

IMAGING ENHANCEMENT OF MALIGNANCY BY CYCLOPHOSPHAMIDE: SURPRISING CHEMOTHERAPY OPPOSITE EFFECTS

Kensuke Yamauchi¹, Meng Yang², Katsuhiko Hayashi^{1,2,3}, Ping Jiang², Mingxu Xu²,
Norio Yamamoto¹, Hiroyuki Tsuchiya¹, Katsuro Tomita¹, A.R. Moossa³, Michael Bouvet³,
Robert M. Hoffman^{2,3}

¹ Dept. of Orthopaedic Surgery, School of Medicine, Kanazawa University, 13-1 Takaramachi,
Kanazawa, Ishikawa, 920-8641 Japan

² AntiCancer, Inc., 7917 Ostrow Street, San Diego, CA 92111-3604, USA.

³ Dept. of Surgery, University of California, San Diego, 200 West Arbor Drive, San Diego, CA
92103-8220

ABSTRACT

Although side effects of cancer chemotherapy are well known, “opposite effects” of chemotherapy which enhance the malignancy of the treated cancer are not well understood. We have observed a number of steps of malignancy that are enhanced by chemotherapy pre-treatment of mice before transplantation of human tumor cells. The induction of intravascular proliferation, extravasation, and colony formation by cancer cells, critical steps of metastasis was enhanced by pretreatment of host mice with the commonly-used chemotherapy drug cyclophosphamide. Cyclophosphamide appears to interfere with a host process that inhibits intravascular proliferation, extravasation, and extravascular colony formation by at least some tumor cells. Cyclophosphamide does not directly affect the cancer cells since cyclophosphamide has been cleared by the time the cancer cells were injected. Without cyclophosphamide pretreatment, human colon cancer cells died quickly after injection in the portal vein of nude mice. Extensive clasmocytosis (destruction of the cytoplasm) of the cancer cells occurred within 6 hours. The number of apoptotic cells rapidly increased within the portal vein within 12 hours of injection. However, when the host mice were pretreated with cyclophosphamide, the cancer cells survived and formed colonies in the liver after portal vein injection. These results suggest that a cyclophosphamide-sensitive host cellular system attacked the cancer cells. This review describes an important unexpected “opposite effects” of chemotherapy that enhances critical steps in malignancy rather than inhibiting them, suggesting that certain current approaches to cancer chemotherapy should be modified.

Keywords: cancer cells, green fluorescent protein, red fluorescent protein, in vivo single cell imaging, nude mice

1. 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 following 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 (TNF) 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 administered 24 hours before tumor-cell injection. Since the half-life of cyclophosphamide is only 17 minutes, the drug did not affect viability of tumor cells injected 24 hours later (9). Carnel et al. (10) suggested that cyclophosphamide induced changes within the pulmonary microvasculature where the injected tumor cells must lodge in order to form metastases. Cyclophosphamide apparently killed macrophages or prevented the macrophages from killing the endothelium-trapped cancer cells. Iwamoto et al. (11) demonstrated cyclophosphamide-induced endothelial damage using monolayer cell cultures *in vitro*.

2. MATERIALS AND METHODS

2.1 Establishment of fluorescent protein-labeled human cancer cells. In order to establish dual-color human cancer cells, the cells were transfected with retroviral RFP and histone H2B-GFP. In brief, the Hind III /Not I fragment from pDsRed2 (Clontech Laboratories, Inc., Palo Alto, CA) 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 precipitated mixture of LipofectAMINE reagent (Life Technologies, Inc. Grand Island, NY), 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 200-1000 µg/ml G418 (Life Technologies, Inc.), increased stepwise, for 7 days (12).

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 histone H2B-GFP retroviral vector, the pLHCX histone H2B-GFP vector containing the hygromycin (HYG)-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 200-400 µg/ml HYG., increased stepwise, such that they produced high amounts of the H2B-GFP vector (12).

For RFP and H2B-GFP gene transduction, clones of cancer cells expressing RFP in the cytoplasm were established. The cells were incubated with retroviral supernatants of PT67-RFP cells for 72 h. Cells were cultured in selective medium, which 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 HYG for 72 h. The level of HYG was increased stepwise up to 400 µg/ml. Bright dual-color cells were thereby obtained (12).

2.2 Drugs and animals. Cyclophosphamide (CPA) was obtained from Sigma-Aldrich (St. Louis, Missouri, United States). Five female 4-week-old athymic CD-1 nude mice, were used in this study. Mice were kept in a barrier facility under HEPA filtration.

2.3 Pretreatment of animals with cyclophosphamide. Host mice were pretreated with cyclophosphamide (100 mg/kg) in the portal vein 6 days and 1 day before injection of HCT-116-GFP-RFP human colon cancer cells or 200 mg/kg ip 24 hours before injection of HT1080-GFP-RFP human fibrosarcome cells in the epigastric cranialis vein (please see below) (13).

2.4 Mice. Mice were fed with autoclaved laboratory rodent diet (Tecklad LM-485, Western Research Products, Orange, CA). 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 (12).

2.5 Mouse model for *in vivo* imaging intravascular behavior of cancer 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 H₂O s.c.). For cell injection, an arc-shaped incision was made in the abdominal skin, and subcutaneous 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. HT1080-GFP-RFP 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 (12).

