Urokinase-mediated Extracellular Matrix Degradation by Human Prostatic Carcinoma Cells and Its Inhibition by Retinoic Acid

Mukta M. Webber and Anuradha Waghry
Departments of Medicine and Zoology, Michigan State University, East Lansing, Michigan 48824-1312

ABSTRACT

Both normal and malignant prostatic epithelial cells in culture secrete urokinase-type plasminogen activator (u-PA) into the culture medium. u-PA has been shown to have a direct association with invasive and metastatic potential of many types of cancers. We propose that prostate cancer has the intrinsic ability to invade and metastasize because of its inherent ability to secrete the serine protease u-PA. We further propose that in prostate cancer, u-PA is the key enzyme which occupies a place at the apex of the proteolytic cascade and initiates the degradative process. Subsequently, collagenases are recruited after activation of procollagenases by another serine protease plasmin formed by the activation of plasminogen by u-PA. Extracellular proteolysis involving plasmin can cause massive degradation of the extracellular matrix. We show that u-PA alone can use fibronectin as a substrate and degrade it, but u-PA alone did not degrade laminin. Serum-free conditioned medium from DU-145 human prostatic carcinoma cells has the ability to degrade both fibronectin and laminin. However, treatment of cultures with 1 μM all-trans retinoic acid (RA) for 48 h reduced the ability of serum-free conditioned medium to cause u-PA-mediated degradation of fibronectin and laminin. Thus, RA had a protective effect on these extracellular matrix glycoproteins. Treatment of cells with RA also decreased their ability to invade Matrigel in the in vitro invasion assay in a dose-dependent manner. RA at the 0.5, 1, and 10 μM level reduced invasion to 65.7%, 46.7%, and 34.3% of control, respectively. RA reduced extracellular proteolysis and thus inhibited extracellular matrix degradation and invasion. These results may also explain one mechanism by which retinoids inhibit invasion and metastasis in vitro and in vivo. These studies have important translational value in the chemoprevention of progression of prostatic intraepithelial neoplasia to invasive carcinoma.

INTRODUCTION

The primary cause of death from prostate cancer is invasion and metastasis. In the early stages of tumor development, cells with a metastatic phenotype may exist in the heterogeneous tumor cell population in a primary tumor. PIN is considered to be equivalent to carcinoma in situ. During progression from PIN to invasive carcinoma, tumor cells cross tissue boundaries and invade the surrounding stroma. Invasion of the BM is an active process involving cell adhesion to the BM, degradation of the ECM, and migration (1). Localized degradation of ECM takes place in areas where the ratio of proteolytic enzymes to their natural inhibitors, present in the surrounding ECM, shifts in favor of proteolysis.

Invasion is a critical initial step in the metastatic cascade. Degradative enzymes involved in invasion and metastasis include serine proteases, metalloproteases, cathepsins, and heparanases. A cascade including all or some of these is probably involved in the invasion process; however, in different tumors, one type of enzymes may dominate the process. A correlation between invasion and metastatic potential and increased expression of ECM degrading proteases has been demonstrated (1). However, little is known about the interactions of normal and malignant prostatic epithelial cells with their ECM, or about the mechanisms involved in the degradation of the BM and ECM during tumor progression, invasion, and metastasis in prostate cancer. An understanding of the mechanisms regulating these interactions is essential for a complete understanding of tumor progression and for devising ways by which these degradative processes could be inhibited while still in the early PIN stage of tumor development.

Retinoids play an important role in the control of normal epithelial cell proliferation and differentiation, and they inhibit carcinogenesis, growth, invasion, and metastasis of certain tumors both in vivo and in vitro (2, 3). A reduction in the incidence of primary prostate cancer and metastases induced by an initiation-promotion protocol involving methyl nitrosourea and testosterone in Lobund-Wistar rats and inhibition of angiogenesis in such tumors by N-(4-hydroxyphenyl)retinamide has been reported (4, 5). Furthermore, inhibition of melanoma cell invasion in vivo and in vitro by RA has also been shown (6). It is interesting to note that a recent epidemiological study showed an increased risk for prostate cancer in men with low serum vitamin A levels (7).

