A Three-Dimensional Tumor Cell Defect in Activating Autologous CTLs Is Associated with Inefficient Antigen Presentation Correlated with Heat Shock Protein-70 Down-Regulation

Virginie Dangles-Marie, Sophie Richon, Mohamed El Behi, Hamid Echchakir, Guillaume Dorothée, Jérôme Thiery, Pierre Validire, Isabelle Vergnon, Jeanne Menez, Moncef Ladjimi, Salem Chouaib, Dominique Bellet, and Fathia Mami-Chouaib


ABSTRACT

We described previously a CTL clone able to lyse the autologous carcinoma cell line IGR-Heu after specific recognition of an HLA-A2-mutated α-actinin-4 peptide complex. Here, we used IGR-Heu, cultured either as standard two-dimensional monolayers or as three-dimensional spheroids, to further analyze the influence of target architecture on CTL reactivity. Interestingly, we found that changes in the tumor structure from two- to three-dimensional induced a dramatic decrease in its capacity to activate autologous CTL, as measured by IFN-γ and tumor necrosis factor-α secretion. These functional alterations were attributable neither to MHC class I expression nor to tumor antigen (Ag) down-regulation, because IGR-Heu, cultured as two- or three-dimensional, expressed similar levels of HLA-A2 and α-actinin-4. More importantly, incubation of three-dimensional cells with synthetic epitope completely restored cytokine release by CTL. This defective Ag presentation correlated with a decrease in heat shock protein (hsp)70 expression by three-dimensional tumors compared with two-dimensional cells. Furthermore, transfection of the tumor cells with hsp70 cDNA completely restored the Ag-presenting potential of spheroids and, therefore, cytokine production by T cells. These data strongly suggest that hsp70 down-regulation in three-dimensional cells may result in tumor resistance to the immune response.

INTRODUCTION

Immunotherapy represents a promising approach to the treatment of malignant human cancers (1, 2). Therapeutic vaccines based on the genetic identification of tumor Ag have been used recently in clinical trials. Many of these trials were based on patient immunization with recombinant viruses that encode cancer Ag. Nevertheless, T-cell responses are only sporadically generated by these immunization approaches. In contrast, peptide vaccines using MHC class I-restricted epitopes from tumor Ag can readily lead to the generation of tumor-reactive CD8+ CTL. Unfortunately, clinical responses are limited and apparently do not correlate with the induction of a specific CTL response in peripheral blood (3). One possible parameter for explaining in vitro readily identifiable CTL responses in patients with growing tumors may lie in the cancer cell architecture. Indeed, solid cancers display three-dimensional geometry, whereas CTL efficacy is usually tested in standard in vitro assays using conventional two-dimensional monolayer tumor cells. It is now commonly admitted that the in vitro model of multicellular tumor spheroids more accurately reflects the tumor microenvironment than the two-dimensional monolayer model (4, 5). Indeed, spheroids mimic in vivo solid tumors in many characteristics, including morphology, compacted organization, growth dynamics, and the capacity to develop a necrotic core. Furthermore, from a therapeutic point of view, cell interactions in tumor spheroids display radiation multidrug resistance similar to that observed in a clinical setting. Therefore, this model is primarily used to study tumor resistance to radio and chemotherapy (6). Thus far, new insights into gene expression (7, 8) and intercellular adhesion have been offered by three-dimensional tumor studies (9). Furthermore, the dramatic influence of cell organization on tumor resistance to apoptosis was recently reported (10–12). Indeed, Weaver et al. (11) demonstrated that polarized three-dimensional architecture confers tumor cell resistance to apoptosis-inducing agents, including chemotherapy drugs and ligation of cell death receptors. Because tumor spheroids mimic avascular tumor stages and micrometastases, they have greatly contributed to the understanding of the role of the cellular microenvironment in tumor biology. However, the influence of tumor architecture on the antitumor immune response and Ag presentation remains poorly investigated.

We demonstrated previously that changes in the architecture of a bladder tumor cell line, from two- to three-dimensional, dramatically impaired its capacity to induce cytokine release by an autologous T-cell line (13). In the present study, we further study the influence of tumor architecture on specific TIL reactivity, and we attempt to determine the mechanisms underlying the defective T-cell activation elicited by the three-dimensional target. For this purpose, we used a lung carcinoma cell line cultured as either standard monolayers (two-dimensional) or spheroids (three-dimensional) and an HLA-A2-restricted CD8+ CTL clone able to lyse autologous cancer cells after recognition of a tumor-specific antigenic peptide (14). Our data point to an alteration in Ag presentation associated with hsp70 down-regulation in the defective capacity of three-dimensional tumor cells to activate autologous CTL.

