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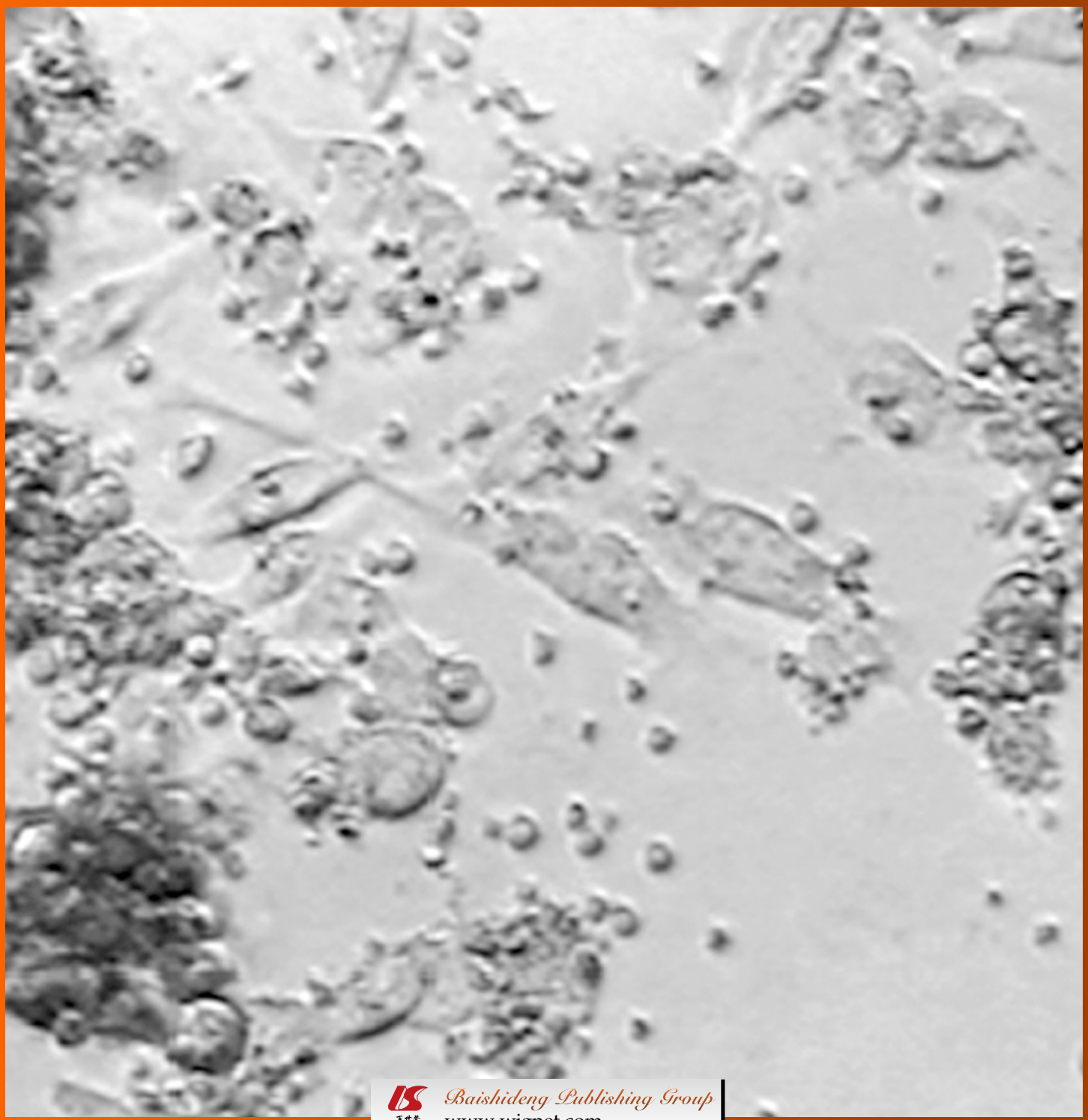

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No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: 0086-10-8538-1892
Fax: 0086-10-8538-1893
E-mail: baishideng@wjgnet.com
http://www.wjgnet.com

EDITING
Editorial Board of *World Journal of Clinical Oncology*,
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: 0086-10-5908-0036
Fax: 0086-10-8538-1893
E-mail: wjco@wjgnet.com
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Fax: 00852-3115-8812
Telephone: 00852-5804-2046
E-mail: baishideng@wjgnet.com
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Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
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EDITORIAL OFFICE
Na Ma, Director
World Journal of Clinical Oncology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: 0086-10-8538-1892
Fax: 0086-10-8538-1893
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EDITORIAL

What is the purpose of launching *World Journal of Clinical Oncology*?

Lian-Sheng Ma

Lian-Sheng Ma, World Series Journals, Baishideng Publishing Group Co., Limited, Beijing 100025, China
Author contributions: Ma LS solely contributed to this paper.
Correspondence to: Lian-Sheng Ma, President and Editor-in-Chief, World Series Journals, Baishideng Publishing Group Co., Limited, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China. l.s.ma@wjgnet.com
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Abstract

The first issue of *World Journal of Clinical Oncology* (*WJCO*), whose preparatory work was initiated on December 20, 2009, will be published on November 10, 2010. The *WJCO* Editorial Board has now been established and consists of 315 distinguished experts from 33 countries. Our purpose of launching *WJCO* is to publish peer-reviewed, high-quality articles via an open-access online publishing model, thereby acting as a platform for communication between peers and the wider public, and maximizing the benefits to editorial board members, authors and readers.

Key words: Maximization of personal benefits; Editorial board members; Authors; Readers; Employees; *World Journal of Clinical Oncology*

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INTRODUCTION

I am very pleased to announce that the first issue of *World Journal of Clinical Oncology* (*World J Clin Oncol*, *WJCO*, online ISSN 2218-4333, DOI: 10.5306), whose preparatory work was initiated on December 20, 2009, will be published on November 10, 2010. The *WJCO* Editorial Board has now been established and consists of 315 distinguished experts from 33 countries. What is the purpose of launching *WJCO*? And what is the scope and how are the columns designed?

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. To realize these desired attributes of a journal and create a well-recognized journal, the following four types of personal benefits should be maximized.

MAXIMIZATION OF PERSONAL BENEFITS

The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others.

Maximization of the benefits of editorial board members

The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution.

Maximization of the benefits of authors

Since *WJCO* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJCO* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading.

Maximization of the benefits of readers

Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion[1].

Maximization of the benefits of employees

It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal[2,3]. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

CONTENTS OF PEER REVIEW

In order to guarantee the quality of articles published in the journal, *WJCO* usually invites three experts to comment on the submitted papers. The contents of peer review include: (1) whether the contents of the manuscript are of great importance and novelty; (2) whether the experiment is complete and described clearly; (3) whether the discussion and conclusion are justified; (4) whether the citations of references are necessary and reasonable; and (5) whether the presentation and use of tables and figures are correct and complete.

SCOPE

The aim of *WJCO* is to report rapidly new theories, methods and techniques for prevention, diagnosis, treatment, rehabilitation and nursing in the field of oncology. *WJCO* covers etiology, epidemiology, evidence-based medicine, informatics, diagnostic imaging, endoscopy, tumor recurrence and metastasis, tumor stem cells, radiotherapy, chemotherapy, interventional radiology, palliative therapy, clinical chemotherapy, biological therapy, minimally invasive therapy, physiotherapy, psycho-oncology, comprehensive therapy, oncology-related traditional medicine, integrated Chinese and Western medicine, and nursing. *WJCO* covers tumors in various organs/tissues, including the female reproductive system, bone and soft tissue, respiratory system, urinary system, endocrine system, skin, breast, nervous system, head and neck, digestive system, and hematologic and lymphatic system. The journal also publishes original articles and reviews that report the results of applied and basic research in fields related to oncology, such as immunology, physiopathology, cell biology, pharmacology, medical genetics, and pharmacology of Chinese herbs.

COLUMNS

The columns in the issues of *WJCO* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide Guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Articles: To report innovative and original findings in oncology; (9) Brief Articles: To briefly report the novel and innovative findings in oncology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJCO*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of oncology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on the research oncology.

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EDITORIAL

Cytotoxic T-cells as imaging probes for detecting glioma

Ali Syed Arbab

Ali Syed Arbab, Cellular and Molecular Imaging Laboratory, Department of Radiology, Henry Ford Hospital, Detroit, MI 48202, United States
Author contributions: Arbab AS solely contributed to this paper.
Supported by NIH grants, No. R01CA122031 and No. 1R21-CA129801
Correspondence to: Ali Syed Arbab, MD, PhD, Cellular and Molecular Imaging Laboratory, Department of Radiology, Henry Ford Hospital, 1 Ford Place, 2F, Box 82, Detroit, MI 48202, United States. saali@rad.hfh.edu
Telephone: +1-313-8744435 Fax: +1-313-8744494
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Abstract

Tumor vaccination using tumor-associated antigen-primed dendritic cells (DCs) is in clinical trials. Investigators are using patients' own immune systems to activate T-cells against recurrent or metastatic tumors. Following vaccination of DCs or attenuated tumor cells, clinical as well as radiological improvements have been noted due to migration and accumulation of cytotoxic T-cells (CTLs). CTLs mediated tumor cell killing resulted in extended survival in clinical trails and in preclinical models. Besides administration of primed DCs or attenuated or killed tumors cells to initiate the generation of CTLs, investigators have started making genetically altered T-cells (CTLs) to target specific tumors and showed *in vivo* migration and accumulation in the implanted or recurrent tumors using different imaging modalities. Our groups have also showed the utilization of both *in vivo* and *in vitro* techniques to make CTLs against glioma and used them as imaging probes to determine the sites of tumors. In this short review, the current status of vaccination therapy against glioma and utilization of CTLs as *in vivo* imaging probes to determine the sites of tumors and differentiate recurrent glioma from radiation necrosis will be discussed.