In the present study, we examined: (a) the ability of u-PA alone and that of CM from DU-145 human prostatic carcinoma cells to degrade ECM glycoproteins fibronectin and laminin; (b) the effects of RA on the degradation of ECM in prostatic carcinoma; and (c) the effects of RA on in vitro invasion by
DU-145 cells. In conducting these studies, one objective was to identify agents which would decrease or block extracellular activity of degradative proteases and thus inhibit the process of ECM degradation and invasion.

MATERIALS AND METHODS

Materials. The materials used were: human prostatic carcinoma cell line DU-145 HTB 81 from American Type Culture Collection; RPMI 1640 medium 320-1875AJ, antibiotic/antimycotic mixture 600-5240AG from Gibco; FBS from Interge; HEMA-3 stain 122-911 from Curtin Matheson; human fibronectin 4008, mouse laminin 40232, and Matrigel 40234 from Collaborative Research; human urokinase 128 from American Diagnostica; RA R 2625, human plasminogen 5661, mAb to human fibronectin F-7387 and to mouse laminin L-8271 from Diagnostica; aprotinin 236-624 from Boehneger Mannheim; Centriflip 10, 4304 filter units for concentrating conditioned medium from Amicon; 4-15% gradient gels from Joule; for gel electrophoresis and Western blot analysis, 4-15% gradient gels were transferred to Immobilon-P membrane IPUH-304 FO from Millipore.

Cell Culture. Stock cultures of DU-145 cells were maintained in RPMI 1640 medium containing 2% glutamine, 100 units penicillin, 100 μg streptomycin, 0.25 μg Fungizone/ml medium, and 5% FBS. Cells were subcultured once per week.

Collection of SF-CM. Cells (2.5 million) were plated in 150-mm culture plates and allowed to grow for 24 h in RPMI 1640 medium containing 10% FBS. Subsequently, the cultures were washed thoroughly with three changes of PBS, and 15 ml serum-free RPMI 1640 medium were added per dish. For cultures to be treated with RA, the SF-CM also contained the appropriate concentration of RA dissolved in absolute ethanol. The final concentration of ethanol in the culture medium was 0.1%. Cells were treated with RA for 48 h in all experiments. For assays involving degradation of fibronectin and laminin by SF-CM, the medium was concentrated using filter units with a molecular weight cutoff of 10,000.

Gel Electrophoresis and Western Blot Analysis. SDS-PAGE was performed according to Laemmli (8; also Ref. 9). Samples of pure fibronectin and laminin were incubated with pure urokinase or with CM from 48-h RA-treated and control cultures. Concentrated samples of SF-CM from cultures were standardized for SDS-PAGE on the basis of a fixed cell number (12,000 cells/lane). Samples were run on 4–15% gradient gels at 200 V at 8°C for 45 min to separate fibronectin and laminin fragments after incubation. For Western blots, samples from 4 to 15% gradient gels were transferred to Immobilon-P membrane and immunoblotted with mAb to fibronectin or laminin and stained using the Vectastain ABC kit as described (9) to detect fibronectin and laminin and their degradation fragments.

Degradation of Fibronectin and Laminin. Pure samples of human fibronectin (2.5 μg) were mixed with 400 milliunits of pure urokinase dissolved in water and incubated for 18 h at 37°C. The reaction was stopped with the addition of sample buffer without β-mercaptoethanol, while the tubes were kept on ice. Nonreduced samples were loaded on 4–15% gradient gels without heating. Only the molecular weight markers were reduced. The gels were stained with Coomassie blue and destained in methanol:acetic acid:water (3:1:6). For laminin, 2.5-μg samples of pure laminin were mixed with 500 milliunits of urokinase and incubated as described above. The sample buffer for laminin samples contained β-mercaptoethanol, and these samples were reduced by heating for 5 min at 95°C. Sample mixtures requiring plasminogen contained 1.5 μg plasminogen. Aprotinin, an inhibitor of plasmin, was used to block any plasmin activity in the CM in order to examine degradation of fibronectin or laminin caused by urokinase alone. Ten units of aprotinin in PBS were added to the conditioned medium sample and incubated for 2 h. Fibronectin or laminin was then added, and the mixture was incubated for 18 h at 37°C and processed as described above.