MATERIALS AND METHODS

Tumor Cell Line and CTL Clone. The IGR-Heu tumor cell line was established from a patient suffering from a large cell carcinoma of the lung (15). This cell line was stably transfected with the pcDNA3 expression vector encoding the hsp70 protein. Multicellular spheroids were prepared by the liquid overlay technique (5). In brief, 96-well tissue culture plates were coated...
with 75 µl of 1% agarose in milliQ aqua. Tumor cells were resuspended with trypsin, and 10^4 cells/microwell were seeded in 150 µl of culture medium to obtain, after 5 days, microwells containing a single spheroid. The number of seeded cells and culture time conditions was critically determined to avoid the formation of a necrotic core.

CTL clone Heu127 was isolated from autologous TIL and cultured as described previously (14, 16).

**Immunohistochemical Analysis of T-cell Infiltrates.** Immunohistochemical staining with UCHT-1 mAb (anti-CD3; Ventana, Strasbourg, France) was performed in paraffin-embedded sections of IGR-Heu spheroids incubated with T cells, as described previously (13).

**Cytokine Release.** Tumor cells (1 × 10^5) displayed either as monolayer or as spheroids were cocultured with T cells in 150 µl of culture medium, supplemented with 10% heat-inactivated human AB serum. In peptide pulsing or blocking experiments, target cells were incubated for 30 min with synthetic peptide or anti-HLA-class I mAb W6/32 before adding effector cells. Each culture condition was carried out in triplicate, and culture supernatants were collected for cytokine measurements. TNFα was detected by measuring the cytotoxicity of the culture medium on the TNF-sensitive WEHI-164-clone with a 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide colorimetric assay (17). IFNγ production was measured with the human IFNγ Cytoset kit (Biosource, Clinisciences, Montrouge, France).

**Flow Cytometric Analysis.** The surface expression of MHC class I, HLA-A2, and CD58 molecules on IGR-Heu and H24 cells in both spatial conformations was assessed by flow cytometry on a FACScalibur (Becton Dickinson, San Jose, CA), using W6/32, MA2.1, and anti-LFA-3 mAb, respectively (13). Data were processed using Cell Quest software (Becton Dickinson). Spheroid disaggregation treatment did not alter the expression of cell surface molecules (18).

**Western Blot Analysis.** Total cellular extracts were prepared from two- and three-dimensional tumor cells (10^6) after cell lysis in SDS sample buffer consisting of 4 M deionized urea, 2% (w/v) SDS, 62.5 mm Tris-HCl (pH 6.8 at 21°C), and 1 mm EDTA. Protein concentration was determined using the detergent-compatible protein assay (Bio-Rad, Marnes la Coquette, France), and 10 µg of protein/lane were separated on a 12% SDS polyacrylamide gel. Proteins were blotted onto a nitrocellulose membrane (Millipore, Saint Quentin en Yvelines, France) and subsequently hybridized with anti-actin-specific Ab (C-11; Santa Cruz Biotechnology, Tebu, Le Perray, France) or hsp70 (C92F3A-5; Stressgen, San Diego, CA). Western blots were developed by secondary Ab and enhanced by chemiluminescence (enhanced chemiluminescence; Amersham, Courtaboeuf, France). The densitometry analysis of the signal was performed with Quantity One software (Bio-Rad).

**RT-PCR Assay.** Total tumor RNA was isolated from two- and three-dimensional cells by the acid-phenol guanidium method, using Rnaple (Eurobio, Les Ulis, France). cDNA was prepared from 1 µg of total RNA by a standard method using reverse transcriptase and an oligo-dT primer. A constant amount of cDNA corresponding to the reverse transcription of 50 ng of total RNA was used as the template for PCR amplification using the ACTN4 primers ACTN4-F (5' - ATGGGCGACTACATGCACG - 3') and ACTN4-R (5' - CGTGGGCGGCAGGTTTCA - 3'). The mixture was subjected to 35 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C.