2.6 Injection of cancer cells in the portal vein. The nude mice were anesthetized with a ketamine mixture (10 μ L ketamine HCl, 7.6 μ L xylazine, 2.4 μ L acepromazine maleate, 10 μ L H₂O) injected into the peritoneal cavity. Human HCT-116-GFP-RFP colon cancer cells (0.125 - 1.0×10^6 cells/50 μ L) were injected in the portal vein of nude mice during open laparotomy (13).

2.7 Fluorescence optical imaging and data analysis. The OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan) containing an MT-20 light source (Olympus Biosystems, Planegg, Germany) and DP70 CCD camera (Olympus Corp., Tokyo, Japan) was used for real-time cellular imaging in live mice. High-resolution images were captured directly on a PC (Fujitsu Siemens computers, Munich, Germany). Images were processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 software (12).

2.8 Visualization of cancer cell trafficking in liver of living mice. At laparotomy, a glass slide was put on the exteriorized liver of the mice to regulate motion. The mice were observed with the Olympus OV100 imaging system. The images were taken immediately after cell injection, and 0.5, 1, 6, 12, and 24 hours, and daily thereafter (13).

3. RESULTS AND DISCUSSION

3.1 Intravascular proliferation, extravasation, and micrometastasis in cyclophosphamide- pretreated mice. In contrast to other cancer cell types such as the Lewis lung carcinoma or mouse mammary tumor cells (MMT060562), HT1080 human fibrosarcoma cells only rarely extravasated in nude mice. Even at 24 hours after injection, the majority of the HT1080 cells remained in vessels without extravasation, which were expanding apparently in order to maintain blood flow. In mice pretreated with cyclophosphamide, HT1080 cells could proliferate extensively intravascularly, extravasate, and subsequently form colonies. The colonies increased in size and were visualized even at low-magnification. In contrast, extravasated colonies were not formed in non-pretreated mice (12).

3.2 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. 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 (12).

3.3 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 vessels' outer surface. However, by one week after cyclophosphamide pretreatment of the mice, the colonies decreased in size. While 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 (12).

3.4 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 appeared to be pushed out to peripheral vessels by 14 days after cell injection. These peripheral intravascular colonies did not decrease in size one week after cyclophosphamide pretreatment, in contrast to the extravascular colonies which eventually regressed. Seven days after HT1080-GFP-RFP cell injection, peripheral vessels with many cancer cells were visualized. The mass of HT1080 cells appeared 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. In narrow capillaries, the HT1080 cells migrated single file. In contrast, the number of HT1080 cells decreased in the vessels of non-pretreated mice. There were only occasionally small colony-like tumor thrombi, which contained fragmented dying tumor cells, which seem to remain in vessels. The vessels expanded due to these tumor thrombi.

Al-Mehdi et al. (14) also observed apparent intravascular tumor cell proliferation in mouse lungs perfused *ex vivo*. The current results demonstrate this phenomenon *in vivo*. The ability of cancer cells to proliferate intravascularly has been a

controversial issue (15). The results in this report demonstrate definitive intravascular proliferation of tumor cells in vivo. This phenomenon has great implications for metastasis.

The results in this study indicate cyclophosphamide, which was administered 24 hours before cancer cell injection, enhanced intravascular proliferation, extravasation, and subsequent colony formation of the HT1080 human fibrosarcoma.

3.5 Entry of cancer cells in the portal vein circulation. After the HCT-116-GFP-RFP human colon cancer cells were injected into the main portal vein, cells accumulated in the terminal portal veins. Initially, many HCT-116-GFP-RFP cells impacted into sinusoids just after injection. The diameter size of the sinusoids was $\sim 5 \mu\text{m}$. Most HCT-116-GFP-RFP cells remained in the sinusoids near the terminal portal vein (13).

3.6 Time course of cancer cell death in the portal vein area. Thirty minutes after injection of HCT116-GFP-RFP in the portal vein, 70% of the cells were viable. However, by 2 hours, only 50% of the cells were viable, and by 6 hours, only 10% of the cells were viable. Viability was readily observed as the dead cells were stripped of their RFP-expressing cytoplasm leaving only the GFP nucleus. The number of apoptotic cells rapidly increased within the portal vein within 12 hours of injection. Fragmentation of GFP nuclei could be clearly visualized (13).

3.7 Efficacy of cyclophosphamide pretreatment on portal vein viability of cancer cells. When the host mice were pretreated with cyclophosphamide, the HCT-116-GFP-RFP cells also survived and formed colonies in the liver after portal vein injection, in striking contrast to their rapid cell death in the untreated animals. These results suggest that a host cellular system attacked the HCT-116-GFP-RFP cells. Most HCT116-GFP-RFP cells survived 24 hours after injection in the cyclophosphamide-treated mice and subsequently formed metastasis in the liver in three of five mice compared with none in the non-cyclophosphamide-treated mice. Some of the HCT116-GFP-RFP micrometastases became vascularized by day 14 (13).

Morris et al. (16) previously described clasmatosis of cells in the liver. We could visualize this phenomenon more clearly by using bright dual-color fluorescent cancer cells with the cytoplasm labeled with RFP and the nucleus labeled with GFP. HCT-116-GFP-RFP cells injected into the portal vein were all dead within 12 hours. Rapid cell death was also reported by Morris et al. (16). The liver may contain an innate rapid local defense for cells entering the liver portal veins. Our results suggest that such a host defense is sensitive to cyclophosphamide.

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.

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