RGD-Tachyplesin Inhibits Tumor Growth

Yixin Chen, Xueming Xu, Shuigen Hong, Jinguo Chen, Ningfei Liu, Charles B. Underhill, Karen Creswell, and Lurong Zhang

Department of Oncology, Lombardi Cancer Center, Georgetown University Medical School, Washington, D.C. 20007 [X-M. X., J. C., N. F., C. B. U., K. C., L. Z.], and The Key Laboratory of China Education Ministry on Cell Biology and Tumor Cell Engineering, Xiamen University, Fujian 361005, People’s Republic of China [Y. C., S. H.]

Abstract

Tachyplesin is an antimicrobial peptide present in leukocytes of the horseshoe crab (Tachylepis tridentatus). In this study, a synthetic tachyplesin conjugated to the integrin homing domain RGD was tested for antitumor activity. The in vitro results showed that RGD-tachyplesin inhibited the proliferation of both cultured tumor and endothelial cells and reduced the colony formation of TSU prostate cancer cells. Staining with fluorescent probes of FITC-annexin V, JC-1, YO-PRO-1, and FITC-dextran indicated that RGD-tachyplesin could induce apoptosis in both tumor and endothelial cells. Western blotting showed that treatment of cells with RGD-tachyplesin could activate caspase 9, caspase 8, and caspase 3 and increase the expression of the Fas ligand, Fas-associated death domain, caspase 7, and caspase 6, suggesting that apoptotic molecules related to both mitochondrial and Fas-dependent pathways are involved in the induction of apoptosis. The in vivo studies indicated that the RGD-tachyplesin could inhibit the growth of tumors on the chorioallantoic membranes of chicken embryos and in syngeneic mice.

Introduction

Tachyplesin, a peptide from hemocytes of the horseshoe crab (Tachylepis tridentatus), can rapidly inhibit the growth of both Gram-negative and -positive bacteria at extremely low concentrations (1, 2). Tachyplesin has a unique structure, consisting of 17 amino acids (KWCFRCYGICYRRCC) with a molecular weight of 2,269 and a pI of 9.93. In addition, it contains two disulfide linkages, which causes all six of the basic amino acids (R, arginine; K, lysine) to be exposed on its surface (3). The cationic nature of tachyplesin allows it to interact with anionic phospholipids present in the bacterial membrane and thereby disrupt membrane function (4, 5).

The structural nature of tachyplesin suggested that it might also possess antitumor properties. Tachyplesin can interact with the neutral lipids in the plasma membrane of eukaryotic cells (4, 5). More importantly, because it can interact with the membranes of prokaryotic cells, it is likely that tachyplesin can also interact with the mitochondrial membrane of eukaryotic cells. Indeed, these membranes are structurally similar because mitochondria are widely believed to have evolved from prokaryotic cells that have established a symbiotic relationship with the primitive eukaryotic cell (6). Recent studies have indicated that mitochondria play a critical role in regulating apoptosis in eukaryotic cells (7). The disruption of mitochondrial function results in the release of proteins that normally are sequestered by this organelle. The release of factors, such as cytochrome c and Smac, can activate caspases that, in turn, trigger the apoptotic cascade (8, 9). Along these lines, Ellerby et al. (10) have found that a cationic antimicrobial peptide (KLAKLAKKLAKLAK) conjugated with a CNGRC homing domain exhibits antitumor activity through its ability to target mitochondria and trigger apoptosis. Because the proapoptotic peptide and tachyplesin belong to the same category of cationic antimicrobial peptide, it seems possible that tachyplesin could have similar antitumor activity.

To explore this possibility, we have examined a chemically synthesized preparation of tachyplesin that was linked to a RGD sequence, which corresponds to a homing domain that allows it to bind to integrins on both tumor and endothelial cells and thereby facilitates internalization of the peptide (11, 12). We found that this synthetic RGD-tachyplesin could inhibit the proliferation of TSU prostate cancer cells and B16 melanoma cells as well as endothelial cells in a dose-dependent manner in vitro and reduce tumor growth in vivo.

