Induction of Cancer Metastasis by Cyclophosphamide Pretreatment of Host Mice: An Opposite Effect of Chemotherapy

Kensuke Yamauchi,[1,2,3] Meng Yang,[1] Katsuhiro Hayashi,[1,2,3] Ping Jiang,[1] Norio Yamamoto,[3] Hiroyuki Tsuchiya,[3] Katsuro Tomita,[3] Abdool R. Moossa,[2] Michael Bouvet,[2] and Robert M. Hoffman[1,2]

[1]AntiCancer, Inc.; [2]Department of Surgery, University of California, San Diego, California; and [3]Department of Orthopedic Surgery, School of Medicine, Kanazawa University, Ishikawa, Japan

Abstract

Although side effects of cancer chemotherapy are well known, "opposite effects" of chemotherapy that enhance the malignancy of the treated cancer are not well understood. In this report, we describe the induction of intravascular proliferation, extravasation, and colony formation by cancer cells, critical steps of metastasis, by pretreatment of host mice with the commonly used chemotherapy drug cyclophosphamide. In contrast, in the unpretreated mice, most cancer cells remained quiescent in vessels without extravasation. HT1080 human fibrosarcoma cells, labeled in the nucleus with green fluorescent protein and red fluorescent protein in the cytoplasm for imaging, were injected into the epigastric cranialis vein of nude mice. Twenty-four hours before cancer cell injection, cyclophosphamide was given i.p. Double-labeled cancer cells were imaged at the cellular level in live mice with the Olympus OV100 Small Animal Imaging System with variable magnification. Cyclophosphamide seems to interfere with a host process that inhibits intravascular proliferation, extravasation, and extravascular colony formation. Cyclophosphamide does not directly affect the cancer cells because cyclophosphamide has been cleared by the time the cancer cells were injected. This report shows an important unexpected "opposite effect" of chemotherapy that enhances critical steps in malignancy rather than inhibiting them, suggesting that certain current approaches to cancer chemotherapy should be modified. [Cancer Res 2008;68(2):516–20]

Introduction

Although side effects of cancer treatment are well known, "opposite effects," which enhance the malignancy of the treated cancer, are not well understood. In this regard, there have been several reports that chemotherapy or irradiation can enhance metastases. For example, tumor cell localization and metastatic growth in the lung were enhanced after the administration of Bleomycin, which caused endothelial cell injury (1). Monoclonal antibodies to pulmonary endothelial cells enhanced lung tumor colony formation (2). The induction of endothelial damage and exposure of the subendothelial basal lamina may enhance lung colony formation. Kerbel et al. (3, 4) have shown that toxic chemotherapy increases tumor angiogenesis by mobilizing endothelial cell precursors in the bone marrow to migrate to the tumor. Tumor necrosis factor has also been reported to enhance experimental metastases *in vivo* (5). In radiation studies, if the lungs of mice are irradiated before cancer cells are injected into the tail vein of the mice, the number of experimental pulmonary metastases increased (6). Other studies have shown that prophylactic local irradiation to the lung of tumor-bearing mice increased the incidence of spontaneous pulmonary metastases (7).

Van Putten et al. (8) reported that cyclophosphamide enhanced formation of pulmonary metastases up to 1,000-fold in a mouse model. In their study, cyclophosphamide was given 24 h before tumor-cell injection. Because the half-life of cyclophosphamide is only 17 min, the drug did not affect viability of tumor cells injected 24 h later (9). Carnel et al. (10) suggested that cyclophosphamide induced changes within the pulmonary microvasculature where the injected tumor cells must lodge to form metastases. Cyclophosphamide apparently killed macrophages or prevented the macrophages from killing the endothelium-trapped cancer cells. Iwamoto et al. (11) showed cyclophosphamide-induced endothelial damage using monolayer cell cultures *in vitro*.

We have previously observed that when host mice were pretreated with cyclophosphamide, HCT-116 human colon cancer cells survived and formed colonies in the liver after portal vein injection. In unpretreated mice, these cells rapidly died after portal vein injection. These results suggested that a cyclophosphamide-sensitive host cellular system attacked the HCT-116 cells (12).