Key words: Cellular magnetic resonance imaging; Cytotoxic T-cells; Glioma; Glioma associated antigen; Imaging probes; Magnetic resonance imaging; Tumor vaccine

Peer reviewer: Domenico Rubello, MD, Professor, Director of the Department of Nuclear Medicine, PET/CT Centre, Radiology, Medical Physics, Santa Maria della Misericordia Hospital, Via Tre Martiri 140, ZIP 45100, Rovigo, Italy

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CELL BASED VACCINATION THERAPIES FOR GLIOMA

Tumor vaccination therapy has been used in active investigations for the last 50 years. Different strategies have been employed to activate or sensitize host T-cells against tumor antigen to eradicate malignant tumors. Investigators have used attenuated or irradiated intact tumor cells with or without adjuvant to initiate an immunogenic reaction[1-3]. These attenuated cells were injected subcutaneously or in the food pad of animals to initiate priming of antigen presenting cells (APC/s) and production of cytotoxic T-cells (CTLs) in the nearby draining lymph nodes. To attract more T-cells for the purpose of sensitization, different cytokines have already been tried during administration of attenuated cells or cell lysate[4-7].

In vitro priming of APCs is another popular way to activate immune systems against tumor antigens. These APCs are collected from peripheral blood, bone marrow or from cord blood[8-12]. APCs represent macrophages and mononuclear cells such as monocytes. These cells are converted to immature and mature dendritic cells (DC/s). During the process of making DCs, tumor specific antigen, either in the form of tumor cell lysate, apoptotic tumor cells or tumor-associated antigenic peptide or proteins are added to the media and then these cells, after priming, are administered systemically or locally to initiate the production of CTLs. Investigators have reported a varying degree of suc-

cess in treating recurrent, metastatic or primary malignant tumors of different origins both in preclinical models and in clinical trials.

Malignant glioma is one of the most aggressive tumors with a poor prognosis despite available treatments including surgery, chemotherapy and radiation therapy[13]. Standard treatment procedures, consisting of surgery and radiation therapy (followed by adjuvant chemotherapy), very often fail due to the inability to accurately delineate tumor margins[14-16], and the median survival time for patients with recurrent glioblastoma multiforme (GBM) is less than 1 year[17]. The infiltrative nature of GBM is considered to be one of the main factors impeding the complete removal of tumor mass by surgical procedure[18]. Following radiation therapy or surgery, recurrence is common and almost invariably occurs within < 2 cm of the prior resection line, which is due to leftover tumor or tumor cells. Dendritic cell-based vaccination therapy against recurrent glioma that utilizes the patient's own DCs which are pulsed, *ex-vivo*, with the derived glioma cell-lysate is currently in clinical trials[19-22]. In experimental glioma models, an increase in the number of CTLs compared to control or pre-vaccination levels is observed following the administration of glioma cell-lysate-pulsed DCs. Investigators have identified specific glioma-associated antigens, which are being used to pulse DCs[23-25]. There are many (at least 10) active clinical trials running, sponsored by the National Cancer Institute, and targeting primary as well as recurrent glioma that utilize primed DC-based vaccination (www.cancer.gov/clinicaltrials). Apoptotic tumor cells, tumor cell lysate and glioma-associated antigens (peptides) are being used to prime the DCs in these clinical trials.

GLIOMA ASSOCIATED ANTIGENS AND PRIMING OF DENDRITIC CELLS

Making DC-based vaccine using tumor cell lysate is relatively non-specific and primed DCs may initiate CTL production which may attack other normal cells and tissues in the body. Investigators have tried to find specific antigens that could be used to make primed DCs and specific CTLs after vaccination. Different antigens have been isolated from different malignant tumors which are expressed specifically for the types of tumors. Brugger *et al*[26] used MUC1 derived HLA-A2 restricted peptides to pulse DCs and produced CTLs *in vitro* to target blasts of acute myeloid leukemia (AML). The generated CTLs showed *in vitro* effectiveness against AML and multiple myeloma cell lines. Other investigators used leukemia-associated antigens as well as apoptotic cells to generate antigen-primed or tumor cell-primed DCs, respectively, for vaccination therapy in AML[27-31]. Malignant melanoma is one of the most widely investigated tumors for cell-based vaccination therapy[32-34]. The most widely used proteins/peptides to prime DCs are human melanoma antigen-A3 (MAGE-3), melanoma antigen MART-1/Melan-A (MART-1), gp100 and tyrosinase[35-37].

The method of priming DCs using tumor-associated antigens (TAA) is more specific than priming with whole tumor cell lysate. Investigators have identified tumor specific antigens (peptides) and these antigens can be used to pulse DCs to initiate antigen specific CTLs when administered into hosts. Zhang *et al*[25] have profiled the antigens in 20 different types of human glioma cell lines and concluded that all the cells exhibited multiple TAA which can be used to prime DCs to initiate production of CTLs. The authors identified a few important antigens, such as, melanoma-2 (Aim-2), B-cyclin, EphA2, GP100, h1, 6-N-acetylglucosaminyltransferaseV (GnT-V), IL13Ra2, Her2/neu, hTert, Mage, Mart-1, Sart-1, and survivin. Based on their results, Dr. Okada's group (at UPMC) and other investigators have identified three important antigens (EphA2, IL13Ra2, survivin) for priming DCs and used as vaccines for glioma treatment[38,39]. NCI sponsored clinical trials are underway to make GAA-pulsed DCs for vaccination in patients with recurrent glioma. In these proposals, the investigators prime the autologous DCs with specific peptides by simple incubation during the conversion of adherent peripheral blood mononuclear cells to mature DCs.

It is obvious from the above discussions that DC-based vaccine is about to be used in clinical practice as an adjuvant therapy for the treatment of different malignant tumors with immunogenic properties. Patient-specific or tumor-specific personalized DC-based vaccine can also be designed using mRNA loading[23,40,41]. However, there has been no discussion or report indicating how to utilize the DC-based technique to make tumor-specific CTLs *ex vivo* and use them as probes for imaging to identify recurrent or metastatic tumors or to differentiate glioma from radiation necrosis in the brain. The following paragraphs will discuss the methods to make CTLs *ex vivo* and to utilize them as probes for detecting tumors and differentiating from radiation necrosis.

MAKING OF CTLs AS PROBES FOR IMAGING

The making of CTLs *ex vivo* involves different stages of experimental procedures. First, autologous or allogeneic primed DCs need to be generated and then collected T-cells should be sensitized against target antigens or peptides. These CTLs can then either be tagged with radioisotopes for tracking with nuclear medicine imaging techniques or can be labeled with different MRI contrast agents to be tracked by MRI. The following describe the procedures for making primed DCs and CTLs for the purpose of using these CTLs as probes for imaging.

Making of primed dendritic cells

There are many ways to make primed DCs-pulsed with tumor-specific antigens. The most widely used method is to pulse autologous or allogeneic DCs with the tumor lysate that are generated from tumor tissues collected from patients[19,42,43]. In this process, the patent's peripheral blood mononuclear cells are collected and DCs are generated from either selecting CD14 positive cells or by selecting adherent cells in culture dishes or plates. These cells are cultured in the presence of granulocyte colony