Materials and Methods

Synthesis of RGD-Tachyplesin. Two peptides were chemically synthesized. The test peptide was RGD-tachyplesin (CRGDCGGKWCVRFCYRGICYRRCC), and the control peptide was a scrambled sequence with a similar molecular weight and pI. To impede enzymatic degradation, the NH2-terminal of the peptide was acetylated, and the COOH-terminal was amidated. Before use, the peptides were dissolved in dimethylformamide and 1% acetic acid, diluted with saline to a concentration of 1 mg/ml, and sterilized by boiling for 15 min in a water bath.

Cell Lines. The TSU human prostate cancer cells, B16 melanoma, Cos-7, and NIH-3T3 were maintained in 10% calf serum and 90% DMEM. The human umbilical vein endothelial cells and ABAE1 cells were cultured in 20% fetal bovine serum and 80% DMEM containing 10 mg/ml fibroblast growth factor 2 and vascular endothelial growth factor, respectively.

Cell Proliferation Assay. Aliquots of complete medium containing 5000 cells were distributed into a 96-well tissue culture plate. The next day, the media were replaced with 160 µl of fresh media and 40 µl of a solution containing different concentrations of the peptides. One day later, 30 µl of 0.3 µCi of [3H]thymidine in serum-free media were added to each well, and after 8 h, the cells were harvested, and the amount of incorporated [3H]thymidine was determined with a beta counter.

 Colony Formation Assay. TSU cells (2 × 104) were suspended in 1 ml of 0.36% agarose in 90% DMEM and 10% calf serum containing 100 µg/ml control peptide or RGD-tachyplesin and then immediately placed on the top of a layer of 0.6% solid agarose in 10% calf serum and 90% DMEM in 6-well plates. Two weeks later, the number of colonies larger than 60 µm in diameter was determined using an Omnicom Image Analysis system (Imaging Products International Inc., Chantilly, VA).

Analysis of Tachyplesin-damaged Cells by Flow Cytometry. Cultures of TSU cells at 80% confluence were treated overnight with 50 µg/ml control peptide or RGD-tachyplesin. The next day, the cells were harvested with 5 mm EDTA in PBS, washed, resuspended in 10% calf serum and 90% DMEM, and then stained with the fluorescent dyes annexin V and propidium iodide, JC-1, and PI.

Received 11/22/00; accepted 1/30/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by National Cancer Institute/NHG Grant R29 CA71545; United States Army Medical Research and Materiel Command Grants DAMD17-99-1-9031, DAMD17-98-1-8099, DAMD17-00-1-0881, and PC970502; and Susan G. Komen Breast Cancer Foundation (C. B. U. and L. Z.). Y. C. was a recipient of China Scholarship Council, and L. Z. was a recipient of funding from the visiting scholar foundation for key laboratory at Xiamen University, China.

2 To whom requests for reprints should be addressed, at Department of Oncology, Lombardi Cancer Center Georgetown University Medical School, 3970 Reservoir Road, NW, Washington, D.C. 20007. Phone: (202) 687-6397; Fax: (202) 687-7505; E-mail: Zhangl@georgetown.edu.

3 The abbreviations used are: ABAE, adult bovine aorta endothelial; FADD, Fas-associated death domain, CAM, chorioallantoic membrane.
YO-PRO-1, and FITC-dextran, according to manufacturer’s instructions (Molecular Probes, Eugene, OR).

**Western Blotting.** Cultures of TSU and ABAE cells at approximately 80% confluence were treated overnight with 100 μg/ml peptides and then harvested with 1 ml of lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.5 μg/ml leupeptin, 1 mM EDTA, 1 μg/ml pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride). The protein concentration was determined by the BCA method (Pierce, Rockford IL), and 20 μg of protein lysate were loaded onto 4–12% BT NuPAGE gel (Invitrogen, Carlsbad CA), electrophoresed, and transferred to a nitrocellulose membrane. The loading and transfer of equal amounts of protein were confirmed by staining with Ponceau S solution (Sigma, St. Louis, MO). The membranes were blocked with 5% nonfat milk and 1% polyvinylpyrrolidone in PBS for 30 min and then incubated for 1 h with 1 μg/ml antibodies to Fas ligand, FADD, caspase 9, caspase 8, caspase 3, caspase 7, and caspase 6 (Oncogene, Boston, MA). After washing, the membrane was incubated for 1 h with 0.2 μg/ml of peroxidase-labeled antirabbit IgG followed by a chemiluminescent substrate for peroxidase and exposed to enhanced chemiluminescence Hyperfilm MP (Amersham, Piscataway, NJ).