We previously showed major differences in the ability of different cancer cells to extravasate and form metastases (13). The Lewis lung carcinoma (LLC) and mouse mammary tumor (MMT), both mouse cancer cell lines, could extravasate from vessels in nude mice and subsequently form colonies. In contrast, in untreated mice, most HT1080 human fibrosarcoma cells still remained in vessels 24 h after injection. The cells were mostly quiescent and rarely extravasated.

We report here that the ability of fluorescent protein–labeled HT1080 cells to proliferate intravascularly, extravasate, and form extravascular colonies was greatly enhanced by the pretreatment of the host nude mouse with cyclophosphamide, a powerful opposite effect of this cancer drug.

Materials and Methods

Establishment of fluorescent protein–labeled HT1080 human fibrosarcoma cells. To establish dual-color HT1080 human fibrosarcoma (HT1080) cells, the cells were transfected with retroviral red fluorescent protein (RFP) and histone H2B-green fluorescent protein (GFP) as previously described (14, 15). In brief, the Hind III/Not I fragment from pDsRed2 (Clontech Laboratories, Inc.) was inserted into the Hind III/Not I site of pLNCX2, which also contains the neomycin-resistance gene (*neo*; Clontech Laboratories, Inc.). For vector production, PT67, an NIH3T3-derived packaging cell line (Clontech Laboratories, Inc.), was incubated with a

Requests for reprints: Robert M. Hoffman, AntiCancer, Inc., San Diego, CA 92111. Phone: 858-654-2555; Fax: 858-268-4175; E-mail: all@anticancer.com.