**RESULTS**

Defective CTL Clone Activation by Three-dimensional Tumor Cells. To study the capacity of three-dimensional tumor cells to stimulate autologous T lymphocytes, we used a lung carcinoma cell line, IGR-Heu, and a specific CTL clone, Heu127, which recognized, at the tumor cell surface, an HLA-A2/mutated α-actinin-4 peptide complex (14). Initial experiments were carried out to examine T-cell infiltration of IGR-Heu spheroids incubated with the Heu127 CTL clone. Immunohistochemical analysis, performed with the use of an anti-CD3 mAb, indicated that T cells homogeneously infiltrated the three-dimensional tumor and showed an absence of any cell damage (Fig. 1A). Furthermore, this analysis showed that the tumor cells were viable and uniform and presented a spherical symmetry with no necrotic core.

![Fig. 1. Three-dimensional spheroids less efficiently activate the CTL clone than two-dimensional monolayers, despite homogenous infiltration. Paraffin-embedded sections of IGR-Heu spheroids containing 10,000 cells incubated for 24 h with 5,000 Heu127 cells are immunohistochemically stained with anti-CD3 mAb (A). TNFα and IFNγ releases are measured in culture supernatants (B-E); 10,000 IGR-Heu cells displaying either two-dimensional (●) or three-dimensional (○) architecture are incubated with CTL at indicated E:T ratios. Culture supernatants are collected at 24 h in B–D and at 24, 48, and 72 h in E. Graphed data represent mean ± SD from triplicate wells. One representative experiment out of three is shown. *, P < 0.05 when two-dimensional target was compared with three-dimensional target for a given E:T ratio (t test).](cancerres.aacrjournals.org)
We then investigated the influence of target architecture on the capacity of tumor cells to activate specific TIL. For this purpose, we measured TNFα and IFNγ production by the Heu127 clone after incubation of effector cells with target cells displaying either two- or three-dimensional conformations. For comparative concerns, all of the cocultures were conducted with an identical number of stimulator cells (10,000 cells) and at least two E:T ratios. As depicted in Fig. 1, B and C, high amounts of IFNγ and TNFα were detected in the supernatants of TIL cocultured with two-dimensional cells. In contrast, a dramatic decrease in cytokine secretion was observed when Heu127 cells were stimulated with three-dimensional targets (Fig. 1, B and C). Similar results were obtained with two additional CTL clones, Heu171 and Heu161 (16, 19), displaying, respectively, the same or a distinct Ag specificity (data not shown). This cytokine secretion decrease was not attributable to protein sequestration in the spheroids, because sonication of the cell complex did not result in enhancement of the IFNγ and TNFα levels (data not shown). Furthermore, kinetic experiments indicated that the cytokine release defect was not correlated with a delayed T-cell activation by spheroids. Indeed, the IFNγ produced in 24-, 48-, and 72-h coculture supernatants was always lower when T cells were incubated with three-dimensional rather than with two-dimensional targets (Fig. 1D). In addition, higher E:T ratios did not restore cytokine secretion by CTL after their stimulation with spheroids (Fig. 1E). It was noteworthy that IFNγ produced by 20,000 effector cells stimulated by the three-dimensional tumor never reached that secreted by 2,000 T cells activated by two-dimensional tumor cells (Fig. 1E).

Alteration of Antigenic Peptide Presentation by the Three-dimensional Tumor. To determine the mechanism involved in differential CTL activation by three- versus two-dimensional targets, we first studied the expression level of a variety of molecules involved in cell-to-cell interactions at the surface of tumor cells cultured in both architectures. Flow cytometry analyses showed that the expression level of MHC class I molecules on the surface of the tumor cells was not affected by their spatial conformation. Indeed, cells cultured in both architectures expressed similar levels of HLA-A2 antigenic peptide presenting elements (Fig. 2A). Similar results were obtained with the CD58 (LFA-3) adhesion molecule. In contrast, CD54 (intercellular adhesion molecule-1) adhesion and B7.1 (CD80) and B7.2 (CD86) costimulatory molecules were not expressed by tumor cells displaying either architecture (data not shown and Ref. 20).