**Effect of RGD-Tachyplesin on TSU Tumor Growth on the Chicken CAM.** TSU cells (2 × 10^7) were mixed with equal amounts of control peptide or RGD-tachyplesin (100 μg in 200 μl of saline) and immediately placed on top of the CAMs of 10-day-old chicken embryos (15 eggs/group) and incubated at 37.8°C. Every other day thereafter, 200 μl of PBS containing 100 μg of the peptides were added tropically to the xenografts on the CAMs. Five days later, the xenografts were dissected from the membrane, photographed, and weighed.

**Effect of RGD-Tachyplesin on B16 Tumor Growth in Mice.** B16 melanoma cells were injected s.c. into the flank of 5-week-old male C57BL/6 mice (5 × 10^7 cells/site; 5 mice/group) and allowed to establish themselves for 2 days. Every other day thereafter, 250 μg of the control peptide or RGD-tachyplesin was injected i.p. into the mice. At the end of 2 weeks, the mice were sacrificed, and the tumor xenografts were removed, photographed, and weighed.

**Statistical Analysis.** The mean and SE were calculated from the raw data and then subjected to Student’s t test. P < 0.05 was regarded as statistical significance.

**Results**

**RGD-Tachyplesin Inhibits the Growth of Tumor and Endothelial Cells in Vitro.** Because both tumor and endothelial cells play an important role in determining tumor progression, we initially examined the effects of RGD-tachyplesin on the proliferation of both of these cells in vitro. As shown in Fig. 1A, RGD-tachyplesin inhibited the growth of the cultured cells in a dose-dependent manner, with an EC_{50} of about 75 μg/ml for TSU tumor cells and 35 μg/ml for the endothelial cells. In contrast, the scrambled peptide had no obvious effect on the proliferation of the cells at 100 μg/ml. This effect was also reflected in the morphology of the cells. After exposure to 50 μg/ml RGD-tachyplesin for 12 h, a significant fraction of treated cells had become rounded and detached, whereas few cells did so after treatment with the control peptide (data not shown).

To determine whether nontumorigenic cells were also affected by RGD-tachyplesin, the immortalized cell lines, Cos-7 and NIH-3T3, were tested in the [3H]thymidine incorporation assay. As shown in Fig. 1B, when treated with 50 μg/ml RGD-tachyplesin, the extent of inhibition of Cos-7 or NIH-3T3 (0–20%) was less than that of tumor or proliferating endothelial cells (40–75%), indicating that nontumorigenic cells are less sensitive to RGD-tachyplesin.

Next, we examined the effects of the peptides on the growth of TSU cells in soft agar. The ability of cells to grow under such anchorage-independent conditions is one of the characteristic phenotypes of aggressive tumor cells. As shown in Fig. 1C, RGD-tachyplesin inhibited the ability of TSU cells to form colonies as compared to the groups of control peptide and vehicle alone.

**Treatment with RGD-Tachyplesin Alters Membrane Function.** We then examined the mechanism by which RGD-tachyplesin inhibited the proliferation of the tumor and endothelial cells. One possibility was that RGD-tachyplesin damages cell membranes, and this damage, in turn, induces apoptosis.

To examine the extent of apoptosis, TSU cells that had been treated for 1 day with the test or control peptides were stained with FITC-annexin and propidium iodide. FITC-annexin V binds to phosphatidylserine, which is exposed on the outer leaflet of the plasma membrane of cells in the initial stages of apoptosis, whereas propidium iodide preferentially stains the nuclei of dead cells, but not living cells. Fig. 2A shows that treatment with RGD-tachyplesin induced apoptosis (annexin V positive, propidium iodide negative) in a greater number of cells than did treatment with the vehicle or control peptide.