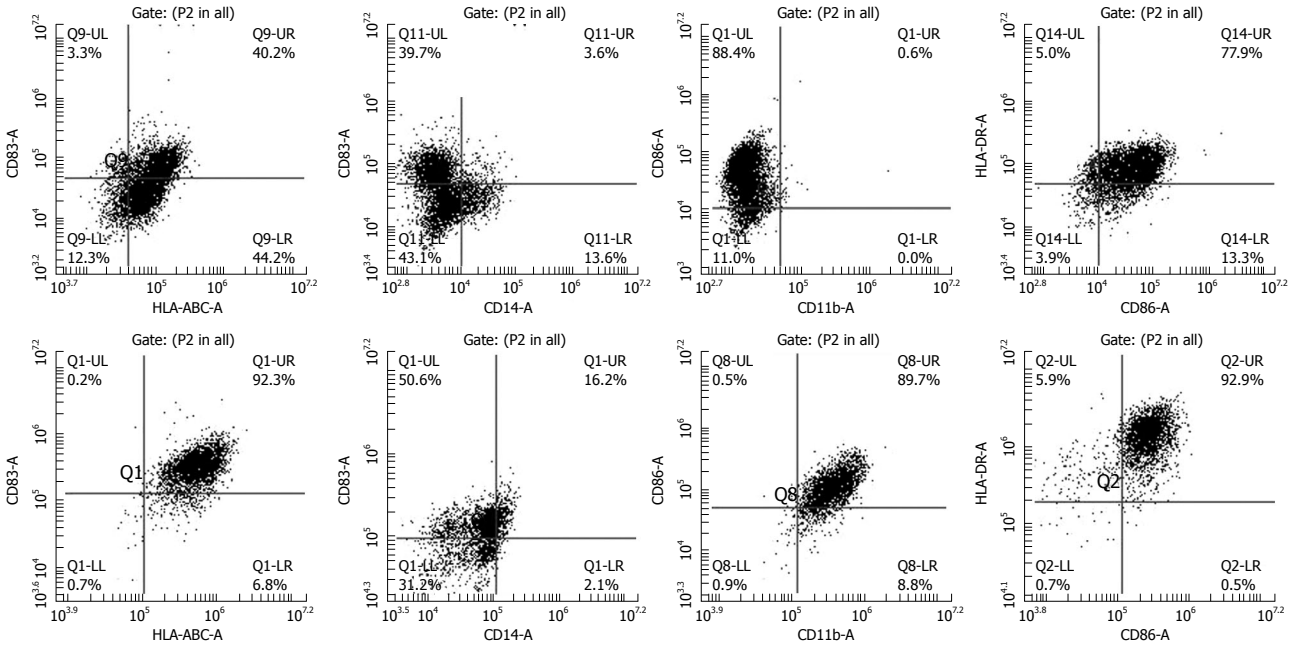

Figure 1 Phenotypic analysis of immature (upper panel) and mature dendritic cells (lower panel). CD14+ cells collected from cord blood were incubated in immature dendritic cell (DC) media (RPMI-1640 plus 10% FBS) containing granulocyte colony stimulating factor (G-SCF) and IL-4 for 8 d and then cells were incubated with tumor necrosis factor (TNF)-α in addition to G-CSF and IL-4. Cells show typical monocytic DC markers (low CD14, high HLA-ABC, HLA-DR, CD86, CD83 and CD11b). Note the increase in the population of CD83 positive cells, a marker of mature DCs, after addition of TNF-α.

stimulating factor (G-SCF) and interleukin-4 (IL-4), which makes immature DCs. To convert these immature DCs (which show high expression of CD86, HLA-DR, HLA-ABC and low expression of CD14) to mature DCs, cells are then cultured in the presence tumor necrosis factor (TNF)-α in addition to G-CSF and IL-4. During the process of making DCs, tumor lysate is added to the cultures. We have extensively studied the method of making tumor lysate-pulsed DCs to make CTLs *ex vivo* using cord blood derived CD14+ and CD2/3+ cells, respectively. Figure 1 shows the phenotypical changes that happen to CD14+ cells during the process of making tumor lysate-pulsed mature DCs. One of the phenotypical markers of mature DCs is CD83. DCs can also be primed using autologous or allogeneic killed or apoptotic tumor cells[44,45]. DCs can phagocytose the tumor cells and eventually express the antigen on the surface. Based on this idea, investigators also made hybrid cells combining DCs and tumor cells, and administered them into the host to initiate production of CTLs[46,47]. For quick and optimal expression of target antigen by DCs, electroporation methods are also used to prime the DCs[40,41,43]. From the clinical point of view, the electroporation method could be suitable for any good manufacturing practice (GMP) grade production of primed DCs for tumor vaccine therapy. A viral-based transfection technique has also been applied to transduce tumor mRNA into DCs for the purpose of vaccine[48-50].

Making of CTLs

CTLs sensitized to specific tumor antigen can be produced both *in vivo* and *in vitro* conditions. All of the clinical trials and animal experiments that utilized tumor specific antigen-pulsed DCs for vaccination, in fact, relied on the *in vivo* production of CTLs. The production of tumor-specific CTLs has been detected in all patients and animals. Investigators have pointed out that the tumor specific CTLs express different T-cells markers, such as CD25, CD4, and CD8[51-54]. We also have analyzed splenocytes collected from tumor (9L gliosarcoma) bearing Fisher-344 rats by flow cytometry, and increases in the population of CD4, CD8, CD80 and CD86 positive cells were observed[55]. The number of these cell populations was significantly different from the splenocytes collected from control (non-tumor bearing) rats, indicating the production of CTLs in tumor bearing rats. By co-incubating primed DCs with collected CD2/3 positive cells, tumor antigen specific CTLs can also be produced *ex vivo*. Our recent investigations showed that CTLs can be used to make imaging probes to detect tumors[55]. However, there has been no report showing the utilization of *ex vivo* produced CTLs for the purpose of diagnostic imaging to detect recurrent or residual tumors. Investigators have used genetically modified CTLs (*in vitro* manipulated) to detect the migration and accumulation in residual GMB[56] in humans and in a rat model of glioma[57]. Kircher *et al*[58] utilized B16-OVA-specific CD8+ to detect the migration and accumulation of magnetically labeled CTLs to the sites of B16-OVA melanoma. All these investigators used CTLs as probes for imaging, which were not collected from the host. We routinely produce CTLs *ex vivo* using cord blood derived primed DCs pulsed with tumor cell lysate and T-cells for the purpose of imaging to detect and differentiate different diseases. The following are the brief descriptions of our procedures: CD14+ and CD2+/CD3+ cells: Both types of cells are isolated from human cord blood under the approved IRB protocol. CD14+ positive cells are separated from other mononuclear cells (cord

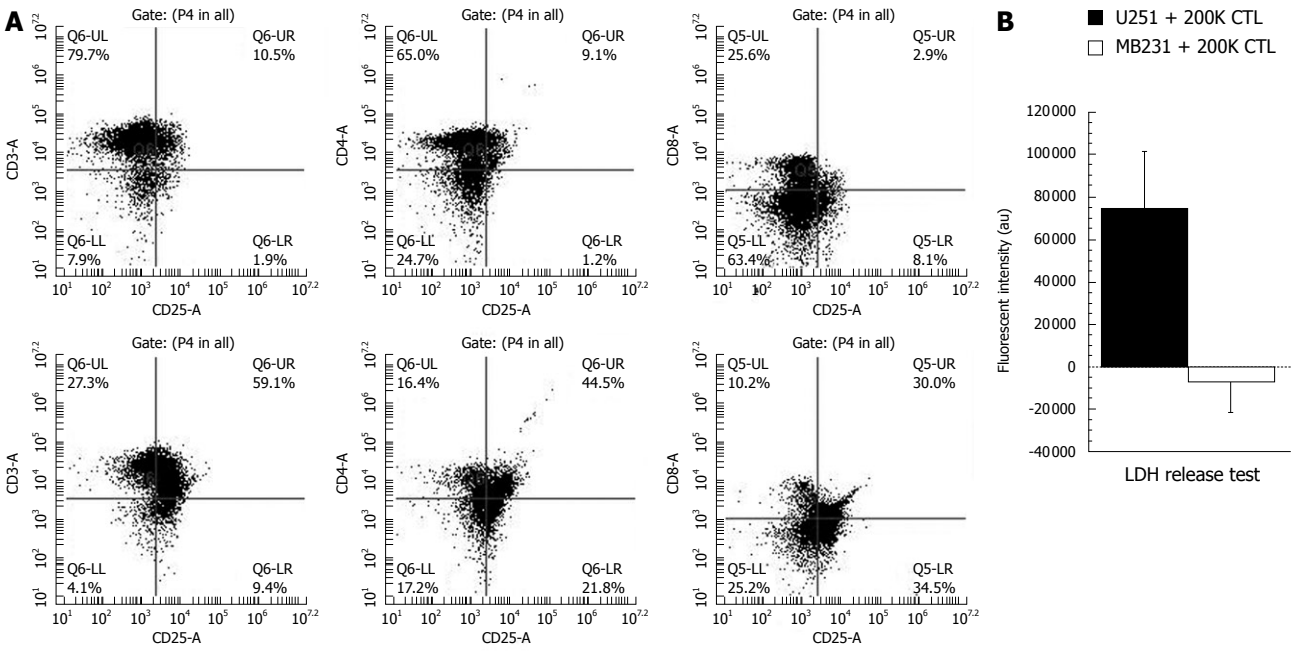

Figure 2 Phenotypic analyses of sensitized T-cells (cytotoxic T-cells) and cytotoxic activity of produced cytotoxic T-cells. A: Analysis of T-cell markers. Phenotypic analysis of control T-cells (upper panel) and T-cells co-incubated with tumor lysate-pulsed irradiated mature dendritic cells (DCs) for 4 d (lower panel). Note the increased number of CD25+ cells (activated T-cells) after sensitizing them with DCs; B: To determine the cytotoxic specificity of the produced cytotoxic T-cells (CTLs) to U251 cells, 200 000 (200K) CTLs (sensitized to U251 cell lysate) were co-cultured overnight with U251 (100 000 cells) or human breast cancer cells (MBA-MD-231, 100 000) and the released lactate dehydrogenase (LDH) was determined by a commercially available membrane integrity assay kit (Cyto Tox-ONE, Promega Corp, WI, USA). LDH levels indicate cytotoxicity since LDH is released to the media once cell membranes are damaged. Note the significantly ($P \leq 0.05$) increased LDH release from U251 cells indicating the specificity of produced CTLs.

blood mononuclear cells are obtained using a Ficoll gradient separation technique) by magnet activated cell sorter (MACS) using magnetic beads conjugated with anti-CD14 antibodies. CD14 depleted cells were further incubated with anti-CD2 antibodies conjugated to magnetic beads to separate CD2+/CD3+ cells. Collected CD14+ cells were further differentiated into DCs (as described below).