Invasion Assay. Cell invasion was assayed in Boyden blind well chambers containing Matrigel-coated filters, as described by Albini et al. (10). One million cells were plated per 100-mm culture plate. Twenty-four h later, cells were washed three times with PBS and treated with RA in 10 ml serum-free medium for 48 h and released from culture plates using 1 mM EDTA, suspended in RPMI 1640 medium containing 0.1% BSA, and counted. Cells were resuspended in medium with BSA at 1 million cells/ml. The lower chamber was loaded with 220 μl CM (chemoattractant) from human lung fibroblasts grown for 24 h in serum-free medium containing 50 μl ascorbic acid. In the upper chamber, 200,000 cells were plated in 650 μl RPMI 1640 with 0.1% BSA on the Nuclepore filter coated with 500 μg/ml Matrigel. The cells were allowed to migrate for 5 h in the incubator at 37°C. The filters were processed according to a method described by Grotendorst (11). Briefly, the migrated cells were fixed, stained with HEMA-3, and counted. Cells were processed according to a method described by Grotendorst (11). Briefly, the migrated cells were fixed, stained with HEMA-3, and allowed to hydrate in distilled water. Nuclear stain was extracted for 15 min with 0.1 N HCl, and absorbance was measured at 620 nm using a Titertek microplate reader. Three replicate filters were prepared per treatment, and the mean values for three such experiments were plotted.

RESULTS

Urokinase Degrades Fibronectin. To determine whether urokinase has the ability to degrade human fibronectin, pure samples of fibronectin and urokinase were incubated for 18 h and analyzed by immunoblotting. Blots treated with mAb to fibronectin show (Fig. 1) that u-PA has the ability to degrade fibronectin into smaller fragments with molecular weights between ~200,000 and 25,000. However, in the presence of plasminogen, which is activated by urokinase to plasmin, further degradation of fibronectin was observed with some fragments having molecular weights different from those of fragments produced by urokinase alone.

Urokinase Does Not Degrade Laminin. Experiments similar to those described above were conducted using laminin as a possible substrate for urokinase. Immunoblots treated with mAb to laminin show (Fig. 2) that pure urokinase does not degrade laminin. However, when plasminogen is added to the sample mixture, degradation of laminin occurs with the major band of laminin fragment seen at M1 ~97,000 and some smaller fragments. The intensity of high molecular weight laminin bands is concomitantly decreased.
CM from DU-145 Cells Degrades Fibronectin. Having established that pure urokinase has the ability to degrade fibronectin, we then examined the ability of CM from DU-145 cells to degrade fibronectin. Immunoblots treated with mAb to fibronectin show that CM can degrade fibronectin with the loss of the $M_t \approx 440,000$ and $\sim 220,000$ bands (Fig. 3). To determine whether the observed fibronectin degradation was indeed caused by urokinase, aprotinin was added to the SF-CM mixture to block plasmin activity, before adding fibronectin. In the presence of aprotinin, there was some recovery of the high molecular weight fibronectin bands (Fig. 3), indicating that some degradation of fibronectin is attributed to urokinase secreted by DU-145 cells. Inhibition of some degradation by aprotinin indicates the presence of low levels of plasmin activity in the SF-CM.

CM from DU-145 Cells Degrades Laminin. The ability of CM from DU-145 cultures to degrade laminin was then examined. Immunoblots treated with mAb to laminin show that (Fig. 4) degradation of laminin occurred, resulting in several laminin fragments with molecular weights between $\sim 200,000$ and $\sim 97,000$. This degradation could be blocked by the addition of aprotinin, indicating that plasmin but not u-PA degraded laminin.

Degradation of Fibronectin Is Inhibited by CM from Cultures Treated with RA. Cultures of DU-145 cells were treated with RA for 48 h in serum-free medium. Samples of pure fibronectin were incubated with concentrated CM and run on 4–15% gradient gels. Results show (Fig. 5) that fibronectin is degraded by CM from untreated or ethanol-treated control cul-
758 Inhibition of Matrix Degradation in Prostate Cancer

Fig. 4 Western blot analysis of the degradation of laminin (LN) by serum-free CM from DU-145 cells, and inhibition of degradation by aprotinin. Two and one-half μg laminin were used in each sample. Samples were run on a 4–15% gradient gel, transferred to Immobilon-P membrane, and immunoblotted with mAb to laminin. Left, molecular weight markers. Lane LN, laminin alone. Laminin bands at Mr ~400,000 and 220,000 are shown. Lane LN + CM, laminin was mixed with CM and incubated for 18 h at 37°C. Lane LN + CM + Ap, laminin was mixed with CM pretreated for 2 h with 10 units aprotinin (Ap) to block plasmin activity. kDa, kilodalton.