©2008 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-07-3063

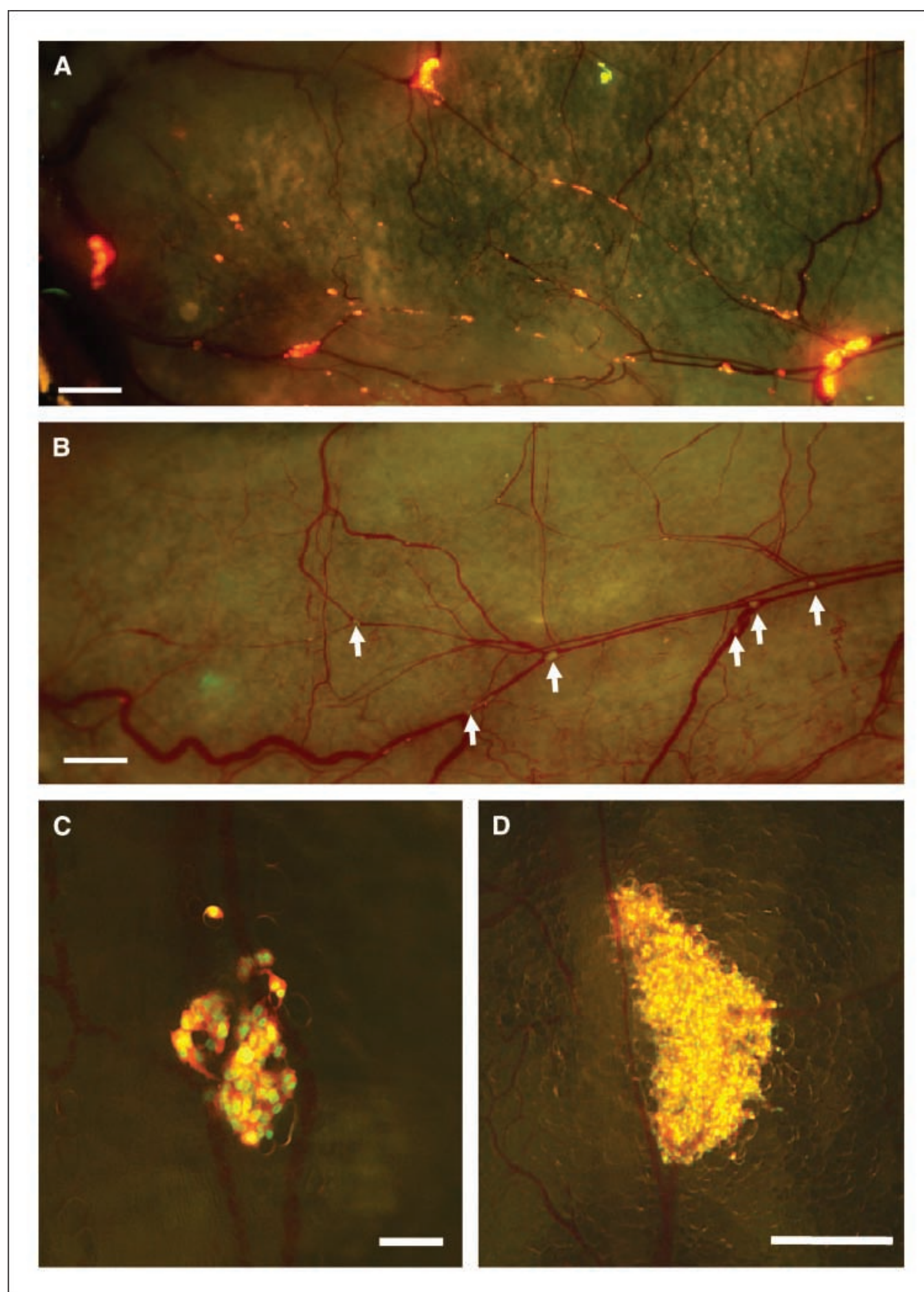

Figure 1. Comparison of intravascular and extravascular tumor colonies in cyclophosphamide pretreated and untreated mice. *A,* 48 h after dual-color HT1080 cells were injected in cyclophosphamide-pretreated mice, many fluorescent intravascular and extravascular colonies are visualized. *B,* in untreated mice, there are only several small colony-like tumor thrombi in the vessels (*arrows*). *Bar,* 5 mm. *C,* in cyclophosphamide-pretreated mice, HT1080 cells have formed a small metastatic colony after extravasation. All cells have exited the vessels. Although some cells have extended their cytoplasm, most cells are round or oval. *Bar,* 100 μm. *D,* in cyclophosphamide-pretreated mice, numerous extravasated HT1080 cells formed a colony around blood vessels. *Bar,* 500 μm.

precipitated mixture of Lipofectamine reagent (Life Technologies, Inc.) and saturating amounts of pLNCX2-DsRed2 plasmid for 18 h. For selection of a clone producing high amounts of an RFP retroviral vector (PT67-DsRed2), the transfected PT67 cells were cultured in the presence of 200 to 1,000 μg/mL G418 (Life Technologies, Inc.), increased stepwise, for 7 days (16).

The histone H2B-GFP fusion gene was inserted at the *Hind* III/*Cal* I site of the pLHCX (Clontech Laboratories, Inc.). To establish a packaging cell clone producing high amounts of a histone H2B-GFP retroviral vector, the pLHCX histone H2B-GFP vector containing the hygromycin-resistance gene (*hyg*) plasmid was transfected in PT67 cells, using the same methods described above for constructing PT67-DsRed2. The transfected cells were cultured in the presence of 200 to 400 μg/mL hygromycin and increased stepwise, such that they produced high amounts of the H2B-GFP vector.

For RFP and H2B-GFP gene transduction, clones of HT1080 expressing RFP in the cytoplasm were initially established. The cells were incubated with retroviral supernatants of PT67-RFP cells for 72 h. Cells were cultured in selective medium that contained 200 μg/mL G418. The level of G418 was increased stepwise up to 800 μg/mL. The cells were then incubated with retroviral supernatants of PT67 H2B-GFP cells. To select for double transformants, cells were incubated in selective medium with hygromycin for 72 h. The level of hygromycin was increased stepwise up to 400 μg/mL (16). Bright dual-color cells were thereby obtained.

Drugs and animals. Cyclophosphamide was obtained from Sigma-Aldrich. Five 4-week-old female athymic CD-1 nude mice were used in this study. Mice were kept in a barrier facility under HEPA filtration. Mice were fed with autoclaved laboratory rodent diet (Tecklad LM-485; Western Research Products). All animal studies were conducted in accordance with the principals and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals under assurance number A3873-1.