Additional experiments were then performed to investigate whether the defective effector cell activation was caused by a down-regulation in the native Ag expression by the three-dimensional target. RT-PCR analysis indicated that two- and three-dimensional cells expressed similar levels of ACTN4 mRNA transcripts (Fig. 3A). In addition, a comparable α-actinin-4 protein level was detected in both target architectures as shown by Western blot analysis carried out using a specific polyclonal Ab (data not shown). We then analyzed whether IGR-Heu tumor spheroids efficiently process and/or transport the antigenic peptide at their surface. For this purpose, we incubated two- and three-dimensional targets with the specific α-actinin-4 peptide (FLASNGVKLV) before addition of the autologous CTL. Three peptide concentrations were tested, and cytokine production by Heu127 CTL was measured (Fig. 3, B and C). Interestingly, incubation of three-dimensional cells with the exogenous mutated α-actinin-4 peptide (FLASNGVKLV) completely restored TNFα and IFNγ secretion in a peptide dose-dependent manner. These results clearly indicate a defect in antigenic peptide presentation by the three-dimensional target. Note that the cytokine level secreted by the three-dimensional target incubated with <10 nm synthetic peptide reached that secreted by the two-dimensional target incubated with the same peptide concentration.

Down-Regulation of hsp70 Expression in the Three-dimensional Tumor Correlated with the Ag Presentation Defect. It has been reported previously that hsp70 plays a crucial role in Ag presentation by MHC class I molecules by chaperoning antigenic peptides generated within the cells (21). Therefore, we analyzed hsp70 expression in both two- and three-dimensional architectures by Western blot.
analysis using a specific mAb. Unexpectedly, IGR-Heu three-dimensional cells expressed at least two times less hsp70 protein than two-dimensional cells (Fig. 4). This result suggests that the decrease in hsp70 expression in spheroid cells may be implicated in their defective Ag presentation to autologous CTL. It should be noted that hsp70 down-regulation in the IGR-Heu three-dimensional tumor was not an isolated observation and was also detected in distinct lung cancer cell lines (data not shown). This down-regulation may result from a decrease in the tumor cell growth rate in three-dimensional tumors as compared with two-dimensional. Indeed, cells cultured in spheroids exhibit a much slower growth rate (10 times less) than tumor cells cultured in monolayers. The observed slower cell cycling did not result from oxygen deficiency, because no hypoxic fraction was detected in the spheroids as measured by flow cytometry using 2-nitroimidazole (data not shown).

To further assess whether hsp70 down-regulation is directly implicated in the alteration of peptide presentation by three-dimensional targets, we stably transfected IGR-Heu tumor cells with the pcDNA3-hsp70 expression vector. One clone, H24, was selected for neomycin resistance and hsp70 overexpression. Indeed, this clone expressed a 2-fold higher level of hsp70 than the parental cell line, as demonstrated by Western blot analysis (Fig. 4). The H24 clone was then cultured as either a two- or three-dimensional architecture and tested for its capacity to activate Heu127 autologous CTL. Interestingly, reestablishment of hsp70 expression in H24 three-dimensional cells completely restored their capacity to induce IFNγ secretion by autologous CTL, which reached that observed by the two-dimensional target (Fig. 5A). In contrast, pcDNA3 empty vector-transfected cells (HN-pC3), used as controls, had no effect on cytokine production by effector cells (Fig. 5A). This cytokine release up-regulation was not correlated with an increase in cell growth and viability, nor with an enhancement of HLA-A2 expression by H24 stimulator cells. Indeed, hsp70-transfected and nontransfected spheroids exhibited a similar growth rate and showed similar cell viability, as measured by propidium iodide staining (data not shown).

In addition, immunofluorescence analysis indicated that H24 and IGR-Heu cells cultured either as two- or three-dimensional expressed similar levels of MHC class I molecules (Fig. 2B). This reestablishment of T-cell activation was directly correlated with Ag presentation by HLA-class I molecules,
because cytokine production by Heu127 effectors was completely inhibited by anti-MHC class I mAb (Fig. 5B). These data further argue for a role of hsp70 in peptide chaperoning. It is noteworthy that H24 hsp70-transfected cells, used as two-dimensional conventional architecture, were able to stimulate the specific CTL clone as efficiently as untransfected IGR-Heu two-dimensional cells (Fig. 5A). This result underlines the moderate hsp70 overexpression in H24 and suggests that the hsp70 expression level observed in parental IGR-Heu tumor cells corresponds to the minimal amount sufficient for efficient Ag presentation to autologous CTL.

