO6 injected animals were thin and few. Therefore, the angiogenic potential of tumor cells correlates with hyaluronidase expression in two different tumor cell lines.

Inhibition of Tumor Induced Angiogenesis by a Known Hyaluronidase Inhibitor. If indeed hyaluronidase is responsible for the induction of angiogenesis, inhibition of hyaluronidase activity should prevent angiogenesis. Apigenin is a hyaluronidase inhibitor *in vitro* and *in vivo* (27, 28). We investigated whether apigenin could inhibit angiogenesis induced by SMMU-2 cells. Tumor cells were mixed with different concentrations of apigenin. Coinjection of tumor cells with apigenin resulted in inhibition of angiogensis in a dose-dependent manner (Fig. 4). Apigenin is not toxic for SMMU-2 cells at tested doses (D.L., unpublished data). Thus, tumor induced angiogenesis can be inhibited *in vivo* by a hyaluronidase inhibitor.

DISCUSSION

The compositions of extracellular matrix are different depending on the tissue. Therefore, tumor cells arising in different tissues would encounter different barriers. Tumor cells must use diverse molecular mechanisms to destroy the surrounding tissues, depending on the nature of the tumors and the surrounding tissues (1, 2). While hyaluronidase has been postulated to be important in tumor growth and metastasis for decades (29), to our knowledge, this is the first report that provide molecular evidence of the presence of hyaluronidase on metastatic tumor cells. Furthermore, our results suggest

Table 2. Hyaluronidase mRNA is present in colorectal carcinoma but not in normal colonic mucosa from the same patient

Patient	Stage of disease	PH-20 expression: Normal mucosa	PH-20 expression: Tumor
451	Metastasis	No	Yes
432	Duke's B2	No	Yes
456	Duke's B2	No	Yes
454	Metastasis	No	Yes

Table 3. Induction of angiogenesis with hyaluronidase+ SMMU-2 or VACO 5 tumor cells

No.		Neovascularization*						
		Day 1	Day 2	Day 3	Day 5	Day 7	Day 9	Day 15
1	SMMU-2	0.5	1	1.5	1.5	2	2.5	3.0
2	SMMU-2	0.5	1	1.5	1.5	1.5	2	3.0
3	SMMU-2	0	0	1	1.5	2	2.5	2.5
4	SMMU-2	0	0	1	1.5	2	2.5	2.5
5	SMMU-2	0	0.5	1	1.5	1.5	2.0	2.5
6	SMMU-2	0	0	1	1.5	2	2.5	2.5
7	SMMU-2	0	0.5	1	1.5	1.5	2.0	2.5
1	SMMU-1	0	0.5	1	1	1	1	0
2	SMMU-1	0	0	0	1	1	0	0
3	SMMU-1	0	0	0	0.5	1	1	0
4	SMMU-1	0	0	0	0	0	0	0
5	SMMU-1	0	0	0	0	0	0	0
		Day 1	Day 3	Day 7	Day 10	Day 14	Day 18	Day 21
1	VACO5	0.5	1	1.5	1.5	2.5	3.0	3.0
2	VACO5	0.5	1	1.5	1.5	2.5	3.0	3.0
3	VACO5	0	0	1	1.5	2	3.0	3.0
4	VACO5	0	0	1	1.5	2	3.0	3.0
5	VACO5	0	0	0	0	0	0	0
1	VACO6	0	0	1	1	2.5	2.5	2.5†
2	VACO6	0	0	0	1	1	1.5	1.5†
3	VACO6	0	0	0	0.5	1	1	1†
4	VACO6	0	0	0	0	0	0	0
5	VACO6	0	0	0	0	0	0	0

Tumor cells were loaded into a syringe with a 33-gauge needle and injected into the corneas of mice with the aid of an ophthalmologic microscope. Each BALB/c mouse received 1×10^5 tumor cells in 10 μl. All the mice were coded and the degree of neovascularization was determined by slit lamp examination. A color photograph of each cornea was taken at each time point. The degree of angiogenesis was determined by two independent individuals based on the neovascularization revealed in the photographs. The score presented is the average of the two scores. The SEM for each time point is >0.5. Maximum neovascularization received a numerical score of 3, indicating the formation of new blood vessels all the way from the limbus of the cornea to the center of the pupil. If the maximal migration of blood vessels was between the limbus and the center of the pupil, the animal received a score of 1.5. If no blood vessel outgrowth occurred, the score was 0.