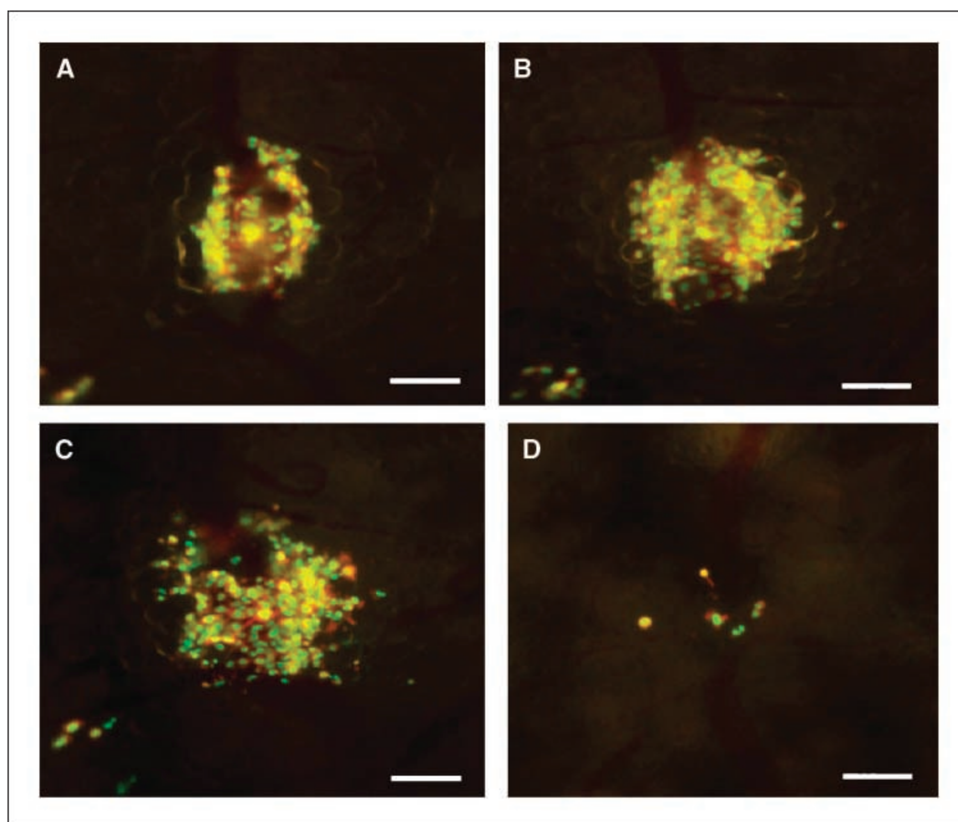

Figure 2. Time course imaging of HT1080 colony formation and regression after extravasation in cyclophosphamide-pretreated mice. The tumor colonies that formed outside of the vessel after extravasation increased in size by ~1 wk after cyclophosphamide administration. After 1 additional wk, the colonies decreased in size. *A,* 4 d after HT1080 cell injection, hundreds of cells formed a metastatic colony around vessels after extravasation. Most cells are surrounding the vessel. *B,* by day 6, the cells proliferated and invaded around the vessel so that the colony increased in size. *C,* a large fraction of the cells disappeared and many cells become round at 7 d. The colony seems to be scattered. *D,* at day 9, there are only seven cells left, which are round. *Bar,* 200 μm for *A–D*.