Preparation of tumor cell lysate-pulsed mature dendritic cells

CD14+ cells are resuspended at the concentration of 3-5 $\times 10^5$ cells/mL in RPMI 1640 media containing 10% FBS, 25 ng/mL of IL-4, 50 ng/mL of G-CSF (granulocyte colony-stimulating factor) and incubated in 5% CO_2/95% air at 37℃ in a humidified incubator for 4 d to make immature DCs. During this incubation period 1/2 of the media is replaced with 2/3rd of fresh media containing cytokines on day 3. On day 5, suspended and loosely adherent cells are collected, centrifuged and resuspended in fresh RPMI 1640 media containing 10% FBS, 25 ng/mL of IL-4, 50 ng/mL of GM-CSF and 50 μg/mL of tumor cell lysate at 5 $\times 10^5$ cells/mL. The cells are thoroughly mixed and further incubated for 4 d. At the end of the 4 d priming, cells are collected and resuspended in fresh media containing 10% FBS, 25 ng/mL of IL-4, 50 ng/mL of GM-CSF and 100 ng/mL of TNF-α and incubated for an additional 4 d. Expression of different markers specific for DCs (such as CD14, CD86, CD83, CD11b, HLA-ABC and HLA-DR) are assessed by flow cytometry before and after addition of TNF-α.

Sensitization of isolated T-cells

Either fresh or cryopreserved T-cells that are isolated from cord blood are cultured overnight in RPMI 1640 media containing 10% FBS, sodium pyruvate, non-essential amino acids, L-glutamine and 10 ng/mL of IL-2 and then co-cultured with irradiated (35 Gy) cell lysate-pulsed mature DCs for 5-6 d. The initial ratio of T-cell to DC was 10:1. T-cell proliferation is monitored every day and based on the cell density; fresh media is added to the co-culture. Phenotypical expression of different T-cell markers (CD3, CD4, CD8, and CD25) is determined by a flow cytometer before and after sensitization, as well as after the magnetic labeling of CTLs. Specificity of CTLs is also determined by a lactate dehydrogenase (LDH) release assay. Figure 2 shows the markers of CTLs and their cytolytic specificity.

Making of CTLs as probes

Commercially available, FDA-approved super paramagnetic iron oxides (SPIO) ferumoxides suspension (Feridex Ⅳ ®, Bayer-Schering Pharmaceuticals Inc, Wayne, NJ, USA) contains particles that are approximately 80-150 nm in size and has a total iron content of 11.2 mg/mL (11.2 μg/μL of iron). Protamine sulfate (American Pharmaceuticals Partner Inc. Schaumburg, IL, USA), supplied at 10 mg/mL, was prepared as a fresh stock solution of 1 mg/mL in distilled water at the time of use. We magnetically label CTLs using our published method, in brief ferumoxides (100 μg/mL) is directly added to the cell suspension in serum free media and then protamine sulfate (3 μg/mL) is added[59]. The FePro complexes are formed

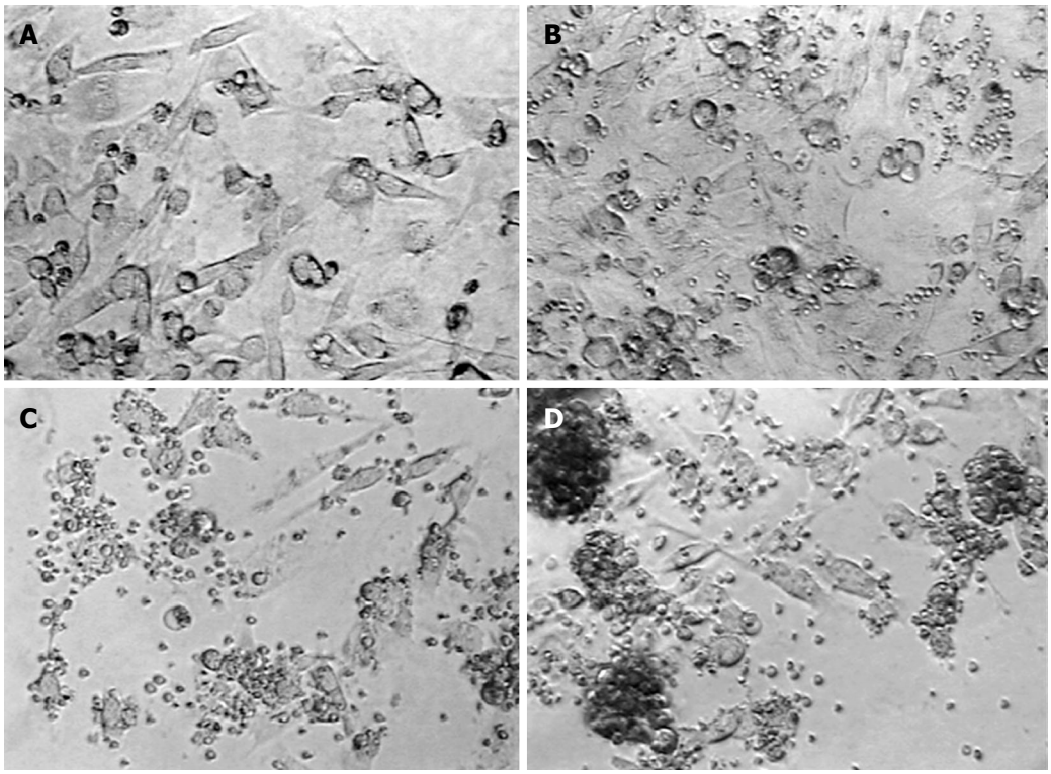

Figure 3 The interaction of cytotoxic T-cells (labeled and unlabeled) with U251 glioma cells. Cytotoxic T-cells (CTLs) were produced using U251 cell lysate-pulsed irradiated mature dendritic cells. A: Normal U251 cells; B: Control T-cells; C:Unlabeled CTLs targeting U251 cells; D:Labeled CTL U251 cells,ferumoxide-protamine sulfate (FePro) labeled CLTs were co-incubated with U251 overnight.

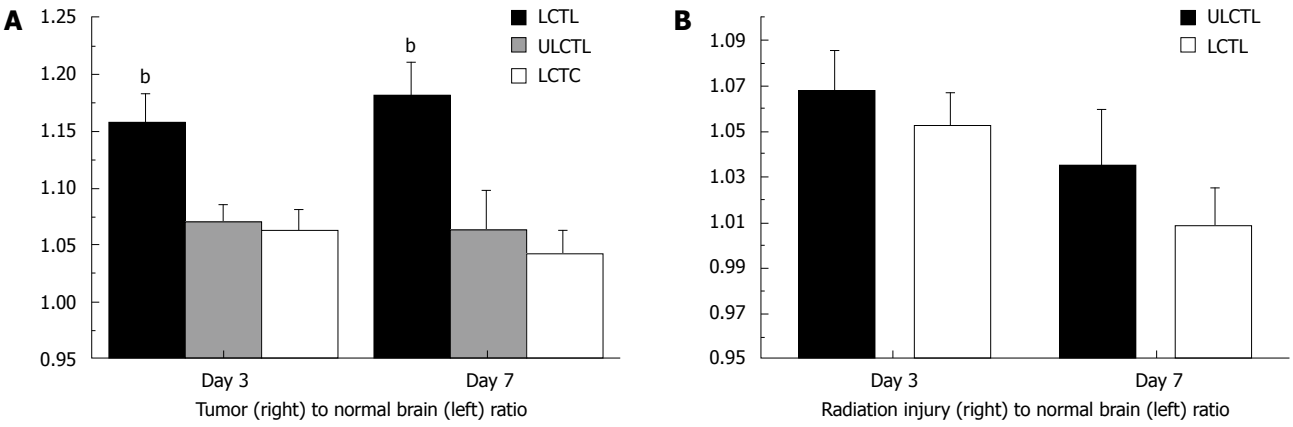

Figure 4 Accumulation of labeled cytotoxic T-cells in implanted U251 tumor and radiation injured sites in rat brain. A: Analyses of R2* values normalized to contralateral normal hemisphere (indirect indicator of the accumulation of iron positive cells) showed significantly higher ($^{b}P \leqslant 0.001$) accumulation of iron positive cells in tumor that received labeled cytotoxic T-cells (LCTL) compared to that of labeled control T-cells (LCTC) and unlabeled CTLs (ULCTL). The number of accumulated cells was higher at both day 3 and 7; B: Similar analyses of R2* values in radiation injured brain normalized to contralateral normal hemisphere showed no difference between the groups of animals that received labeled and unlabeled CTLs.

in the cell suspension. After 15 min of incubation in serum free media, an equal volume of complete media (containing serum) is added to the cell suspension and further incubated for 4 h.

Determination of specificity of labeled and unlabeled CTLs

To determine whether the produced CTLs have the specificity to target tumor cells *in vitro* and to determine whether FePro labeling alters this specificity, a specific number of CTLs (labeled or unlabeled) and control T-cells were co-incubated with target tumor cells. The interaction (accumulation of the added T-cells around tumor cells) of the added CTLs or T-cells with tumor cells was photomicrographed at 0 and 18 h of co-culture. Figure 3 shows the interaction of tumor cells and CTLs. We have not seen any changes in the specificity of CTLs following labeling with FePro for imaging purposes.