†The blood vessels developed in these mice are qualitatively very different from the blood vessels developed in animals injected with VACO5. In these mice only one or two very thin blood vessels can be seen in each animal. The degree of neovascularization remained the same in these mice at 28 days after tumor cell injection. In VACO5-injected animals angiogenesis involved many blood vessels and the blood vessels were much larger.

that hyaluronidase on tumor cells may participate in the induction of angiogenesis and spreading of tumor cells.

We presented molecular evidence that the hyaluronidase present in SMMU-2 tumor cells is similar to the PH-20 hyaluronidase present on human sperm. The expression of hyaluronidase may be more complex in human tumor cells. In contrast to human sperm in which only one single size mRNA was present (20), under lower stringent hybridization conditions two additional mRNA species, a 4-kb and a 9-kb mRNA can be detected in SMMU-2 but not in SMMU-1 tumor cells. The nature of these two cross-hybridized mRNA are not known.

The exact mechanisms by which hyaluronidase bearing tumor cells induce angiogenesis and facilitate tumor growth and metastasis are not known. Since only hyaluronidase+ tumor cells but not hyaluronidase− tumor cells induce angiogenesis; therefore, it is unlikely that the neovascularization observed in the cornea was immune mediated. Hyaluronic acid is a polymer consisting of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid (15, 16). Fragments of hyaluronic acid have been reported to stimulate the proliferation of endothelial cells and induce angiogenesis *in vitro* (25, 26). Therefore, tumor cells expressing hyaluronidase can depolymerize the extracellular matrix. Hydrolytic products of hyaluronic acid will then stimulate the proliferation of endothelial cells, which is an essential process in the induction of angiogenesis.

Hyaluronic acid is known to play a role in determining the location of blood vessels in the embryo (30). Limb buds of chicken embryos contain within the peripheral mesoderm an avascular zone that is rich in hyaluronic acid. Epithelial tissues that synthesize a large amount of hyaluronic acid relative to other glycosaminoglycans caused avascularity when implanted into normally vascular wing mesoderm. Epithelia that synthesize little hyaluronic acid did not cause avascularity (30). Therefore, intact hyaluronic acid may be a barrier for neovascularization. Digestion of hyaluronic acid by hyaluronidase bearing tumor cells may remove this barrier to allow the growth of new blood vessels. Proteoglycans bind growth factors (31, 32). Binding of growth factors to proteoglycans in the extracellular matrix can concentrate or protect growth factors from degradation. Digestion of extracellular matrix by tumor hyaluronidase may therefore free growth factors stored in the microenvironment, thus enhancing their availability for target cells. Some of these factors may be essential for angiogenesis and/or tumor growth.

The development of angiogenesis during tumor growth has been studied extensively (2, 3). However, whether angiogenic factors were produced solely by tumor cells or by host tissue or both remained controversial. Blood vessel density within tumors vascular was always higher at the periphery of the tumor than the center of the tumor (33). It is not clear why vascular development is most prominent in the periphery of the tumor if postulated angiogenic factors are presumably released within by (O.K.) all the tumor cells. Thompson and Smith (33) postulated that biologically meaningful angiogenic factors must require interactions between tumor and adjacent stromal components. Our observation that tumor hyaluronidase may play a role in angiogenesis provides indirect support for Thompson and Smith's hypothesis.