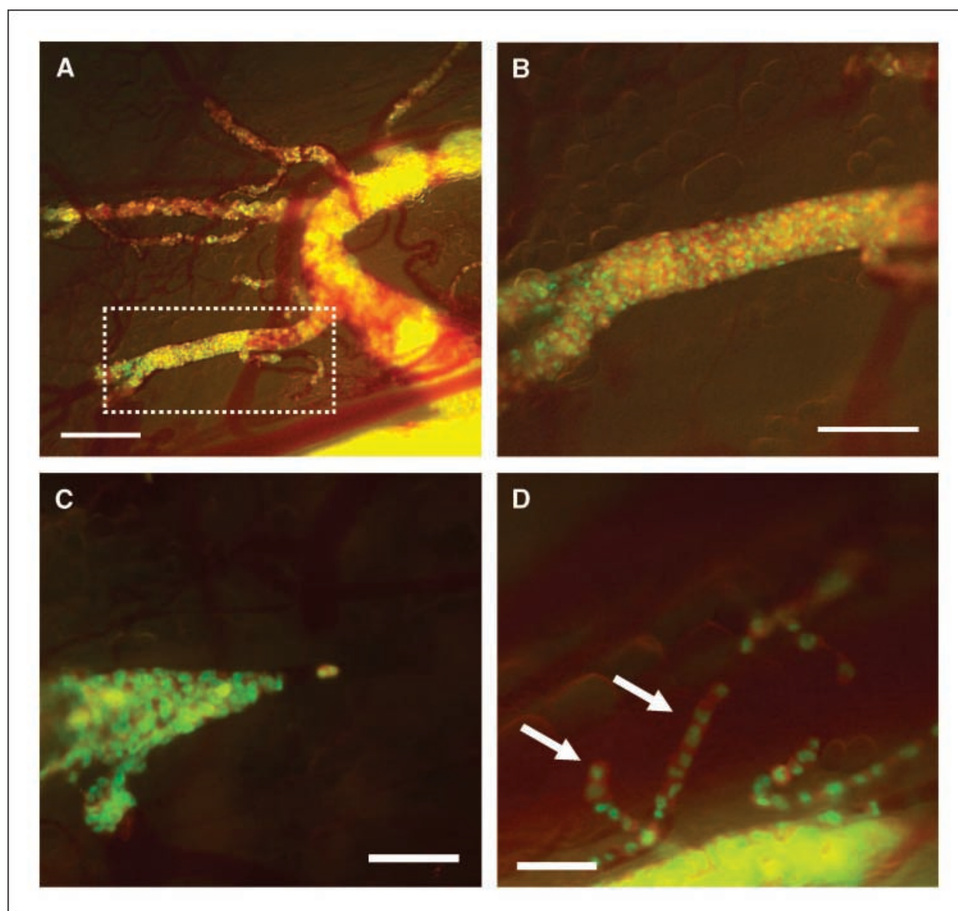

Figure 3. Time course imaging of intravascular proliferation and intravascular spread of HT1080 cells in cyclophosphamide-pretreated mice. *A,* 14 d after cell injection, HT1080 cells invaded and proliferated in the vessels without extravasation. The growing colonies enlarged the vessel diameter. *Bar,* 2 mm. *B,* magnified image of part of *A* enclosed in the square. The vessel is filled with HT1080 cells with slight expansion. *Bar,* 500 μm. *C,* at day 7 after HT1080 cell injection, many cells have filled the vessel. The vessel is narrow, and the mass of HT1080 cells seems to block blood flow. *Bar,* 200 μm. *D,* the HT1080 cells migrated within a narrow vessel. Many cells lined up single file and migrated in capillaries (*arrows*). *Bar,* 100 μm.

Mouse model for *in vivo* imaging of dual-color HT1080 cells. Nude mice were anesthetized with a ketamine mixture (10 μL ketamine HCl, 7.6 μL xylazine, 2.4 μL acepromazine maleate, and 10 μL H_2O s.c.). Cyclophosphamide (200 mg/kg) was injected into the i.p. cavity of the mice 24 h before cell injection. For cell injection, an arc-shaped incision was made in the abdominal skin, and s.c. connective tissue was separated to free the skin without injuring the epigastric cranialis artery and vein. The skin flap was spread and fixed on a flat stand. Dual-color fluorescent HT1080 cells (1×10^6) were injected in the epigastric cranialis vein. The skin flap was closed immediately after injection. Cancer cells, which were inside of the skin flap, were directly imaged every 24 hr. The skin flap was completely reversed after each repeated observation.

Fluorescence optical imaging and data analysis. The OV100 Small Animal Imaging System (Olympus Corp.) containing an MT-20 light source (Olympus Biosystems) and DP70 CCD camera (Olympus Corp.) was used for real-time cellular imaging in live mice. High-resolution images were captured directly on a PC (Fujitsu Siemens computers). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 software.