**DISCUSSION**

In the present study, we further investigated the impact of tumor architecture on specific T lymphocyte reactivity in a human lung carcinoma model, which included a tumor cell line, cultured either as a two- or three-dimensional structure, and an autologous CTL clone recognizing a mutated α-actinin-4 tumor epitope presented in an HLA-A2.1 context (14). Our results provide evidence that three-dimensional tumor cells stimulate the specific CTL clone less efficiently than two-dimensional cells. Indeed, tumor cells cultured as spheroids displayed a tremendous decrease in their capacity to induce cytokine secretion by the specific CTL. T-cell hyporeactivity was not correlated with impaired antigenic peptide presentation. Indeed, spheroids incubated with the exogenous mutated α-actinin-4 peptide completely restored their capacity to stimulate the autologous CTL clone in a peptide dose-dependent manner. These results further confirm that tumor cells are viable. Alteration in tumor Ag presentation was not caused by native Ag down-regulation, because viable multicellular spheroids were homogeneously infiltrated by TIL, and surface molecules, including HLA-A2 and LFA-3, were similarly expressed on three- and two-dimensional cells. This influence of tumor cell architecture on T-cell reactivity was not an isolated phenomenon and would be extended to distinct human tumor models (13).

Interestingly, the present data indicate for the first time that the defect in the capacity of three-dimensional tumor cells to activate the specific CTL was correlated with impaired antigenic peptide presentation. Indeed, spheroids incubated with the exogenous mutated α-actinin-4 peptide completely restored their capacity to stimulate the autologous CTL clone in a peptide dose-dependent manner. These results further confirm that tumor cells are viable. Alteration in tumor Ag presentation was not caused by native Ag down-regulation, because three-dimensional tumor cells expressed similar levels of α-actinin-4 protein as two-dimensional cells. It is now established that efficient presentation of antigenic peptides to CD8+ T cells is dependent on the generation of antigenic peptides by the proteasome and their transport into the endoplasmic reticulum, where they are assembled into an HLA class I/β2m dimer. Our initial experiments indicated that two- and three-dimensional cells expressed similar amounts of proteasome components, including the 20S proteasome subunit α2 (HC3), excluding its implication in the observed Ag presentation defect (data not shown).

A key role of hsp70 in Ag presentation by MHC class I molecules has been reported recently (22, 23). Trafficking of processed peptides in the cytosol for MHC class I presentation does not occur by passive diffusion but requires hsp70 chaperoning. Analysis of hsp70 in two- and three-dimensional structures indicated a significant decrease in expression of this chaperone protein in three-dimensional tumor cells, strongly suggesting its implication in defective spheroid cell recognition by the specific CTL clone. Hsp70 down-regulation does not correlate with hypoxia, because no hypoxic fraction was detected in IGR spheroids. Hypoxia is usually detected in spheroids >500 μm in diameter (24–26). The decrease in hsp70 expression may result from a slower cell growth rate in tumor spheroids than in monolayers. Indeed, tumor cells cultured in three-dimensional architecture proliferate much more slowly than cells cultured in two-dimensional. Similar differential changes in cell cycling were reported to occur during culture of tumor cells as monolayers versus small spheroids (27). Furthermore, the growth rate in multicellular spheroid cultures has been reported to be influenced by glucose supply (28). It is noteworthy that nonproliferating but viable tumor cells were also identified in vivo (29).

Interestingly, transfection of the tumor cells with hsp70 before spheroid formation completely restored the three-dimensional target capacity to efficiently stimulate autologous CTL. These results suggest that the level of hsp70 in untransfected three-dimensional lung carcinoma was too low to support optimal Ag presentation, and they dramatically support a crucial role of hsp70 in Ag presentation by MHC class I molecules. A higher dose of hsp70 in three-dimensional tumors may be required for presentation of weakly concentrated tumor peptide by low-MHC class I-expressing cancer cells. It has been reported previously that presentation of tumor Ag by MHC class I molecules on the B16 murine melanoma cell line is significantly enhanced by transfection of the hsp70 gene (21, 30). Along the same lines, treatment of cells with deoxyspergualin, a drug that binds hsp70 and hsp96 with apparent specificity, abrogated the ability of cells to present endogenously generated antigenic peptides by MHC class I (23). However, the use of deoxyspergualin is fraught with practical difficulties precluding its use in such studies. Therefore, spheroids eliciting a more physiological down-regulation of hsp70 expression may provide an in vitro model for studying the role of hsp70 in the endogenous pathway of Ag presentation by MHC class I molecules.