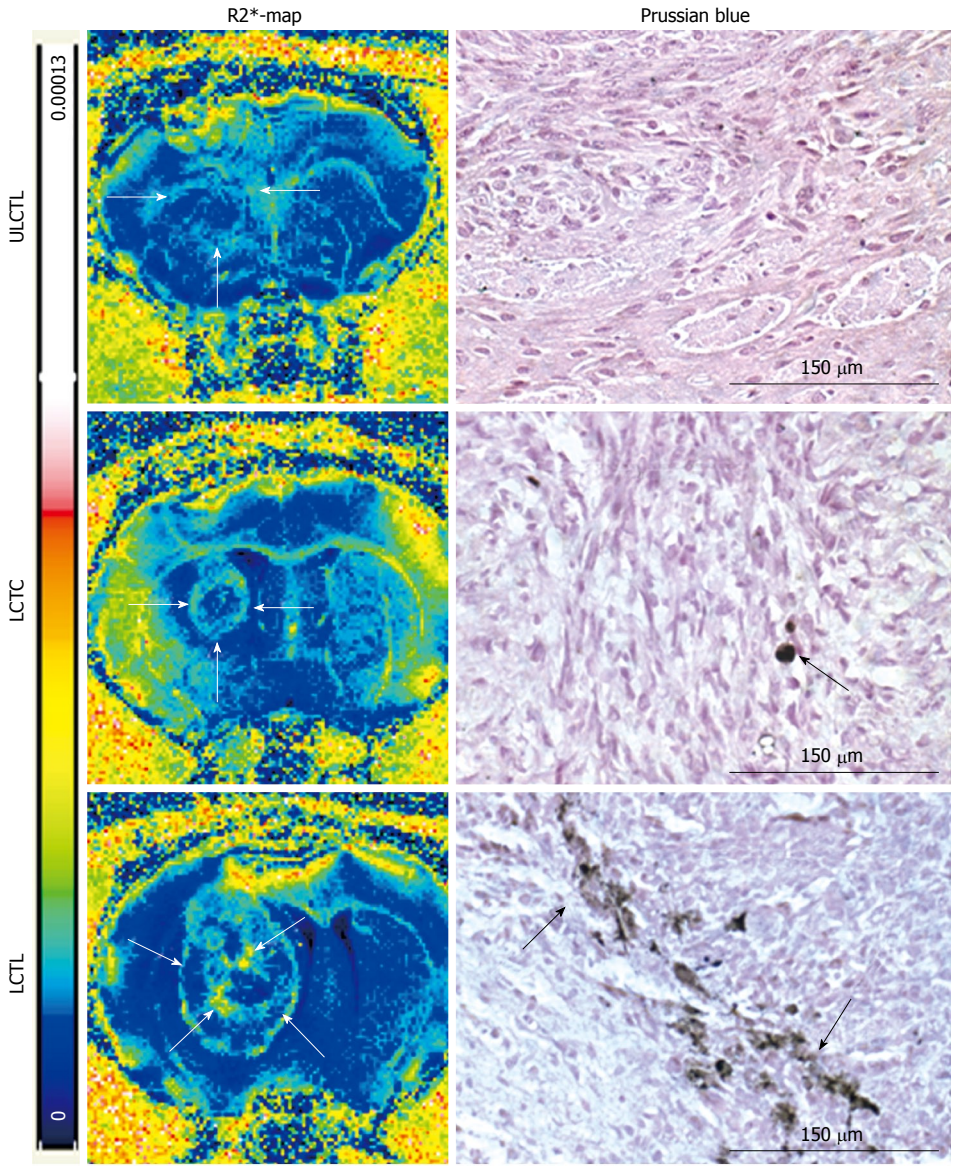

Figure 5 Magnetic resonance imaging relaxivity maps and Prussian blue staining. R2* maps and DAB enhanced Prussian blue staining from representative animals that received unlabeled cytotoxic T-cells (CTLs) (ULCTL, upper row), labeled control T-cells (LCTC, middle row) and labeled CTLs (LCTL, lower row). R2* maps show high signal intensity areas only in tumors that received LCTC and LCTL. Animals that received LCTL show high signal intensity areas both at the peripheral and central part of the tumors (arrows). Corresponding DAB enhanced Prussian blue staining show multiple Prussian blue positive cells in tumors that received LCTL (arrows). There are a few Prussian blue positive cells seen in tumors that received LCTC (arrow). No definite Prussian blue positive cells were seen in tumors that received ULCTL.

Applications of CTLs as imaging probes

Lymphocytes has been in use for decades to detect different disease conditions, such as xenografted tumor in rodents, renal allograft, autoimmune thyroid disease, metastatic melanoma, *etc*[60-63]. Autologous lymphocytes have been labeled with radioactive isotopes and accumulation at specific sites has been detected by nuclear medicine imaging[64,65]. Autologous lymphocyte labeling with radioactive isotopes, such as In-111-oxine and administration into patients are FDA approved procedures for diagnostic purposes. Chin *et al*[66] has reported the utilization of tumor infiltrating lymphocytes collected from resected tumor specimens and expanded *ex vivo* using recombinant IL-2 as imaging probes by labeling with In-111-oxine. However, the authors did not notice any accumulation of In-111 labeled lymphocytes at the sites of metastasis. Lymphocytes have also been labeled with iron oxides to track the migration by magnetic resonance imaging (MRI), however, there has been no report of making tumor specific CTLs *ex vivo* for the detection of tumors by *in vivo* imaging. Previously our group reported the making of sensitized splenocytes (CTLs) *in vivo* in syngeneic Fisher-344 rats by implanting 9L gliosarcoma cells. These CTLs were collected from spleen and used as imaging probes to detect the implanted tumor in another set of rats carrying the 9L glioma in the brain[55]. The *in vivo* produced CTLs showed specificity by accumulating in and around the implanted tumors, whereas splenocytes collected from control rats did not show significant accumulation in the implanted tumors. The CTLs (sensitized splenocytes) were also able

to differentiate implanted tumor from radiation necrosis as there was no accumulation at the sites of radiation injury (necrosis). Based on the results of sensitized splenocytes, we have started making CTLs in the *ex vivo* setting to sensitize T-cells against implanted U-251 glioma using glioma cell lysate-pulsed DCs. Our preliminary results are very encouraging (Figures 4 and 5) and can be translated to clinics after proper IND and FDA approval. In this study, we magnetically labeled *ex vivo* produced CTLs and injected them intravenously into rats bearing U251 glioma or radiation injury. Our main hypothesis was to prove that CTLs would specifically accumulate at the sites of glioma. We used MRI to detect the accumulated CTLs in the tumors. The results showed a significantly higher number of CTLs accumulated in U251 glioma and there was no significant accumulation of CLTs at the sites of radiation injury. The CLTs can be used to differentiate recurrent glioma from radiation necrosis. This study is underway.

CONCLUSION

An *ex vivo* technique at a GMP grade laboratory can be utilized to produce tumor specific CTLs and these CTLs can be used as cellular imaging probes to detect sites of recurrent or residual tumors. Personalized treatment is becoming a key word for current trends in cancer treatment. Because of polymorphism and the chance of mutation in tumor cells in an individual patient, our technique can be utilized to create cellular imaging probes to detect patient-specific recurrent or residual tumors and the treatment strategy can be changed based on the image findings.

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TOPIC HIGHLIGHT

Joanna Skommer, PhD, *Series Editor*

Molecular mechanism of base pairing infidelity during DNA duplication upon one-electron oxidation

Jóhannes Reynisson

Jóhannes Reynisson, Department of Chemistry and Auckland Bioengineering Institute, The University of Auckland, Auckland 1142, New Zealand
Author contributions: Reynisson J solely contributed to this paper.
Correspondence to: Dr. Jóhannes Reynisson, Department of Chemistry and Auckland Bioengineering Institute, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand. j.reynisson@auckland.ac.nz
Telephone: +64-9-3737599 Fax: +64-9-3737422
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Abstract

The guanine radical cation ($G^{\bullet+}$) is formed by one-electron oxidation from its parent guanine (G). $G^{\bullet+}$ is rapidly deprotonated in the aqueous phase resulting in the formation of the neutral guanine radical [$G(-H)^{\bullet}$]. The loss of proton occurs at the N1 nitrogen, which is involved in the classical Watson-Crick base pairing with cytosine (C). Employing the density functional theory (DFT), it has been observed that a new shifted base pairing configuration is formed between $G(-H)^{\bullet}$ and C constituting only two hydrogen bonds after deprotonation occurs. Using the DFT method, $G(-H)^{\bullet}$ was paired with thymine (T), adenine (A) and G revealing substantial binding energies comparable to those of classical G-C and A-T base pairs. Hence, $G(-H)^{\bullet}$ does not display any particular specificity for C compared to the other bases. Taking into account the long lifetime of the $G(-H)^{\bullet}$ radical in the DNA helix (5 s) and the rapid duplication rate of DNA during mitosis/meiosis (5-500 bases per s), $G(-H)^{\bullet}$ can pair promiscuously leading to errors in the duplication process. This scenario constitutes a new mechanism which explains how one-electron oxidation of the DNA double helix can lead to mutations.

Key words: Base pairing; Density functional theory; Deprotonation; DNA duplication; Duplication rate; Guanine neutral radical; Nucleotides; Oxidative DNA damage; Radical lifetime

Peer reviewer: Millie M Georgiadis, Associate Professor, Department of Biochemistry and Molecular Biology Indiana University School of Medicine, 635 Barnhill Dr. Indianapolis, IN 46202, United States

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INTRODUCTION

The aqueous redox chemistry of the nucleosides and nucleotides has been extensively investigated for the last 40 years using pulse radiolysis, laser photolysis, electron spin resonance and other time resolved and steady state techniques[1]. More recently, theoretical methods have been employed in the study of redox damage of DNA[1]. This intense interest in the components of DNA is understandable since it carries our genetic code and if damaged can lead to mutations possibly resulting in cancer[2,3]. Furthermore, oxidative damage of DNA is implicated in aging[4] and bacterial drug resistance[5]. It is now understood that DNA damage initiated by ionising radiation elicits a complicated set of events engaging various signalling pathways in cells[6].