This induction of apoptosis could have been due to the disruption of mitochondrial function. To examine this, we used JC-1 staining, which measures the membrane potential of mitochondria. As shown in Fig. 2, B and C, treatment with RGD-tachyplesin caused a shift in the fluorescence profile from one that was highly red (Fig. 2B) to one that was less red and more green (Fig. 2C). This indicated that the membrane potential of mitochondria was changed by treatment with RGD-tachyplesin.

We also examined the integrity of the plasma membrane and nuclear membrane after treatment with the scrambled peptide and RGD-tachyplesin using two different fluorescent markers. YO-PRO-1 dye can only stain the nuclei of cells with damaged plasma and nuclear membranes. Fig. 2D shows that treatment with RGD-tachyplesin allowed the YO-PRO-1 dye to pass into the nuclei, causing an increase in the fluorescence intensity. Similar results were obtained when the cells were stained with FITC-dextran, which is not taken up by viable, healthy cells but can pass through the damaged plasma membrane of unhealthy cells. Fig. 2E shows that cells treated with RGD-tachyplesin took up a greater amount of FITC-dextran (M_t 40,000) than did those treated with the control peptide. These results indicated that the majority of RGD-tachyplesin-treated cells allowed these big molecules to pass their damaged membranes.

The above-mentioned experiments were also carried out with ABAE cells, and similar results were obtained (data not shown). Presumably, RGD-tachyplesin induces apoptosis in both TSU and ABAE cells by damaging their membranes.

**RGD-Tachyplesin Triggers Apoptotic Pathways.** Apoptosis can be induced by two mechanisms: (a) the mitochondrial pathway; and (b) the death receptor pathway (13). To identify the nature of the apoptotic pathway triggered by RGD-tachyplesin, both TSU and ABAE cells were treated overnight with RGD-tachyplesin and control peptide and then analyzed by Western blotting for the alterations of molecules involved in the mitochondrial and Fas-dependent pathways. Fig. 3 shows that treatment of both TSU tumor cells and ABAE cells with RGD-tachyplesin caused the cleavage of M_r 46,000 caspase 9 into subunits of M_r 35,000 and M_r 10,000, indicating activation of the mitochondrial-related, Fas-independent pathway. In addition, RGD-tachyplesin treatment could up-regulate the expression of upstream molecules in the Fas-dependent pathway, including Fas ligand (M_r 43,000), FADD (M_r 28,000), and activate subunits of caspase 8 (M_r 18,000). Furthermore, the downstream effectors, such as caspase 3 subunits (M_r 20,000), caspase 6 (M_r 40,000), and caspase 7 (M_r 34,000), were also up-regulated by RGD-tachyplesin. These results suggest that RGD-tachyplesin induces apoptosis through both the mitochondrial-related, Fas-independent pathway and the Fas-dependent pathway. However, because there is cross-talk between these two pathways (13), we do not have enough evidence to determine which one is the initiator.
RGD-Tachyplesin Inhibits the Growth of TSU and B16 Tumor in Vivo.

In the final series of experiments, we examined the in vivo effects of RGD-tachyplesin on the growth of TSU or B16 tumor cells in CAM or mouse models. As shown in Fig. 4, the TSU tumor xenografts growing in CAM in the group treated with RGD-tachyplesin (Fig. 4A) were smaller than those in the group treated with control peptide (Fig. 4A). In addition, the average weight of the xenografts in the RGD-tachyplesin-treated group was significantly less than that of the xenografts in the control group (Fig. 4C). Similarly, in the B16 mouse model, the B16 tumor xenografts in the RGD-tachyplesin-treated group (Fig. 4E) were smaller than those in the control group (Fig. 4D), and this difference was statistically significant (P < 0.05; Fig. 4F). It should be noted that RGD-tachyplesin did not appear to be toxic to the mice, as judged by their weights and activity at the end of the experiment. Thus, the results from two models are consistent with each other, indicating that RGD-tachyplesin can inhibit tumor growth in vivo.

