Inhibition of angiogenesis induced by cerebral arteriovenous malformations using Gamma Knife irradiation

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Object. The authors studied the effect of Gamma Knife irradiation on angiogenesis induced by cerebral arteriovenous malformation (AVM) tissues implanted in the corneas of rats.

Methods. Ten AVM specimens obtained from tissue resections performed at Marmara University between 1998 and 2004 were used. A uniform amount of tissue was implanted into the micropocket between the two epithelial layers of the cornea. Gamma Knife irradiation was applied with dose prescriptions of 15 or 30 Gy to one cornea at 100% isodose. Dosing was adjusted so that the implanted cornea of one eye received 1.5 Gy when 15 Gy was applied to the other cornea. Similarly, one cornea received 3 Gy when 30 Gy was applied to the other cornea. Angiogenic activity was graded daily by biomicroscopic observations. Forty-eight other rats were used for microvessel counting and vascular endothelial growth factor (VEGF) staining portions of the experiment. Micropieces of the specimens were again used for corneal implantation. Rats from each group were killed on Days 5, 10, 15, and 20, and four corneas from each group were examined.

Gamma Knife irradiation dose dependently decreased AVM-induced neovascularization in the rat cornea as determined by biomicroscopic grading of angiogenesis, microvessel count, and VEGF expression.

Conclusions. The results suggest that Gamma Knife irradiation inhibits angiogenesis induced by AVM tissue in the cornea angiogenesis model. The data are not directly related to understanding how Gamma Knife irradiation occludes existing AVM vasculature, but to understanding why properly treated AVMs do not recur and do not show neovascularization after Gamma Knife irradiation.

Key WORDS • angiogenesis • arteriovenous malformation • Gamma Knife surgery • irradiation • vascular endothelial growth factor

Arteriovenous malformations have the biological potential to develop angiogenesis.22,30 In recent decades, research has provided information about the molecular fundamentals of the angiogenic process in these malformations.3,10,11,14,28,30,41,42 Clinical reports have provided complementary information to molecular research regarding the growth and de novo appearance of cerebrovascular malformations.2,18,35 Thus, AVMs cannot be completely treated unless angiogenically active AVM tissue is completely removed or biologically transformed into quiescent, angiogenically inactive tissue. In other words, any treatment modality that is used or will be used in the management of AVMs must take into account the angiogenesis inherent in AVM tissue.

After its introduction by Steiner in 1972, GKS has become one of the most commonly used modalities for treating cerebral AVMs.19 Despite the clinically proven effectiveness of GKS in cerebral AVMs, its biological basis is not fully understood. Szeifert and colleagues38,39 and Schneider and colleagues31 have contributed to understanding the histopathology of AVMs after GKS. The effect of gamma irradiation on cultured endothelial cells also has been investigated. However, the role of gamma irradiation in the angiogenic activity of AVMs remains to be elucidated.

Currently, animal CAMs are used most often to investigate neoplastic angiogenesis.17 In these models, tissue with angiogenic potential is implanted in a micropocket created in the cornea, stimulating vessel formation in the avascular corneal matrix. The investigator makes visual observations, and typically uses a standardized grading system to measure angiogenesis induced by the tumor type under investigation. Using this approach, the extent of angiogenesis is assessed in a dynamic way (changes are observed over time),
and effects of different types of tissue can be compared in the same setting, even in the same animal. In a recently published study from our laboratory, we used a rat CAM to test the angiogenic activity of AVMs, cavernous malformations, and venous angioma tissues. Semiquantitative data were recorded as a function of time, and information provided by this dynamic model was compared with previous information acquired with static descriptive methods used in our own laboratory and others.10,11,22

The current study aimed to test the hypothesis that Gamma Knife irradiation inhibits the angiogenic activity induced by AVM tissue in the CAM. It is important to note that the data presented are not directly related to understanding how Gamma Knife irradiation occludes existing AVM vasculature, but are related to understanding why properly treated AVMs do not recur and do not show neovascularization.

Materials and Methods

Patients and Tissue Samples

The current study used AVM tissue specimens that had been obtained from resections performed at the Marmara University Department of Neurosurgery and the Institute of Neurological Sciences between January 1998 and January 2004. All specimens came from tissue samples that had been stored in the liquid nitrogen tissue bank at the Laboratory of Molecular Neurosurgery of the Institute of Neurological Sciences. This tissue bank is strictly regulated, and informed consent was given by each patient or a legally responsible person. Ten AVM specimens were randomly selected from 42 available AVM tissues.