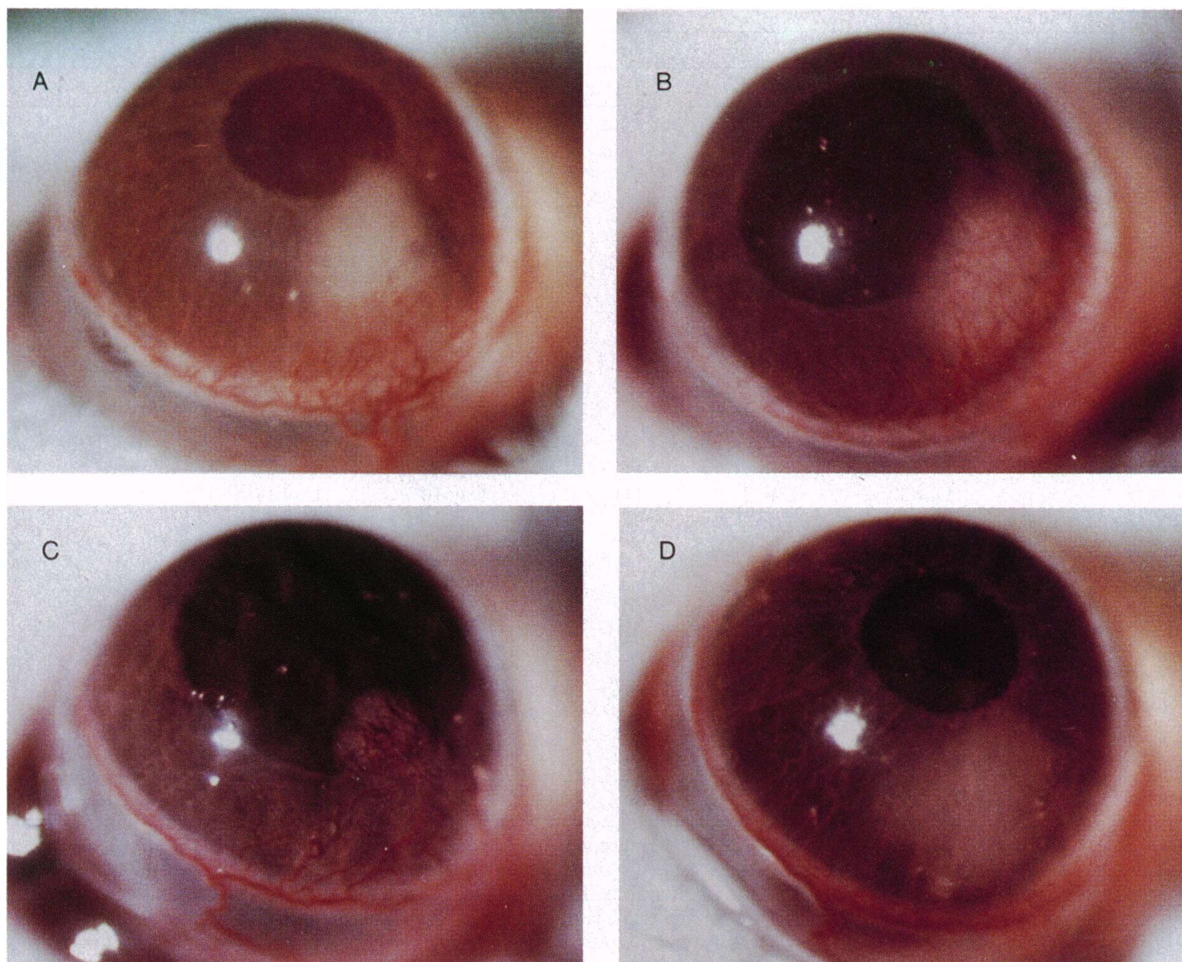

FIG. 3. Photography of angiogenesis induced with hyaluronidase+ SMMU-2 tumor cells. Animals were injected with SMMU-1 or SMMU-2 tumor cells as described in Table 3. (*A*) Threre days after injection of 10^5 SMMU-2. (*B*) Five days after injection of 10^5 SMMU-2. (*C*) Thirteen days after injection of 10^5 SMMU-2. (*D*) Thirteen days after injection of 10^5 SMMU-1.