Discussion

The major conclusion of this study is that RGD-tachyplesin can inhibit tumor growth by inducing apoptosis in the tumor cells and the associated endothelial cells. This conclusion was supported by the following observations. First, RGD-tachyplesin was able to inhibit the growth of B16 tumor cells in syngenic mice. Second, RGD-tachyplesin also blocked the proliferation of both tumor and endothelial cells in culture in a dose-dependent fashion, whereas proliferation was relatively unaffected in nontumorigenic cell lines Cos-7 and NIH-3T3. Third, RGD-tachyplesin induced apoptosis in cultured TSU cells, as indicated by staining with fluorescent markers for apoptosis including FITC-annexin V, which detects exposed phosphatidylserine, and JC-1, which tracks mitochondrial membrane potential. Finally, RGD-tachyplesin stimulated the activation and production of several molecules in the apoptotic cascade in both TSU and endothelial cells, as judged by Western blotting.

---

RGD-TACHYPLESIN INHIBITS TUMOR CELL GROWTH

RGD-TACHYPLESIN INHIBITS TUMOR CELL GROWTH

Our results also suggest that RGD-tachyplesin up-regulates apoptosis related to both the mitochondrial and the death receptor pathways. The involvement of the mitochondrial pathway was suggested by the facts that staining with JC-1 indicated the membrane potential of mitochondria was decreased (Fig. 2, B and C) and that the caspase 9 was activated (Fig. 3) in cells treated with RGD-tachyplesin. Presumably, this resulted from the release of cytochrome c, which, in turn, bound to Apaf-1 and activated caspase 9 and then caspase 3, caspase 7, and caspase 6 (13, 15–17). This is the mechanism by which the peptide described by Ellerby et al. (10) induced apoptosis. In addition, we found that members of the death receptor pathway (Fas ligand, FADD, caspase 8, caspase 3, caspase 2, and caspase 6) were up-regulated. Thus, RGD-tachyplesin may have multiple effects on the target cells. It is difficult at this point to determine what initial event is responsible for the RGD-tachyplesin-induced activation of apoptosis.

There appears to be considerable cross-talk between the mitochondrial apoptotic pathway and Fas-dependent pathway. The caspase 6 activated by the mitochondrial pathway (cytochrome c→Apaf-1→caspase 9→caspase 3) could act on FADD and then on caspase-8, which triggered the Fas-dependent pathway. On the other hand, the caspase 8-activated Fas-FADD pathway could act on BID that stimulates the mitochondrial pathway (15–17). This cross-talk creates positive feedback and enhances the apoptosis cascade.

RGD-tachyplesin also appeared to be relatively nontoxic to cells not associated with tumors. When RGD-tachyplesin was administered at a concentration that inhibited tumor growth, there was no notable side effects on either the chicken embryos or mice with regard to animal body weight and activity at the end of each experiment. In addition, studies on cultured cells indicated that the sensitivity to RGD-tachyplesin varied depending on cell type. In comparison to tumor cells and proliferating endothelial cells, immortalized cells such as Cos-7 (green monkey kidney cells) and NIH-3T3 (fibroblast cells) were less sensitive to RGD-tachyplesin. Taken together, these results suggest that RGD-tachyplesin is a well-tolerated peptide.

RGD-tachyplesin also appears to be more potent than similar cationic peptides. The unique cyclic structure of tachyplesin maintained by two disulfide bonds may make it more effective in targeting membranes than the linear antimicrobial peptides, such as KLAK-LAKKLAKLAK (a proapoptotic peptide; Ref. 10), which is suggested by its lower minimal inhibition concentration on both Escherichia coli and Staphylococcus aureus of 2 versus 6 μM (18, 19). Furthermore, tachyplesin interacts not only with anionic phospholipids of bacterial and mitochondria but also with neutral lipids of eukaryotic plasma membrane (4, 5, 18). Ellerby et al. (10) reported that their proapoptotic peptide inhibited proliferation with an EC₅₀ of about 100 μg/ml for endothelial cells, whereas our results indicated that RGD-tachyplesin had a much stronger efficacy on proliferating cells.