The mean age of the patients who provided the 10 AVM specimens was 30 years (range 18–52 years). Patients who had received radiation therapy with the Gamma Knife or other systems and those who had undergone embolization procedures were excluded. None of the 10 AVM cases had presented with intracranial hemorrhage. For each tissue specimen in the study, after resection the sample was immediately washed in dimethyl sulfoxide (Sigma D-8779) and then placed in liquid nitrogen (−187˚C) within 15 minutes after removal.

Male Sprague–Dawley rats weighing 300 to 400 g were used for this study. All experiments were conducted in accordance with the Animal Care and Use Committee of the Marmara University Faculty of Medicine. Methodological principles of the experimental model have been recently published by Konya et al.23

Corneal Angiogenesis Assay

Four to five hours prior to the experimental procedures, each tissue sample in liquid nitrogen was brought to room temperature, washed with dimethyl sulfoxide, and cut into suitably sized pieces (~2–3 mm in diameter) under a microscope.

Each rat was anesthetized with an intraperitoneal injection of ketamine, and all manipulations were done under the microscope in sterile conditions. Both corneas of each animal were anesthetized with 0.5% of topical proparacaine and each globe was gently proptosed with jeweler forceps. Using an operating microscope, a paracentral intrastromal linear keratotomy (~ 4 mm long and at a right angle to the limbus) was performed with an arachnoid blade. A micropocket was used to form a micropocket in the corneal tissue. A uniform amount of tissue was implanted in the micropocket between the two epithelial layers of the cornea. The date the procedure was performed was recorded as Day 0.

At Day 1, Gamma Knife irradiation was performed in a specially designed stereotactic frame for the rat and fixation device for a 1.5-tesla magnetic resonance unit (General Electric) in tandem with a Lebonoss Gamma Knife Unit (Elekta) containing imaging and radiosurgery application procedures were performed under intraperitoneal ketamine anesthesia. The dose prescription was 15 or 30 Gy (with one 4-mm collimator) to one cornea at 100% isodose. The dose plan was adjusted so that the implanted cornea of the animal’s other eye received 1.5 Gy when 15 Gy was applied to the first implanted cornea. Similarly, one cornea received 3 Gy when 30 Gy was administered to the other cornea.

As a positive control, AVM tissues were implanted and not irradiated in one cornea of 10 animals; as a negative control, a blood clot was implanted into the other cornea of the same 10 animals and not irradiated. Therefore, 10 AVM tissue specimens were tested for dose groups (1.5 and 15 Gy; and 3 and 30 Gy) and for positive (nonirradiated AVM) and negative controls (clot).

Ten rats (20 corneas) were used to test the 1.5- and 15-Gy irradiation doses, 10 rats (20 corneas) were used to test 3-Gy and 30-Gy irradiation doses, and 10 rats (20 corneas) served as controls to test 0 Gy and clot (no irradiation). In summary, 30 rats (60 corneas) were used in the control (clot) and dosing groups (0, 1.5, 3, 15, and 30 Gy) (Table 1).

Biomicroscopic observations were recorded daily for 20 days. If signs of ocular infection appeared in either eye (discharge, redness around the eye), that rat was excluded and a replacement was acquired (with the same procedure and tissue sample). For daily data collection, each of the 60 corneas was photographed using a digital video system (Digital Video Camera 201; Sony) attached to a biomicroscope (Zeiss), and the degree of angiogenesis present was graded by a person who was blinded to the study. Ongoing angiogenic activity was graded daily according to a system based on the number of vessels around the implanted corneal micropocket. The grading scale for angiogenesis was as follows: Grade 0, no evidence of angiogenesis (no visible vessels); Grade 1, low angiogenic activity (< three vessels visible); Grade 2, moderate angiogenic activity (three–eight vessels visible); and Grade 3, high angiogenic activity (> eight vessels visible and/or the corneal tissue between arterioles contained anastomosing microvessels) (Fig. 1).

Microvessel Counting and VEGF Staining Intensity

Forty-eight other rats were used for the microvessel counting and VEGF staining portion of the experiment. Micropieces of the specimens described previously were again used for corneal implantation. For each of the six different cornea groups (nonirradiated clot, nonirradiated AVM, and 1.5-, 3-, 15-, and 30-Gy AVM), three different rat groups (nonirradiated AVM and clot, 1.5 and 15 Gy, and 3 and 30 Gy) composed of 48 rats (96 corneas) were used. Four rats from each group (12 rats, 24 corneas) were killed on Days 5, 10, 15, and 20, and four corneas from each of the six cornea study groups were examined for a microvessel count and VEGF staining (Table 1).