Results and Discussion

Intravascular proliferation, extravasation, and micrometastasis in cyclophosphamide-pretreated mice. In contrast to other cancer cell types such as the LCC or MMT cells (MMT060562), HT1080 human fibrosarcoma cells only rarely extravasated in nude mice (13). Even at 24 h after injection, the majority of the HT1080 cells remained in vessels without extravasation, which were expanding apparently to maintain blood flow. However, in mice pretreated with cyclophosphamide, HT1080 cells could proliferate extensively intravascularly, extravasate, and subsequently form colonies (Fig. 1*A*). The colonies increased in size and were visualized even at low magnification. In contrast, extravasated colonies were not formed in nonpretreated mice (Fig. 1*B*).

Types of metastatic colonies formed in cyclophosphamide-treated mice. There were two types of colonies formed by HT1080 cells in the cyclophosphamide-pretreated mice. One type of colony formed outside of the vessel after HT1080-GFP-RFP cells extravasated (Fig. 1*C* and *D*). The other type of colony formed within the vessels without extravasation. The type of colony formed depended upon the width of the vessels. In narrow vessels, HT1080 cells had a higher extravasation frequency than in wide vessels where HT1080 usually formed colonies within the vessels without extravasation.

Eventual regression of extravasated colonies in cyclophosphamide-treated mice. Within hours after cell injection in the cyclophosphamide-pretreated mice, HT1080 cells extravasated and subsequently grew around the outer surface of the vessels. However, by 1 week after cyclophosphamide pretreatment of the mice, the colonies decreased in size (Fig. 2*A*–*D*). Although the colonies were decreasing in size, the cells became round and brighter. In contrast to the colonies outside of the vessels, the tumor colonies inside the vessels continued to proliferate and increase in size.

Proliferation and spread of intravascular HT1080 cells to peripheral vessels in cyclophosphamide-pretreated mice. After the HT1080 cells proliferated within the vessels of the cyclophosphamide-pretreated mice, some cells seemed to be pushed out to peripheral vessels by 14 days after cell injection (Fig. 3*A* and *B*).