Tumor-induced angiogenesis was inhibited with apigenin, a known inhibitor of hyaluronidase. Apigenin is a flavonoid that exhibits a great variety of pharmacological effects in different biological systems (27, 28). Apigenin is a nontoxic and non-mutagenic flavonoid. Flavonoids are benzo-γ-pyrone derivatives that are ubiquitous in the leaves and stems of vascular plants (34). Epidemiological studies generally have found that the consumption of vegetables and green teas were correlated negatively with the incidence and mortality of stomach, colon, breast, prostate, lung, esophageal, and bladder cancers (35, 36). Apigenin has also been reported to inhibit the proliferation of human tumor cell lines *in vitro* (37). Topical application of apigenin inhibited epidermal ornithine decarboxylase activities and skin tumor promotion in mice (38). In mice treated with apigenin there was a tendency to decrease the conversion of papillomas to carcinomas (39). Tangeretin, another flavonoid, inhibited invasion of mouse tumor cells into embryonic chicken heart *in vitro* (40). Tannic acid is commonly found in food such as green tea and betel nuts. A very low dose of tannic acid in drinking water prevented the induction of colon cancers in rats (41). In addition to other pharmacological effects, many of these flavonoids are inhibitors of hyaluronidase. Dextransulfate inhibited the formation of metastatic tumor in animal models (42). A patient with early human lung cancer has been reported to be successfully treated with dextransulfate and carboquone (43). Dextransulfate is an inhibitor of hyaluronidase (44). It is tempting to speculate that the anti-neoplastic effects of some of these compounds may be partially attributed to their ability to inhibit tumor associated hyaluronidase activity.

Another cell surface molecule that has direct relevance to this study is CD44. Expression of CD44 has been reported to be important in tumor growth and metastasis (45, 46). One of the major ligands for CD44 is hyaluronic acid (45, 46). Binding of soluble hyaluronic acid to CD44 on human breast tumor cell lines *in vitro* resulted in internalization and degradation of hyaluronic acid. Furthermore, binding and degradation of hyaluronic acid correlates with the invasive potential of the tumor cells (47). Potentially, lysosomal hyaluronidase may digest internalized hyaluronic acid. Hyaluronic acid fragments secreted by the tumor cells may induce angiogenesis. Tumor cells may be able to bind and internalize soluble hyaluronic acid *in vitro*. Whether tumor cells can internalize hyaluronic acid present in the extracellular matrix is not known. Furthermore, both VACO5 and VACO6 colon carcinoma cell lines used in our studies expressed high levels of CD44 (result not shown). However, only hyaluronidase positive VACO5 induced angiogenesis.

We presented evidence that a hyaluronidase molecule is present on some but not all long term human tumor cell lines including colon adenocarcinoma. Hyaluronidase mRNA is also present in biopsies from patients with colon carcinomas but not from mRNA isolated from the normal colon tissue of the same patient. Expression of hyaluronidase on tumor cells could enable them to avoid intercellular adhesion, depolymerize the extracellular matrix, release growth factors stored in the matrix, and also induce angiogenesis, thereby facilitating the spread of tumor cells. Experiments are now in progress to transfect cloned hyaluronidase gene into hyaluronidase neg-

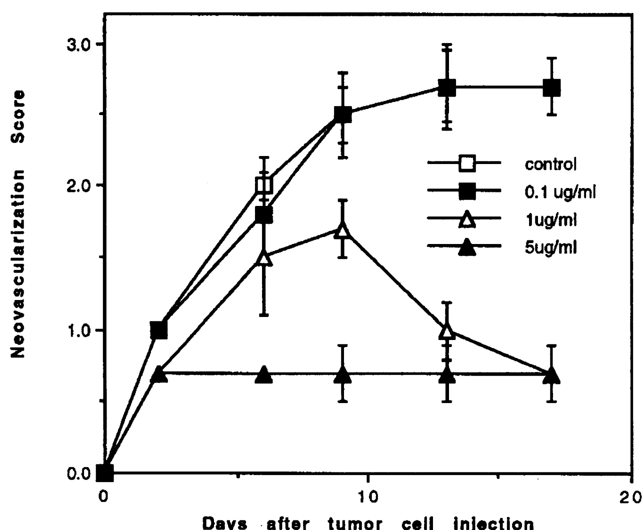

FIG. 4. Inhibition of angiogenesis with the hyaluronidase inhibitor, apigenin. SMMU-2 tumor cells (1×10^5) were mixed with either PBS or different concentrations of apigenin (Sigma) in PBS. Tumor cells were then injected into the corneas of normal mice. Each group consisted of five mice.

ative tumor cell lines to directly address the role of hyaluronidase in tumor metastasis. More importantly, a number of hyaluronidase inhibitors are readily available. These naturally occurring compounds, which are generally nontoxic and nonmutagenic, may be able to disrupt critical functions of hyaluronidase, and may have therapeutic value.

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