Each eye was fixed in formaldehyde solution and embedded in a paraffin block. Full-thickness sagittal sections passing through the corneal micropocket were stained with hematoxylin and eosin, and immunohistochemical stain for VEGF. The procedures for these staining methods have been described previously.20–22

For each specimen, the number of microvessels was counted under a magnification of 10, and the mean for each group was recorded. The intensity of VEGF staining was assessed in a three-tier system: Grade 0, no expression; Grade 1, moderate or focal expression; and Grade 2, strong and diffuse expression. For each tissue group, the mean microvessel count and the mean VEGF staining grade were calculated on postimplantation Days 5, 10, 15, and 20.

Data Analysis

The Statview software package (version 4.5; Abacus Concepts) was used to illustrate the percentage distributions of specimens and to graph the data. The results for corneal angiogenesis grading were interpreted and compared as a function of time using cell line graphs with error bars indicating one standard error of the mean.

Results

Corneal Angiogenesis Assay

The extent of angiogenesis that five different Gamma Knife irradiation dose groups and a clot negative control of AVMs induced in the CAM is shown in Fig. 1. The AVM-
implanted but nonirradiated corneas exhibited high-grade angiogenesis starting from Day 5. Dose groups 1.5 Gy and 3 Gy displayed similar curves reaching Grade 2 after only 20 days. Dose groups 15 Gy and 30 Gy displayed similar curves and did not show more than Grade 1 angiogenesis. Each dose group was evaluated using 10 corneas with tissues from 10 different AVMs.

**Microvessel Counts**

The mean microvessel counts at Days 5, 10, 15, and 20 for each dose group evaluated in the study are shown in Fig. 2. Nonirradiated corneas implanted with AVMs showed the

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**TABLE 1**

Summary of the animal paradigms used in this study

<table>
<thead>
<tr>
<th>Experimental Paradigm</th>
<th>Procedure</th>
<th>No. of Rats &amp; Corneas</th>
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<tr>
<td>CAM (30 rats, 60 corneas)</td>
<td>AVM tissue in 1 cornea &amp; clot in the other</td>
<td>10 rats</td>
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<td></td>
<td>same AVM tissue to both eyes; 1 irradiated by 15 Gy, other by 1.5 Gy in the same procedure</td>
<td>10 rats, 10 corneas have 10 different AVM tissues &amp; no radiation; 10 corneas have blood clot</td>
</tr>
<tr>
<td></td>
<td>same AVM tissue to both eyes; 1 irradiated by 30 Gy, other by 3.0 Gy in the same procedure</td>
<td>10 rats, 10 corneas have 10 different AVM tissues &amp; 15-Gy irradiation; 10 corneas have AVM tissues &amp; 1.5-Gy irradiation</td>
</tr>
<tr>
<td>microvessel count &amp; VEGF staining (48 rats, 96 corneas)</td>
<td>no radiation w/ AVM to 1 cornea &amp; clot to other cornea</td>
<td>10 rats, 10 corneas have 10 different AVM tissues &amp; 30-Gy irradiation; other 10 corneas have AVM tissues &amp; 3.0-Gy irradiation</td>
</tr>
<tr>
<td></td>
<td>same AVM tissue to both eyes; 1 irradiated by 15 Gy, other by 1.5 Gy in the same procedure</td>
<td>Days 5, 10, 15, &amp; 20, 4 rats were killed (16 total); 4 corneas (32 total) from each of the 2 cornea study groups (no radiation &amp; blood clot) were studied</td>
</tr>
<tr>
<td></td>
<td>same AVM tissue to both eyes; 1 irradiated by 30 Gy, other by 3.0 Gy in the same procedure</td>
<td>Days 5, 10, 15, &amp; 20, 4 rats were killed (16 total); 4 corneas (32 total) from each of the 2 cornea study groups (15 &amp; 1.5 Gy) were studied</td>
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**FIG. 1.** Cell line graph showing the extent of angiogenesis in the five different dose groups of AVMs and the clot-negative control group (photographs) tested in the CAM. The AVM-implanted but nonirradiated corneas exhibited high-grade angiogenesis starting at Day 5. Dose groups 1.5 Gy and 3 Gy displayed similar curves reaching Grade 2 after only 20 days. Dose groups 15 Gy and 30 Gy displayed similar curves and did not show more than Grade 1 angiogenesis. Each dose group was evaluated using 10 corneas with tissues from 10 different AVMs.
highest degree of neovascularization in terms of microves- sel counts. At Day 10, microvessel counts were dose de- pendent; that is, the higher the Gamma Knife irradiation applied, the fewer microvessel counts in the CAM were de- tected.