Fig. 5 SDS-PAGE analysis of the effects of RA on the degradation of fibronectin (FN) by SF-CM from DU-145 cells. Two and one-half μg fibronectin were used in each sample. Samples were run on a 4–15% gradient gel. Left, molecular weight markers. Lane FN, fibronectin alone. Fibronectin bands at Mr ~440,000 and 220,000 are shown. Lane FN + CM, fibronectin was mixed with CM from untreated control cultures. Lane ETOH/C, same as Lane C except that CM from ethanol-treated cultures was used. Lane 0.1 μM RA, fibronectin was mixed with CM from cells treated with 0.1 μM RA. Lane 1.0 μM RA, fibronectin was mixed with CM from cells treated with 1.0 μM RA. kDa, kilodalton.

Fig. 6 SDS-PAGE analysis of the effects of RA on the degradation of laminin (LN) by CM from DU-145 cells. Two and one-half μg laminin were used in each sample. Samples were run on a 4–15% gradient gel. Left, molecular weight markers. Lane LN, laminin alone. Laminin bands at Mr ~400,000 and 220,000 are shown. Lane LN + CM, laminin was mixed with CM from untreated control cultures. Lane ETOH/C, same as Lane C except that CM from ethanol-treated cultures was used. Lane 0.1 μM RA, laminin was mixed with CM from cells treated with 0.1 μM RA. Lane 1.0 μM RA, laminin was mixed with CM from cells treated with 1.0 μM RA. kDa, kilodalton.

Laminin is degraded by CM from untreated or ethanol-treated control cultures, with a concomitant decrease in the high molecular weight laminin bands. CM from cultures treated with 0.1 μM RA showed the same pattern of laminin degradation as the controls. However, CM from cells treated with 1.0 μM RA (Fig. 6) resulted in the protection from degradation and partial recovery of the Mr 400,000 laminin band, with the concomitant decrease in lower molecular weight laminin fragments.

Treatment with RA Inhibits Invasion of Matrigel by DU-145 Cells. The ability of DU-145 cells to invade Matrigel-coated filters is impaired by a 48-h pretreatment with RA plus RA treatment during the 5-h invasion assay period. Results show (Fig. 7) that RA treatment reduced invasion of Matrigel by DU-145 cells in a dose-dependent manner. Invasion, expressed as percentage of control, was reduced to 65.7% at 0.5 μM RA, 46.7% at 1 μM RA, and 34.3% at 10 μM RA. These results represent mean values for three separate experiments.

DISCUSSION

Plasminogen activators are considered to play an important role in extracellular proteolysis. Increased u-PA secretion may permit localized proteolysis at the invasive front of the tumor and allow cell detachment, migration, and invasion to occur. Although a transient, local breakdown of the basement membrane and ECM occurs in a number of normal biological processes, such as wound healing and tissue remodeling, the consequences of uncontrolled matrix degradation can be severe, as in tumor invasion and metastasis. A main difference between a benign and a malignant tumor is the ability of the malignant tumor to invade the surrounding normal tissue and metastasize to distant sites. There is a direct correlation between protease activity and metastatic potential, e.g., high plasminogen activator and type IV collagenase levels are associated with invasive and metastatic potential (6, 9, 12, 13). Evidence for the association between increased u-PA expression and invasion and
metastatic ability of cancer cells is accumulating. For example, epidermal growth factor increases u-PA expression and invasive ability of PC3 human prostatic carcinoma cells (14). We have shown that DU-145 cells express five times more extracellular, secreted u-PA activity than the tested normal prostatic epithelial cells (9). In human pulmonary adenocarcinomas, more cells in metastases and advanced invasive human lung carcinomas were positive for u-PA than in low-grade tumors. Therefore, u-PA was used as a prognostic indicator of tumor growth and metastasis (12). Nude mice inoculated with a human squamous cell carcinoma developed invasive tumors expressing high u-PA, and anticalytic mAbs to u-PA inhibited invasion (15).