Several lines of evidence have opened up new perspectives for the clinical application of hsp in cancer therapy. Indeed, it has been shown that hsp70/peptide complexes purified from human melanomas specifically activate HLA class I-restricted antimelanoma T cells when loaded on HLA-matched, Ag-presenting cells, demonstrating a role of hsp as a chaperone of human immunogenic peptides (31). Furthermore, a positive correlation between hsp70 expression and tumor immunogenicity was reported (32, 33). In this context, it has been reported that tumor cells subjected to nonlethal heat shock stress were unable to form tumors in syngenic mice (33). In contrast, tumor variants selected after in vivo growth acquired tumorigenicity and lost the ability to synthesize hsp70 (32). In addition, evidence has been provided, indicating that hsp70-negative esophageal squamous cell carcinoma was correlated with a significantly poor prognosis as compared with hsp70-expressing esophageal tumors (34). Therefore, hsp70 down-regulation by the IGR-Heu three-dimensional tumor does not correspond to a unique feature of this cancer cell and may also occur in vivo. Our data support a mechanism by which hsp70 might enhance tumor immunogenicity by influencing the capacity of tumor cells to process and directly present endogenous Ag to T cells. This optimization of immunogenicity was not correlated with an increase in MHC class I expression by IGR-Heu tumor cells after hsp70 transfection, nor with an increase in tumor cell viability (data not shown). Concordant results indicated enhanced susceptibility to CTL without an increase in MHC class I expression or Ag processing after conditioned overexpression of hsp70 in a human melanoma cell line (35). In contrast, it has been shown that poorly immunogenic melanoma cells, which normally express very low levels of MHC class I, exhibit increased levels of MHC class I Ag on their surface when stably transfected with hsp70 (30). These surface molecules are complexed with peptides, and, unlike the parental melanoma cells, the hsp-expressing tumor cells can process and present Ag directly to specific CTL in vitro (30).

It is clearly established that tumor cells use several mechanisms to adapt their survival by blocking a variety of key regulators of the immune response and circumventing antitumor defense (36). In this regard, spheroids clearly mimic initial avascular stages of solid tumors in vivo, such as tumor aggregates of disseminated tumors in ascites.

3686
fluid or systemic circulation (37). Thereby, the fact that metastasizing tumor cells form tumor emboli by adhering to other tumor cells could be compared with the modification in tumor architecture from two- to three-dimensional and may protect the cells from immune recognition and destruction. Our data suggest that a defect in Ag presentation, consequent to hsp70 down-regulation, may allow three-dimensional tumors to escape from the immune response. The present spheroid model may be extrapolated to in vivo conditions and emphasizes the capacity of hsp70 to enhance tumor immunogenicity. Furthermore, this model may accurately reflect the tumor microenvironment and partially shed light on the paradox observed in vaccination trials of cancer patients with tumor-specific peptides, where the absence of significant tumor regression and measurable clinical benefits may occur despite a strong CTL immune response detected in vitro (3). Therefore, the use of spheroids instead of two-dimensional monolayer cells as targets in in vitro cytotoxicity activity experiments could be useful for the monitoring of vaccination trials.

ACKNOWLEDGMENTS

We thank J. P. Levillain, C. Bovin, and J. L. Janneau for technical assistance and Y. Lecluse and C. Blanc for fluorescence-activated cell sorter analysis. We also thank Dr. D. Gruenwald from the thoracic department of the Institut Mutualiste Montsouris, Paris.

REFERENCES

A Three-Dimensional Tumor Cell Defect in Activating Autologous CTLs Is Associated with Inefficient Antigen Presentation Correlated with Heat Shock Protein-70 Down-Regulation

Virginie Dangles-Marie, Sophie Richon, Mohamed El Behi, et al.


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/63/13/3682

Cited articles This article cites 37 articles, 9 of which you can access for free at: http://cancerres.aacrjournals.org/content/63/13/3682.full.html#ref-list-1

Citing articles This article has been cited by 5 HighWire-hosted articles. Access the articles at: /content/63/13/3682.full.html#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.