Expression of VEGF

The mean VEGF expression grades on Days 5, 10, 15, and 20 for each dose group are depicted in Fig. 3. Nonir- radiated corneas implanted with AVMs showed the highest degree of neovascularization in terms of immunohistochem- ical expression of VEGF. At Day 20, VEGF staining inten- sities were dose dependent; that is, the higher the Gamma Knife irradiation application, the less expression of VEGF was detected in the CAM.

Discussion

Angiogenesis and AVMs

Angiogenesis requires complex interactions among a va- riety of molecules that regulate endothelial cell processes, such as: production of the ECM; endothelial cell invasion, migration and proliferation; and remodeling of the vascular network. In simple terms, the following are required for the angiogenic cascade to occur: (1) a cell in need of angiogenesis, such as an angiogenic endothelial cell in an AVM; (2) signal transduction from this angiogenic cell, such as production of the VEGF ligand; (3) a signal rec-ognition system at the endothelial cell membrane, such as the VEGF-receptor system; (4) intracellular mechanisms (including genetic responses) that answer the signal appropri- pately, such as actions of integrins and immediate early genes; and (5) modulation of the ECM to allow vascu- lar sprouting by substances such as integrins and matrix metalloproteinases. The AVM endothelium, subendothe- lium, smooth-muscle layer, and adventitia show signs of increased angiogenic activity in each of these major steps in the angiogenic cascade.

The expression profile does not follow any particular pat- tern, however, and is not restricted to vessel size, pheno- type, or cerebral location, which implies a nonspecific secondary elevation of angiogenic molecules. Additionally, whether upregulation of angiogenic ligands and receptors is the result of the decreased flow or ischemia within these lesions is not known. The current data support genetic aberrations as the primary causative insult, with sec- ondary resultant phenotypic alterations observed in the expression of growth factors.

Two studies using microarray technology have con- tributed to the field of understanding AVM angiogenesis. Shenkar and colleagues and Hashimoto and coworkers have identified more than 70 genes that are differentially ex- pressed in AVMs. Future investigations based on these find- ings to assess the functional implications of each gene will elucidate this biological network. One recent study from our laboratory tested the angiogenic activity of AVMs dynam- ically in the rat cornea model for the first time. The AVM tissues removed from an in vitro environment (peri-AVM cerebral tissue) were still active angiogenically and were
Inhibition of AVM-induced angiogenesis

able to produce neovascularization in the rat cornea significantly more frequently than in normal brain and arterial tissues. Thus, angiogenic activity is part of the nature of AVMs, whether it is genetic or phenotypic.

One other molecular feature of AVM angiogenesis is its embryological character. For example, the Flk-1 receptor, which is expressed by AVM endothelium, normally is not detected in the adult brain but is detected in the developing human fetal brain. Additionally, although not as immature as cerebral cavernous malformations, AVM tissues express ECM proteins (such as more fibronectin and collagen III than a normal mature arterial wall), integrins (such as increased integrin αvβ3 and αvβ5 expressions), and matrix metalloproteinases II and IX in the pattern that is more similar to fetal brain vasculature than to adult brain vasculature.

De Novo Generation of AVMs

Reports on the presence of enlarged AVMs and recurrence of cerebral AVMs after normal postoperative angiographies, and reappearance of AVMs after total endovascular occlusion, may be signs of the clinical facet of the dynamic angiogenic activity of AVMs observed molecularly in previous sections of this paper. Sonstein and coworkers have shown increased expressions of VEGF in recurrent pediatric and adult AVMs, indicating a dynamic angiogenic state in recurrent AVMs. Sure and colleagues reported an increased level of angiogenesis in partially obliterated AVMs compared with nonembolized AVMs. In a more recent study by Sure et al., the authors found that there was a significantly greater incidence of VEGF and HIF in embolized AVMs. Nonetheless, HIF is expressed in the majority of nonembolized AVMs, so that its actual role regarding the embolization response is unclear. The authors conclude that the results support, at least circumstantially, their hypothesis that embolization induces hypoxia, which in turn results in neoangiogenesis, possibly related to VEGF upregulation. The high incidence of HIF in nonembolized AVMs suggests that there are other mechanisms at work in the pathogenesis and maintenance of AVMs. The idea that AVMs are not static congenital lesions is not new, but the hypothesis is clearly supported by the demonstration of biological mechanisms that could potentially influence growth of new vessels. The embryologically immature molecular character of the AVM vessel wall is reflected clinically by the higher incidence of overt recurrence of AVMs that occurs in younger patients after surgical excision or radiosurgery.