Given that cumulative cancer risk increases with the fourth power of age and is associated with an accumulation of DNA damage, oxidative DNA damage is of great interest regarding early tumorigenesis and eventually cancer. These redox damage mechanisms have a potential role in the initiation, promotion and malignant conversion

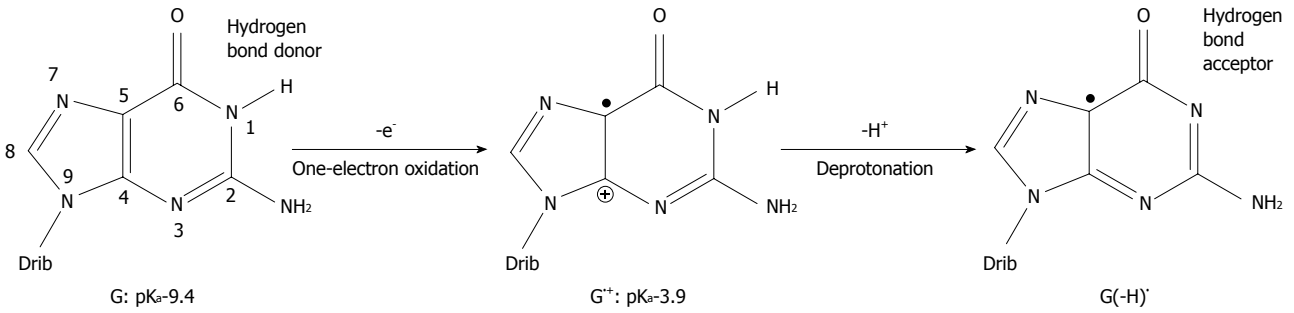

Figure 1 Oxidation of guanine (G) and deprotonation of its radical cation ($G^{\bullet+}$). The pK_a-value of G is drastically lowered upon one-electron oxidation and subsequent deprotonation of the N1 proton changes it from a hydrogen bond donor to a hydrogen bond acceptor. The number of atoms constituting G is shown. Drib: 2'-deoxyribose moiety.

stages of carcinogenesis[2]. Lesions such as 7,8-dihydro-8-oxoguanine (8OG) are established biomarkers of oxidative stress; coupled with their mutagenicity in mammalian cells, this has led to them being proposed as intermediate markers of cancer[2]. A more complete understanding of these oxidative damage processes in DNA is highly desirable in order to find new therapeutic strategies to battle this devastating disease.

BINDING SPECIFICITY ALTERATION OF THE GUANINE BASE

It has been found that when organic molecules are one-electron oxidized in the aqueous phase, a rapid deprotonation occurs from hydrogen bond donors undoubtedly driven by the massive solvation energy of the proton (ΔG_{aq} = -263.9 kcal/mol)[7-9]. As an example, the pK_a-value of cytosine (C) is lowered from 12.15 to between 2 and 4 when C is one-electron oxidized[8,10,11]. With respect to DNA, guanine (G) is its most easily oxidized component[12] and when the π-stack of double stranded DNA loses an electron, the positive charge migrates to G-C rich areas in the double strand[13-16] and the pK_a-value of G is lowered significantly from 9.4 to 3.9 at the nitrogen-1 atom (N1), as depicted in Figure 1[10,17,18]. After departure of the proton from the N1-site, it becomes a hydrogen bond acceptor instead of a hydrogen bond donor. The question has emerged as to whether this event leads to a change in the pairing ability of the G moiety with other bases[19]? In fact, it is a common view that ligand hydrophobicity improves affinity, whereas hydrogen bonding improves specificity for interactions in biochemical systems[20]. Simulating one-electron oxidation and the consequent deprotonation of the central N1-proton for G-C, using the density functional theory (DFT)[21], a new slipped conformation of the base pair was formed as depicted in Figure 2[19]. This slipped configuration, G(-H)•-C, was later independently derived by Bera *et al*[22] using a systematic search for all possible hydrogen bonding configurations between G(-H)• and C. The predicted base pairing energy (BPE) was -18.2 kcal/mol for G(-H)•-C[19,23]. This lies between the BPE's of the adenine-thymine base pair (A-T) at -13.0 kcal/mol and that of G-C at -21.0 kcal/mol[24,25].

DEPROTONATION OF OXIDIZED GUANINE IN DOUBLE STRANDED DNA

Under what circumstances can $G^{\bullet+}$-C in the DNA stack lose the central N1 proton making up one of the Watson-Crick hydrogen bonds? It does not have access to the aqueous phase since it is the central hydrogen bond and is flanked by base pairs on either side in the double stranded DNA helix. It is imperative that N1-H comes into contact with the water phase (water acting as a proton acceptor), i.e. within G-C, the G(N1-H)-C(N3) Watson-Crick hydrogen bond has to be broken for the N1 proton to be lost (Figure 2). The hydrogen bonds between the base pairs may be broken in three situations: First, the "swing-out" of the bases by concerted thermal motions of the DNA strand[26,27]. This mechanism is unlikely since it takes place on the milli- to micro-second time scale and is in competition with further charge migration in the DNA helix and/or with water addition to C8 of $G^{\bullet+}$, which are considerably faster. The rate of charge migration is estimated as 5×10^7/s and 6×10^4/s for the water addition, i.e. in the micro-nanosecond timescale[16,28]. Furthermore, the BPE of $G^{\bullet+}$-C is increased to -40.9 kcal/mol compared to -21.0 kcal/mol of its parent pair, inhibiting the frequency of the breathing motions of the base pair[24,29,30]. Second, when duplication of DNA occurs, the DNA strand is untwisted and the hydrogen bonds between the bases are broken to allow duplication of the strand. Third, during DNA transcription to messenger-RNA, it proceeds in a similar fashion to the duplication of DNA. In addition, it has been suggested that deprotonation occurs from the exocyclic amine group of C in $G^{\bullet+}$-C based on pulse-radiolysis and kinetic isotope experiments[31-33]. The proposed deprotonation mechanism is shown in Figure 3. This reaction cascade can lead to the G(-H)•-C slipped configuration[34].

PAIRING INFIDELITY OF THE DEPROTONATED GUANINE RADICAL

A related question has emerged as to whether it is possible to pair T, A and G itself to G(-H)•? This was investigated using the DFT method and the results are given in Figure 4[19].

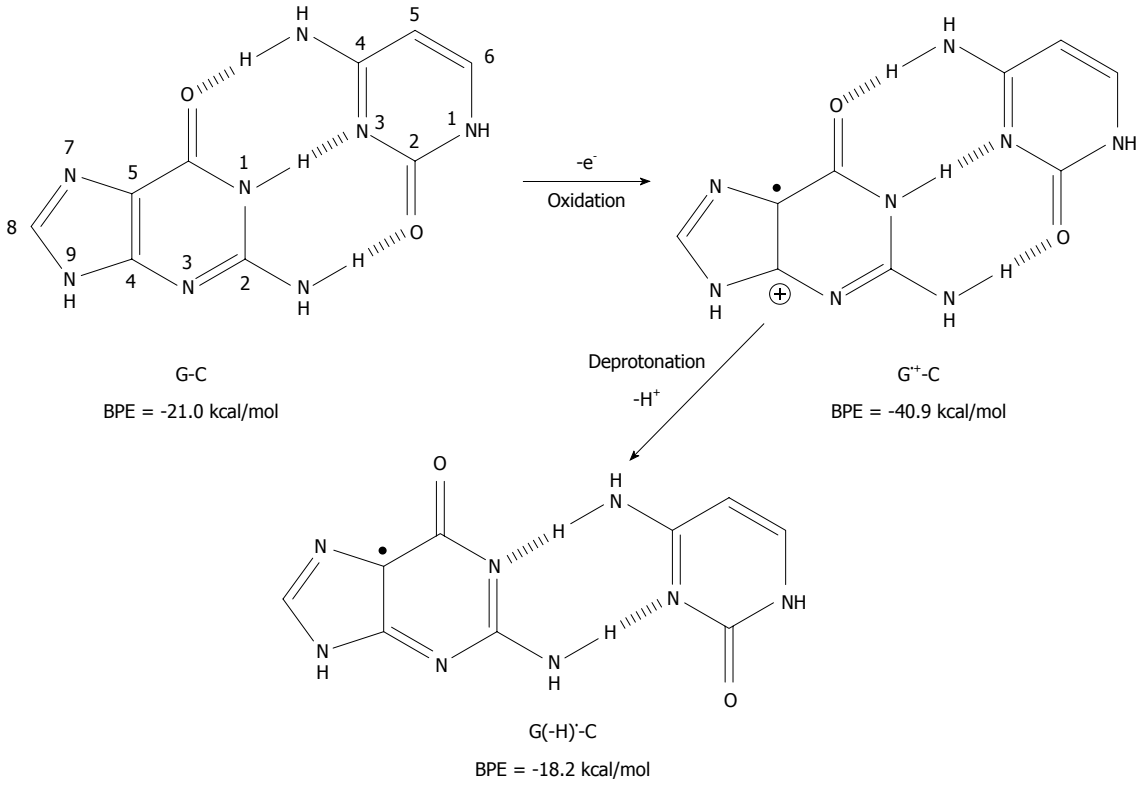

Figure 2 Deprotonation-induced structural change of the G-C base pair initiated by one-electron oxidation leading to the shifted base pair G(-H)• - C. BPE: Base pairing energy.