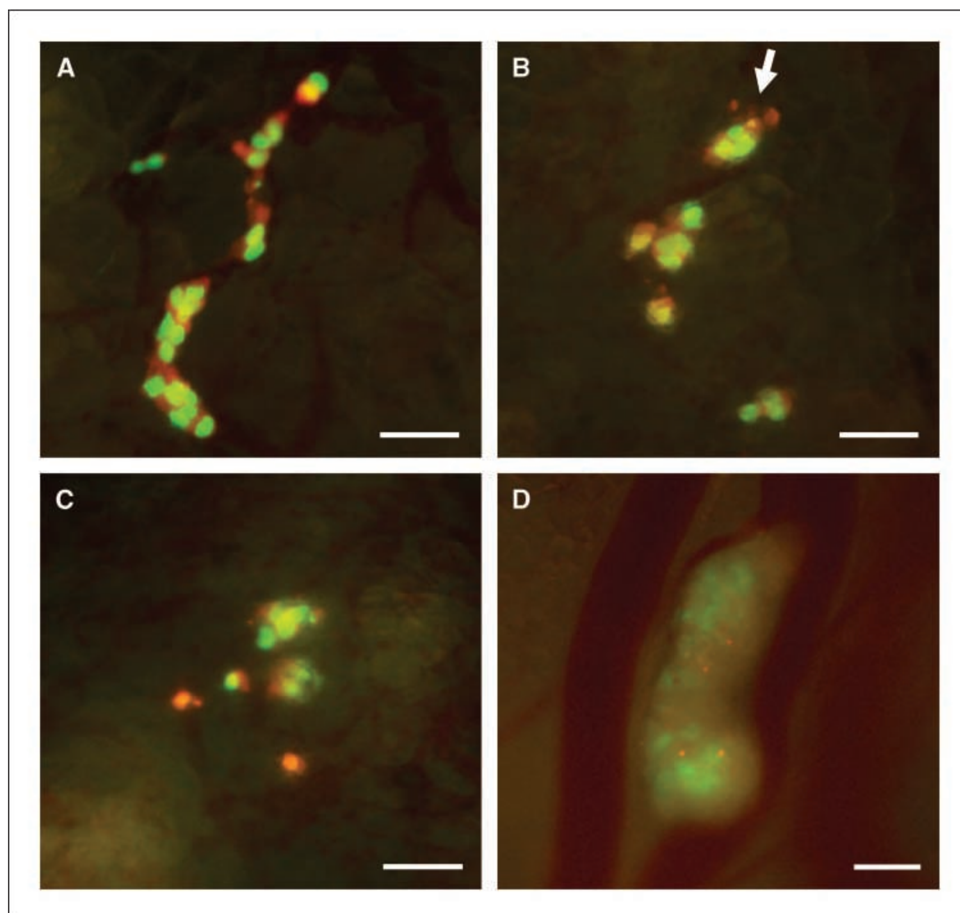

Figure 4. Time course imaging of HT1080 cell death in vessels in nonpretreated mice. *A,* 24 h after cell injection, many HT1080 cells have arrested in the vessel. *Bar,* 200 μm. *B,* at 72 h, more HT1080 cells disappeared and fragmented cytoplasms are visualized (*arrow*). *Bar,* 200 μm. *C,* only some HT1080 cells survived at 96 h. *Bar,* 200 μm. *D,* a large colony is visualized, which contains fragmented dying cells. The green fluorescence is derived from GFP-H2B in the nucleus, and red fluorescence is from RFP-tagged cytoplasm. Note that the vessel expands due to the tumor thrombus. *Bar,* 200 μm.

These peripheral intravascular colonies did not decrease in size 1 week after cyclophosphamide pretreatment, in contrast to the extravascular colonies that eventually regressed. Fig. 3*C* shows time course imaging of i.v. invasion and proliferation of HT1080 cells. Seven days after HT1080-GFP-RFP cell injection, peripheral vessels with many cancer cells were visualized. The mass of HT1080 cells seemed to partially block blood flow and caused the vessel to widen. Thus, the HT1080 cells formed colonies in larger vessels and then spread to peripheral vessels throughout the entire body. When the vessels were too narrow, increasing colony size due to cancer-cell proliferation caused the vessels to widen (Fig. 3*C*). In narrow capillaries, the HT1080 cells migrated single file (Fig. 3*D*). In contrast, the number of HT1080 cells decreased in the vessels of nonpretreated mice (Fig. 4*A–D*). There were only occasionally small colony-like tumor thrombi, which contained fragmented dying tumor cells, which seem to remain in vessels (Fig. 4*D*). The vessels expanded due to these tumor thrombi.

Al-Mehdi et al. (17) also observed apparent intravascular tumor cell proliferation in mouse lungs perfused *ex vivo*. The current results show this phenomenon *in vivo*. The ability of cancer cells to proliferate intravascularly has been a controversial issue (18). The results in this report show definitive intravascular proliferation of tumor cells *in vivo*. This phenomenon has great implications for metastasis.

The results in this study indicate cyclophosphamide, which was given 24 h before cancer cell injection, enhanced intravascular proliferation, extravasation, and subsequent colony formation of the HT1080 human fibrosarcoma. Previous studies had suggested that cyclophosphamide may enhance metastasis due to endothelial cell injury (10, 11). This could be the case in the present report where injured endothelia could allow cells to extravasate more readily, especially cells with an apparent very poor capability to extravasate such as HT1080. However, our previous results showed cyclophosphamide pretreatment of the host mouse greatly decreased cell death of cancer cells in the portal circulation (15). This result, in combination with the enhancement of intravascular proliferation, extravasation, and subsequent colony formation by cyclophosphamide shown in the present study, suggest a host-based cancer cell–killing process is inhibited by cyclophosphamide. The recovery of the host-based cell killing of the extravasated cancer cells a week after cyclophoshamide pretreatment could explain the regression of the extravasated colonies of HT1080 over time. Thus, both cyclophosphamide-induced endothelial injury as well as cyclophosphamide inhibition of an innate cell-based antitumor system could play a role in the "opposite" effect of cyclophosphamide observed in this study.