This summary of different clinical situations of AVM neovascularization supports the argument countering hypotheses that state that AVMs are strictly congenital lesions. One other radiological observation comes from a study by Mullan and colleagues that the current methods of intrauterine and neonatal ultrasonography have been used to identify aneurysms of the vein of Galen at a very early stage, but so far have not aided in the discovery of an equivalent number of cerebral AVMs, even though these anomalies are far more frequently seen in neurosurgical practice.

**Fig. 3.** Bar graph showing the mean VEGF staining grades at Days 5, 10, 15, and 20 for each dose group and the control group evaluated in the study. Photomicrographic examples of microvessels are given for 3 Gy at Day 5 (left), nonirradiated at Day 15 (center), and nonirradiated at Day 20 (right). Nonirradiated corneas implanted with AVMs showed the highest degree of neovascularization in terms of immunohistochemical expression of VEGF. At Day 20, microvessel counts were dose dependent—the higher the level of Gamma Knife irradiation applied, the lower the expression of VEGF detected in the CAM.
Dose-Dependent Effects of Gamma Knife Irradiation on Angiogenesis

The main finding of the present study is that Gamma Knife irradiation reduces neovascularization in the rat cornea around implanted AVM tissue. Additionally, microvessel counting and VEGF staining, performed as part of this experiment, support this finding. Recent data from our group33 show that testing angiogenic potentials of different human cerebrovascular malformations by using the CAM provides quantitative data that allow assessment of neovascularization over time. The dynamic aspect of this method (evaluating angiogenesis over time) is novel, as most published information on angiogenesis in AVMs is based on static (one point in time) descriptive analyses. This comparative analysis of the angiogenic activity of cerebrovascular malformations concludes that AVMs show the highest amount of angiogenic activity, and the angiogenic activity of cavernous malformations is significantly lower than that of AVMs but still higher than that of normal arteries in the brain.

The current study also contributes the data that Gamma Knife irradiation inhibition of AVM-induced angiogenesis is dose dependent. The dose of radiation needed to have an antiangiogenic effect may be different than that needed to have an occlusive effect in AVMs with Gamma Knife irradiation. Our data reveal that 15 Gy and 30 Gy irradiation had a significantly greater antiangiogenic effect than did 1.5 Gy and 3 Gy. However, to elucidate definitive dose escalation curves, further experiments with greater numbers of animals are recommended.

Biological Mechanisms of AVM Antiangiogenic Activity

Although GKS has been used to treat cerebral AVMs for more than 30 years and has been validated clinically worldwide, the biological mechanisms when applied to the AVM tissue that lead to obliteration of the nidus and exclusion from circulation are far from being completely understood. Szeifert and colleagues38–40 and Schneider and coworkers41 have studied the histopathology of AVMs treated with GKS. Two types of histopathological changes were reported in these studies. Changes to the vascular elements were characterized as endothelial damage, vessel wall irregularities, subendothelial proliferation, and thrombus formation. Changes in the supportive tissue of AVMs were observed as granulation tissue formation, proliferation of spindle-shaped cells, accumulation of fibrocytes, and extensive hyaline degeneration and focal calcifications in collagen fibers. This radiation-induced granulation tissue reaction is similar to the response seen in the connective tissue stroma of tumors following ionizing radiation.24,25 One group suggests that, “In view of the contractile activity of myofibroblasts, the proliferation generated by irradiation and the transformation of the resting cells into an activated form could be relevant to the shrinking process and eventual occlusion of AVMs after radiosurgery.”79

These observations are descriptive, however, and more observations and particularly dynamic experiments in animal models are needed to elucidate the mechanisms of AVM obliteration after Gamma Knife irradiation. Its biological processes may be different than the antiangiogenic activity of Gamma Knife irradiation on AVMs, which seems to be more important to the clinical appearances of neovascularization properties of AVMs mentioned previously, that is, the presence of enlarged AVMs and recurrence of cerebral AVMs after normal postoperative angiographies, and reappearance of AVMs after total endovascular occlusion.

Conclusions

Gamma Knife irradiation reduces neovascularization in the rat cornea induced by human AVM tissue. This inhibitory effect of Gamma Knife irradiation on AVM-stimulated angiogenesis is dose dependent. This experiment demonstrates the antiangiogenic property of Gamma Knife irradiation and may be important in understanding why some AVMs that become angiographically negative after Gamma Knife irradiation do not reappear whereas other AVMs that become angiographically negative after endovascular treatment do reappear. Therefore, one may be certain of completely healing AVMs only if all the angiogenic AVM tissue (possibly the AVM endothelium) is surgically removed, or if this first option is not achieved, transformed to a biologically nonangiogenic state.

References