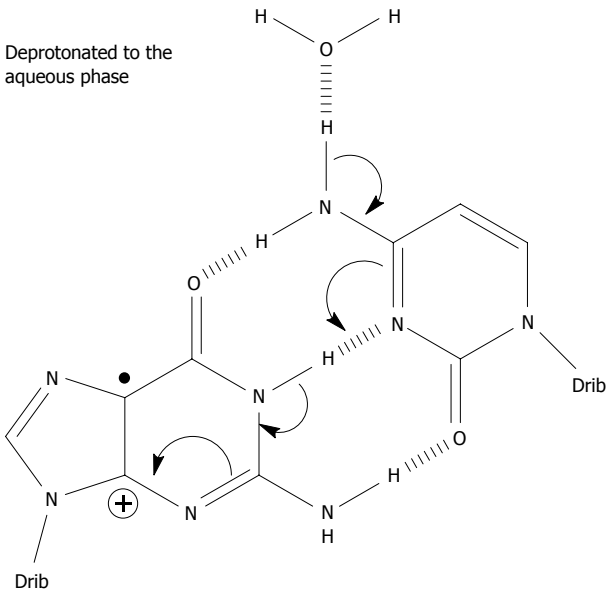

Figure 3 A possible mechanism which involves the exocyclic amine moiety on C as the proton donor of the one-electron oxidized base pair in which the initial charge sits on G, i.e. in the complementary strand. Spin-charge separation between G and C plays a crucial role in the reaction cascade. The depicted deprotonation can lead to the formation of G(-H)•-C[34].

Armed with the knowledge that the G(-H)•-C base pair has only two hydrogen bonds, G(-H)• was paired to T and structurally optimized. The BPE was calculated to be -10.4 kcal/mol for G(-H)•-T, which is comparable to the A-T base pairing energy (-13.0 kcal/mol[24 25,29,35]). The relatively low energy can be explained in terms of the non-planarity of the bases with respect to each other. On the basis of the calculations, they are roughly 25° out of plane, measured at their carbonyl groups, O^6 (G) and O^4 (T). The distance between these oxygen atoms is 3.5 Å, which proximity leads to Coulombic repulsion and hence the non-planar conformation.

The calculated hydrogen bonding energy of the G(-H)•-A base pair is -13.6 kcal/mol, as shown in Figure 4. This binding is somewhat stronger than that for the natural A-T pairing (-13.0 kcal/mol)[24,25,29,35].

The hydrogen bond energy of G(-H)•-G (structure depicted in Figure 4) is similar to that of G-C[36]. This is not surprising as three hydrogen bonds are formed in both structures. A second type of G-G base pair is conceivable between two G(-H)• moieties (G(-H)•-G(-H)•) as shown in Figure 4. For this, the hydrogen bond energy is -18.5 kcal/mol, somewhat lower than for G(-H)•-G, since it has one less hydrogen bond. The Pt(Ⅱ) electrophile coordinates at N7 of G. This acidifies the N1 proton, similar to the oxidation of G. With these Pt-G species, structures similar to G(-H)•-G and G(-H)•-G(-H)• were observed with ^{1}H-NMR and X-ray crystallography[37], which provides experimental evidence of their existence.

ONE-ELECTRON OXIDATION DURING DNA DUPLICATION

Using *in-situ* photolysis electron paramagnetic resonance

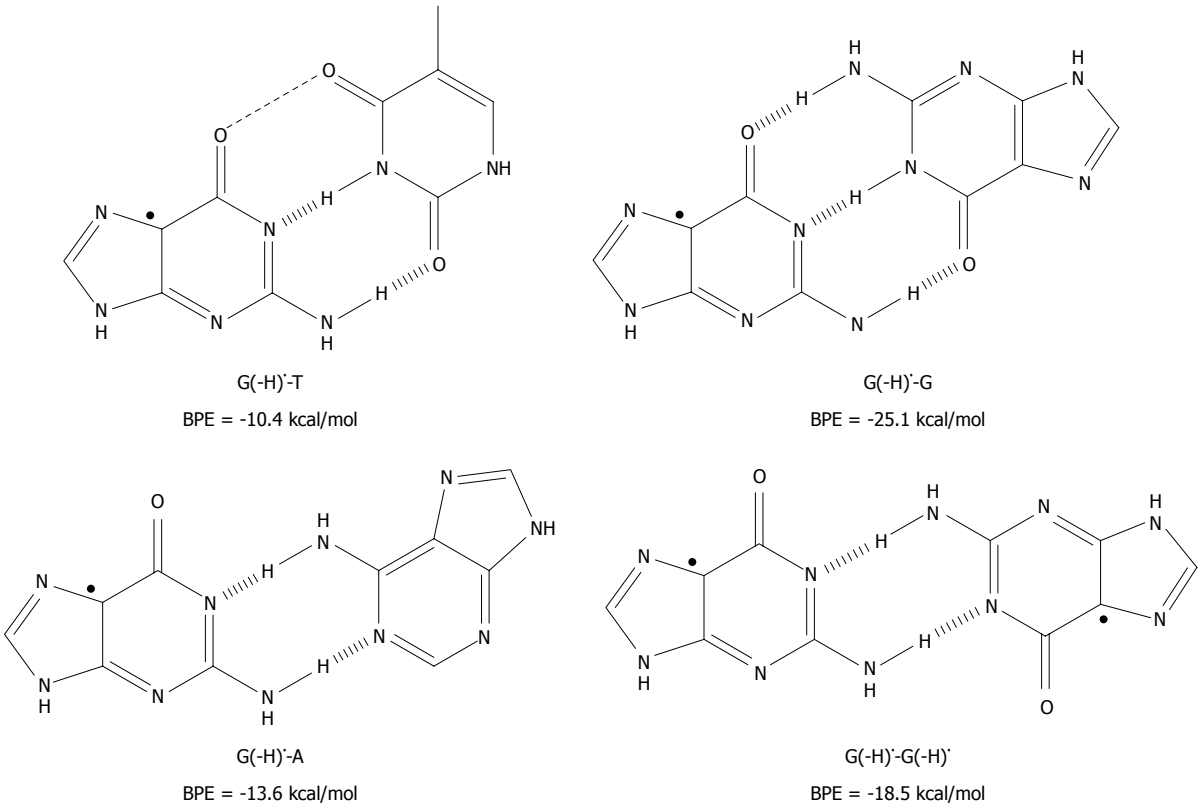

Figure 4 The unnatural base pairs between G(-H)• and the other bases[19]. The substantial base pairing energy (BPE) for the non-classical complexes depicted leads to the conclusion that G(-H)• does not have any specificity for C.

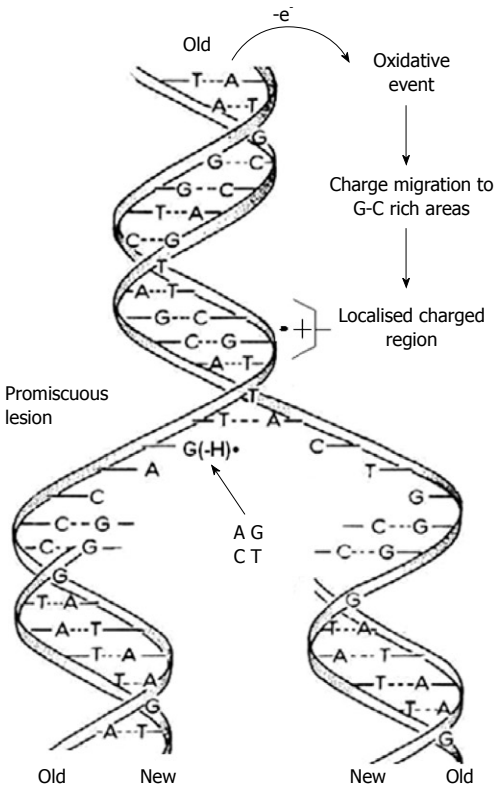

Figure 5 As the two strands of the double helix unwind, each pairs up with the appropriate bases to form a new double helix. The two new helices are identical to each other and to the original. This process is compromised by one-electron oxidation of the π-DNA stack, deprotonation from G•+ and the subsequent formation of G(-H)•, which is promiscuous with regard to base pairing.

(EPR), Hildenbrand and Schulte-Frohlinde, detected a long-lived radical (lifetime 5 s) which was produced only from double stranded DNA when ionised with < 220 nm light in an aqueous solution at pH 7[38]. This radical was assigned to G(-H)•. The rate of DNA duplication was measured to be between 5-500 nucleotides/s depending on the cell type, species and other factors[39,40]. Considering the long lifetime of G(-H)• in double stranded DNA and the rapid DNA duplication rate, it emerges that in the case of one-electron oxidation during mitosis-/-meiosis, G(-H)• is formed when the two strands unwind. As shown in Figure 4, it can form base pairs with all of the nucleotides with binding energies similar to the classical A-T and G-C Watson-Crick base pairs. This means that G(-H)• does not have specific affinity for C, i.e. it is completely promiscuous when it comes to base pairing. Therefore G(-H)• can pair with all of the nucleotides leading to mispairing. A depiction of this scenario is presented graphically in Figure 5.