In human breast cancer, those tumors expressing increased u-PA showed high risk for early recurrence and poor prognosis (13). In primary breast cancer, metastasis-free survival was best predicted by u-PA levels (16). In bladder cancer patients, tumor recurrence and progression were associated with high u-PA content (17). According to one study, the androgen-responsive, human prostate carcinoma cell line LNCaP does not secrete u-PA, lacks u-PA receptor, and shows low metastatic potential, whereas DU-145 and PC-3 cell lines secrete u-PA, express u-PA receptor, and are highly invasive in an in vitro invasion assay (18). These studies demonstrate a close association between u-PA activity and the ability of tumors to invade and metastasize.

Our results show that u-PA can degrade fibronectin, that its further degradation is accomplished by plasmin, and that the ECM components can serve as substrates for u-PA, thereby establishing an important mechanism of localized proteolysis in prostate cancer cell invasion. Thus, u-PA-secreting cells in early foci of invasive carcinoma and prostatic carcinoma could cause massive degradation of ECM glycoproteins fibronectin and laminin, mediated by u-PA and plasmin. Fibronectin degradation by u-PA, in the absence of plasminogen, was also observed by Gold et al. (19), and cleavage products of fibronectin differed when u-PA or plasmin was used. Our results confirm this finding. Urokinase has cleavage specificity for arginine-valine bonds in plasminogen and in synthetic substrates and could similarly cleave such bonds in fibronectin (20). Activation of plasminogen by u-PA is enhanced by the presence of soluble fibronectin (21) and by a laminin peptide (22) which further enhance the degradative proteolytic cascade. It is interesting to note that fibronectin fragments have been found in high concentration in plasma cryoprecipitates from cancer patients (23). We propose that u-PA plays a key role in the proteolytic cascade in prostate cancer. Some evidence for the involvement of a proteolytic cascade independent of metalloproteinases has been recently presented by Mackay et al. (24). These investigators propose that tumor cells possess a mechanism for the degradation of type IV collagen, which is plasminogen-dependent but metalloproteinase-independent, and suggest that plasmin may be responsible for this degradation and that type IV collagenase may not be absolutely required. Because of the architecture of the BM and ECM, laminin and fibronectin protect collagen from degradation, so they must be first removed before collagen degradation will occur (24).

Proteases which degrade the BM play an important role in the progression of carcinoma in situ to invasive carcinoma. Tumor cells that secrete u-PA are able to recruit the widely distributed proteolytic system involving plasminogen, which is a circulating zymogen present in high concentrations in plasma and extracellular fluids. Since plasmin has a wide substrate specificity and since it can activate procollagenases, including type IV collagenase, it is logical to consider that u-PA would play a crucial role in the initial stages of invasion by prostate cancer cells. Even if the amount of u-PA produced by invasive cells is small, the autocalytic nature of pro-u-PA (25) and the relatively high concentration of plasminogen in ECM would yield high local levels of plasmin, an enzyme with the ability to degrade ECM. The observation that invasion and metastasis (15) can be inhibited by antibodies to u-PA provides further credence to the proposition that u-PA plays a significant role in invasion and metastasis.

Treatment of DU-145 cells with RA resulted in the inhibition of fibronectin and laminin degradation by SF-CM. This effect is reflected in the marked decrease in the invasive ability of RA-treated DU-145 cells (Fig. 7). Other evidence also shows that retinoids can inhibit invasion, decrease collagenase and PA activity, and reduce invasive and metastatic potential of human and rodent cells in vitro and in vivo (3, 6). For example, human melanoma cell lines treated with 10 μM RA secreted lower levels of plasminogen activators and type IV collagenase than untreated cells and showed a concomitant decrease in invasion through Matrigel (6). Retinoids also stimulate ECM production, alter cell adhesion properties, modulate transforming growth factor β production, and inhibit angiogenesis (2, 3, 26). Our results show that RA can decrease the extracellular proteolytic activity, ECM degradation, and invasion. Studies are in progress.
to examine the effects of RA on transforming growth factor β and u-PA inhibitor expression.