Fig. 2. The effect of RGD-tachyplesin on the function of TSU cells. TSU cells were treated overnight with 50 μg/ml control peptide or RGD-tachyplesin and then stained with different membrane probes. A, staining with annexin V and propidium iodide for apoptotic cells. The percentage of cells that were positive for FITC-annexin V and negative for propidium iodide was analyzed by flow cytometry. The RGD-tachyplesin-treated cells had a higher percentage of apoptotic cells (P < 0.01). B and C, staining with JC-1 for mitochondrial membrane potential. The cells treated with the control peptide (B) and RGD-tachyplesin (C) were stained with 10 μg/ml JC-1 for 10 min and then analyzed by flow cytometry. The RGD-tachyplesin shifted the spectrum of the cells from high red (B, healthy) to high green and low red (C, loss mitochondrial potential). D, staining with YO-PRO-1 for integrity of nuclei membrane. The peptide-treated cells were stained with 0.1 μg/ml YO-PRO-1 dye, an indicator for damaged nuclei membranes (the first peak represents the dye-stained G₀-G₁-phase cells; the second peak represents the dye-stained S-M-phase cells). The RGD-tachyplesin treated cells shift from right to left, indicating the loss of integrity of the nuclei membrane. E, staining with FITC-dextran for integrity of plasma membrane. The peptide-treated cells were incubated with 50 μg/ml FITC-dextran (M, 40,000) for 30 min and analyzed with flow cytometry. A higher proportion of RGD-tachyplesin-treated cells allowed FITC-dextran to pass through their plasma membrane as compared to the control.

Fig. 3. Effect of RGD-tachyplesin on molecules involved in the apoptosis cascade. Twenty μg of lysate from TSU and ABAE cells treated with 100 μg/ml control peptide or RGD-tachyplesin were loaded onto a 4–12% BT NuPAGE gel, electrophoresed, and transferred to a nitrocellulose membrane. The loading and transfer of equal amounts of protein were confirmed by staining with a Ponceau S solution. After blocking with 5% nonfat milk, the membranes were incubated for 1 h with 1 μg/ml antibodies to caspase 9, Fas ligand, FADD, caspase 8, caspase 3, caspase 7, and caspase 6 followed by horseradish peroxidase-conjugated antirabbit IgG and enhanced chemiluminescence substrate and finally exposed to Hyperfilm MP.

2437
RGD-TACHYPLESIN INHIBITS TUMOR CELL GROWTH

Fig. 4. Effect of RGD-tachyplesin on tumor growth in vivo. A–C, effect of RGD-tachyplesin on the TSU xenografts on CAM. TSU cells (2 × 10⁵) were mixed with either control peptide or RGD-tachyplesin (100 μg) and immediately placed on top of the CAM of 10-day-old chicken embryos and incubated at 37.8°C. Every other day, additional peptides were applied tropically to the TSU xenografts. Five days later, the xenografts were removed from the CAM, photographed, and weighed. The TSU xenografts treated with RGD-tachyplesin were significantly smaller than those treated with the control peptide (P < 0.01). D–F, effect of RGD-tachyplesin on tumor growth in the mouse model. B16 melanoma cells (5 × 10⁵) were injected s.c. into the flank of C57BL/6 mice and allowed to establish themselves for 2 days. Every other day after that, 250 μg of control peptide or RGD-tachyplesin were injected i.p. into the mice. At the end of 2 weeks, the mice were sacrificed, and the xenografts were removed, photographed, and weighed. The B16 xenografts from animals treated with RGD-tachyplesin were significantly smaller than those from animals treated with the control peptide (P < 0.01).

endothelial cells, with an EC50 of about 35 μg/ml. Furthermore, RGD-tachyplesin acts not only on proliferating endothelial cells but also on tumor cells. This dual effect of RGD-tachyplesin will enhance its antitumor function.

In conclusion, this study demonstrates that RGD-tachyplesin can be used as an antitumor agent. By disrupting vital membranes and inducing apoptosis, it inhibits all of the tumor cells tested. Further study of RGD-tachyplesin and its analogues may lead to finding a new category of antitumor drug.

Acknowledgments

The animal protocols were reviewed and approved by the Animal Care and Use Committee of Georgetown University.

References

RGD-Tachyplesin Inhibits Tumor Growth

Yixin Chen, XueMing Xu, Shuigen Hong, et al.

Cancer Res 2001;61:2434-2438.

Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/6/2434

Cited articles  This article cites 19 articles, 9 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/6/2434.full#ref-list-1

Citing articles  This article has been cited by 12 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/6/2434.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.