Our results suggest such opposite effects of cyclophosphamide that can enhance critical steps in metastasis must be taken into consideration for administration of cytotoxic chemotherapy in the clinic.

Acknowledgments

Received 8/9/2007; revised 10/24/2007; accepted 11/16/2007.

Grant support: National Cancer Institute grants CA099258 and CA103563 (AntiCancer, Inc.), NIH grant R21 CA109949-01, and American Cancer Society grant RSG-05-037-01-CCE (M. Bouvet).

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.

References

1. Orr FW, Adamson IYR, Young L. Promotion of pulmonary metastasis in mice by Bleomycin-induced endothelial injury. Cancer Res 1986;46:891–7.
2. Kennel SJ, Lankford TK, Ullrich RL, Jamasbi RJ. Enhancement of lung tumor colony formation by treatment of mice with monoclonal antibodies to pulmonary capillary endothelial cells. Cancer Res 1988;48:4964–8.
3. Munoz R, Man S, Shaked Y, et al. Highly efficacious nontoxic preclinical treatment for advanced metastatic breast cancer using combination oral UFT-cyclophosphamide metronomic chemotherapy. Cancer Res 2006; 66:3386–91.
4. Shaked Y, Ciarrocchi A, Franco M, et al. Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. Science 2006;313:1785–7.
5. Orosz P, Echtenacher B, Falk W, et al. Enhancement of experimental metastasis by tumor necrosis factor. J Exp Med 1993;177:1391–8.
6. Withers HR, Milas L. Influence of preirradiation of lung on development of artificial pulmonary metastases of fibrosarcoma in mice. Cancer Res 1973;33:1931–6.
7. Camphausen K, Moses M, Beecken WD, et al. Radiation therapy to a primary tumor accelerates metastatic growth in mice. Cancer Res 2001;61:2207–11.
8. Van Putten LM, Kram LKJ, Van Direndonck HHC, et al. Enhancement by drugs of metastatic lung nodule formation after intravenous tumor cell injection. Int J Cancer 1975;15:588–95.
9. Kline I, Gang M, Tyrer DD, et al. Duration of drug levels in mice as indicated by residual antileukemic efficiency. Chemotherapy 1968;13:28–41.
10. Carmel RJ, Brown JM. The effect of cyclophosphamide and other drugs on the incidence of pulmonary metastases in mice. Cancer Res 1977;37:145–51.
11. Iwamoto Y, Fujita Y, Sugioka Y. YIGSR, a synthetic laminin peptide, inhibits the enhancement by cyclophosphamide of experimental lung metastasis of human fibrosarcoma cells. Clin Exp Metastasis 1992;10:183–9.
12. Tsuji K, Yamauchi K, Yang M, et al. Dual-color imaging of nuclear-cytoplasmic dynamics, viability, and proliferation of cancer cells in the portal vein area. Cancer Res 2006;66:303–6.
13. Yamauchi K, Yang M, Jiang P, et al. Development of real-time subcellular dynamic multicolor imaging of cancer-cell trafficking in live mice with a variable-magnification whole-mouse imaging system. Cancer Res 2006;66:4208–14.
14. Yamamoto N, Jiang P, Yang M, et al. Cellular dynamics visualized in live cells *in vitro* and *in vivo* by differential dual-color nuclear-cytoplasmic fluorescent-protein expression. Cancer Res 2004;64:4251–6.
15. Jiang P, Yamauchi K, Yang M, et al. Tumor cells genetically labeled with GFP in the nucleus and RFP in the cytoplasm for imaging cellular dynamics. Cell Cycle 2006;5:1198–201.
16. Hoffman RM. The multiple uses of fluorescent proteins to visualize cancer *in vivo*. Nat Rev Cancer 2005;5:796–806.
17. Al-Mehdi AB, Tozawa K, Fisher AB, et al. Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. Nat Med 2000;6:100–2.
18. Luzzi KJ, MacDonald IC, Schmidt EE, et al. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. Am J Pathol 1998;153:865–73.