As far as the proposition that prostatic cancer cells have an inherent ability and predilection of early invasion is concerned, of special interest is PIN, the precancerous lesions which precede microinvasion and progress to invasive carcinoma (27). PIN shows focal proliferation, cellular disorganization and heterogeneity, and disruption of the basal epithelial cell layer followed by disruption of the BM in advanced lesions of high-grade PIN. Changes in the polarity of secretion by epithelial cells may take place as a result of dysplasia and anaplasia. In normal prostatic glands in vivo, polarized secretion of prostatic proteins including u-PA, takes place at the apical end of glandular epithelium. Cells of a rat prostatic carcinoma cell line grown at low density in medium containing 5% FBS showed morphology resembling squamous metaplasia and anaplasia. This resulted in a change in polarized secretion so that now cells secreted urokinase equally at the apical and the basal ends (28). Such morphological changes may be comparable to dysplasia and anaplasia in late PIN and their progression to invasive carcinoma (27). Thus, in vivo, the disorganization of cellular polarity may result in increased secretion of u-PA at the basal end, resulting in localized proteolysis of the ECM in high-grade PIN. Data presented here provide some clues into the mechanisms involved in the intrinsic ability of prostatic cancer cells to degrade the ECM at the transition from high-grade PIN or carcinoma in situ to invasive carcinoma. Inhibition of early lesions such as PIN, before the onset of invasion, is especially important in the control of invasive carcinoma. Since retinoids are considered to be important in chemoprevention (29), it is logical to consider the idea that agents such as RA, which can reduce extracellular protease activity and enhance ECM synthesis and integrity, may play an important role in blocking progression of PIN to invasive carcinoma.

The net ECM proteolysis depends on the ratio between the levels of extracellular or cell-membrane-bound, active urokinase secreted by tumor cells and the levels of plasminogen activator inhibitors, which are u-PA’s natural inhibitors. The balance between the u-PA and plasminogen activator inhibitor must be in favor of active u-PA in order for proteolysis to occur. It has been shown that some tumor cells also produce high levels of protease inhibitors (30). Thus, it will be necessary to study the balance between proteases and their natural inhibitors. The identification of synthetic serine protease inhibitors has important application in prostate cancer prevention and intervention by inhibition of invasion and tumor progression to a metastatic state.

We propose that urokinase plays a pivotal role in and occupies a place at the apex of the proteolytic cascade and initiates the degradative process in prostate cancer invasion. This process subsequently recruits collagenases, after the procollagenases are activated by plasmin, formed by the activation of plasminogen by u-PA. This proteolytic cascade may include activation of pro-u-PA to active u-PA by autocalysis and by plasmin, activation of plasminogen to plasmin by u-PA, direct degradation of fibronectin by u-PA, degradation of fibronectin and laminin by plasmin, and activation of collagenases by plasmin. Thus, u-PA-dependent fibronectin and laminin degradation may be a prerequisite for subsequent collagen degradation. Montgomery et al. (31) have shown that a rapid removal of fibronectin and laminin precedes dissolution of collagens. As a result, focal dissolution of the ECM could take place in advanced PIN followed by invasion and metastasis. The autocatalytic activity of u-PA (25) in itself may be sufficient to initiate the enzymatic cascade.

We propose that due to the constitutive property of prostatic epithelium to secrete urokinase, preinvasive prostatic lesions, such as high-grade PIN, have an inherent ability and predilection to invade and metastasize. This may be the reason why 48% of the patients with prostatic carcinoma already have disseminated disease at first diagnosis by rectal examination (32). This also provides suggestive evidence for early metastasis of prostatic carcinomas. Since degradation of endothelial ECM is necessary for angiogenesis, we suggest that u-PA secreted by cancer cells will promote angiogenesis in very small tumors and enhance invasion and metastasis. Our results show that RA has the ability to decrease the net extracellular proteolytic activity and, thus, decrease ECM degradation and invasion. The mechanisms involved in these RA effects are under investigation. Inhibitors of extracellular proteolytic activity at the cell-matrix interface may have therapeutic value in cancer prevention and inhibition of invasion and metastasis. Our new cell models of immortalized and malignant prostatic epithelial cell lines derived from adult human prostate (33) should facilitate this work.

ACKNOWLEDGMENTS

We thank Dr. Jean Burnett for her critical evaluation of this article and Diano Bello, Beth Roestel, Daniel Edwards, Salmaan Quader, and W. Scott Metcalfe for their invaluable assistance.

REFERENCES


M M Webber and A Waghray


| Updated version | Access the most recent version of this article at: [http://clincancerres.aacrjournals.org/content/1/7/755](http://clincancerres.aacrjournals.org/content/1/7/755) |

**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.