The mechanism presented here is new and an alternative to the scenario that mispairing of DNA bases is mostly caused by oxidative end products such as 8OG[41]. These products are closed shell, i.e. they are not radical species and therefore, have a much longer lifetime than G(-H)•. 8OG is one of the many redox products which is derived from the oxidation, and the subsequent water addition, of G[17,18,42]. It can form *syn-anti* base pairs[43], with all of the nucleotides and these have base pairing energies of -10 kcal/mol[44]. The 8OG-T base pair is depicted in Figure 6 as an example of *syn-anti* base pairs.

So far, the role of DNA polymerase has not been

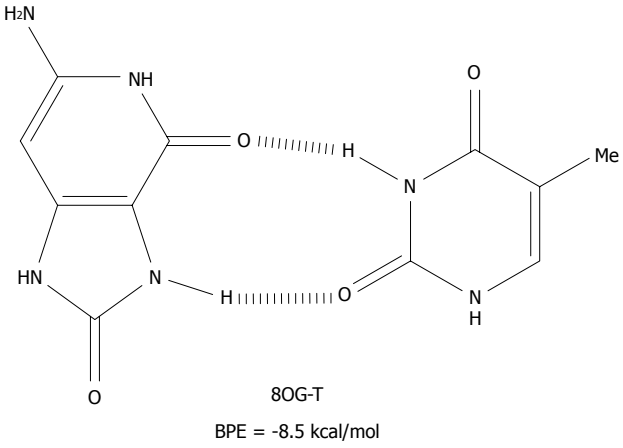

Figure 6 The *syn-anti* base pair of 8-oxoguanine-T. BPE: Base pairing energy; 8OG: 8-oxoguanine.

considered and the DNA bases and base pairs have been treated as *in vacuo* as a model. The structure of DNA polymerase and its steric limitations within the active site are well documented[45-47]. The structure of the binding site in the replicating enzymes will undoubtedly have an effect on the proposed infidelity mechanism based on G(-H)$^{\bullet}$, e.g. the rate of duplication.

CONCLUSION

In this review, an alternative mechanism for promiscuous base pairing during DNA duplication, initiated by one-electron oxidation, is proposed based on theoretical calculations. Some experimental results exist which support the existence of the non-classical base pairs discussed, i.e. the slipped G(-H)$^{\bullet}$-C and the G(-H)$^{\bullet}$-G base pairs. Further experimental and theoretical work is needed to corroborate the mechanism proposed. In particular, experiments conducted with time resolved resonance Raman spectroscopy on model DNA duplication systems are pertinent as well as modelling studies on the effect of DNA polymerase.

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TOPIC HIGHLIGHT

Joanna Skommer, PhD, *Series Editor*

Microfluidics: Emerging prospects for anti-cancer drug screening

Donald Wlodkowic, Zbigniew Darzynkiewicz

Donald Wlodkowic, Auckland Microfabrication Facility, Department of Chemistry, University of Auckland, 1142 Auckland, New Zealand
Zbigniew Darzynkiewicz, Brander Cancer Research Institute, Department of Pathology, New York Medical College, Valhalla, NY 10595, United States
Author contributions: Wlodkowic D and Darzynkiewicz Z contributed to the design and wrote the manuscript.
Correspondence to: Dr. Donald Wlodkowic, MSc, PhD, Assistant Professor, Auckland Microfabrication Facility, Department of Chemistry, University of Auckland, 23 Symonds Street, 1142 Auckland, New Zealand. d.wlodkowic@auckland.ac.nz
Telephone: +64-9-3737599 Fax: +64-9-3737422
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Abstract

Cancer constitutes a heterogenic cellular system with a high level of spatio-temporal complexity. Recent discoveries by systems biologists have provided emerging evidence that cellular responses to anti-cancer modalities are stochastic in nature. To uncover the intricacies of cell-to-cell variability and its relevance to cancer therapy, new analytical screening technologies are needed. The last decade has brought forth spectacular innovations in the field of cytometry and single cell cytomics, opening new avenues for systems oncology and high-throughput real-time drug screening routines. The up-and-coming microfluidic Lab-on-a-Chip (LOC) technology and micro-total analysis systems (μTAS) are arguably the most promising platforms to address the inherent complexity of cellular systems with massive experimental parallelization and 4D analysis on a single cell level. The vast miniaturization of LOC systems and multiplexing enables innovative strategies to reduce drug screening expenditures while increasing throughput and content of information from a given sample. Small cell numbers and operational reagent volumes are sufficient for microfluidic analyzers and, as such, they enable next generation high-throughput and high-content screening of anti-cancer drugs on patient-derived specimens. Herein we highlight the selected advancements in this emerging field of bioengineering, and provide a snapshot of developments with relevance to anti-cancer drug screening routines.

Key words: Microfluidics; Lab-on-a-chip; Cytometry; Cytomics; Cancer; Anti-cancer drugs; Cancer therapy; Drug screening

Peer reviewers: Des R Richardson, BSc, MSc, PhD, DSc, Professor of Cancer Cell Biology, NHMRC Senior Principal Research Fellow, Director, Iron Metabolism and Chelation Program, Department of Pathology, University of Sydney, Sydney, NSW 2006, Australia; E YK Ng, PhD, PGDTHE, Associate Professor, School of Mechanical and Aerospace Engineering, College of Engineering, Nanyang Technological University, 50, Nanyang Avenue, Singapore 639798, Singapore; Shufeng Zhou, MD, PhD, A/Professor, School of Health Sciences, RMIT University, Bundoora, Victoria 3083, Australia

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INTRODUCTION

Validation of potential therapeutic targets in cancer requires the introduction of functional live cell assays that provide both spatial and temporal inter-relationships in signaling networks[1,2]. Many cell signaling pathways are initiated and executed through multiple interconnected signaling cascades that differ in both space and time with-

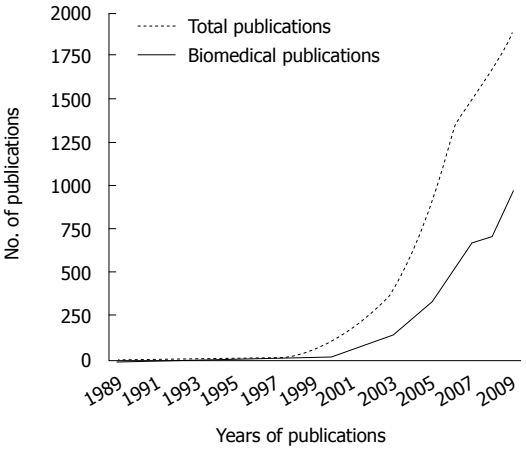

Figure 1 Explosive growth of the microfluidic Lab-on-a-Chip technologies. Note that the last decade brought a dramatic increase in the development of innovative microfabricated technologies aimed at studies of cells and biomolecules. These technologies attract a growing interest within the biomedical community, reflected by a logarithmic growth of publications on the subject. The Scopus database containing citations, abstracts and references covering 16 000 peer-reviewed titles from 4000 publishers was used to perform a bibliographical analysis spanning the period between 1989 and 2009. Study was based on the key word "microfluidic" and included two sets of variables: (dashed line) Total publications (database search from "Biology, Agricultural and Environmental Sciences, Chemistry, Physics, Mathematics and Engineering; Life and Health Sciences" collections), (solid line) Biomedical publications (database search from "Biology and Life and Health Sciences" collections). Analysis included only Articles and Reviews and excluded "In press" articles.

in the same cell population encountering the stimulant or drug[3,4]. Modulation of different signaling pathways can lead to additive, synergistic or antagonistic drug actions. Such levels of complexity, with multiple variables acting at the same time, requires an in-depth investigation of cell populations in real-time at a single cell level[1,5,6]. In this context, functional cytomics is slowly becoming an omnipotent part of the post-genomic drug discovery pipeline[7,8]. Although, it is widely recognized that the validation of therapeutic targets revealed by proteomic and genetic screens requires 4D (3D space plus time) functional cell-based assays, their widespread applications are still underdeveloped[1,9,10]. High-content analysis (HCA) is one of the key platforms that recently improved drug screening routines by collecting content-rich data sets[3]. Surprisingly, however, the commonly used HCA approaches are still based on a static principle, yielding information on cell status at a single time point[1,9,10]. Capabilities of high-speed, multiparameter and real-time analysis of great numbers of isolated cells are as yet profoundly limited[1,9,10]. It is still challenging to record, in high-throughput, time-resolved data on a multitude of diverging cellular outputs.

On the other hand, the cost and time savings play an ever increasing role in industrial perspective drug screening routines[3,11]. Not surprisingly, enabling strategies that reduce expenditures, while increasing throughput and content of information from a given sample, attract mounting interest within the biopharmaceutical community. The last decade has brought many innovations to the field of cytomics and cytometry[10]. Probably the most fascinating are the prospects and explosive development of innovative micro- and nanofluidic Lab-on-a-Chip (LOC) technologies (Figure 1)[10-15]. Transfer of traditional bioanalytical methods to a microfabricated format provides the means to increase both the resolution of analysis and sampling throughput while reducing the cost of a single assay[10-15]. By providing an alternative to expensive instrumentation, such as flow or laser scanning cytometers and sorters, which are unaffordable for small research or clinical laboratories, these miniaturized tools of cytometry can be used more widely and also can be available in underprivileged countries.

EMERGING PROSPECT OF MICROFLUIDICS FOR CANCER RESEARCH

Microfluidics is a new arena of bioengineering aimed at manipulating liquids and particles in ultralow volumes in small channels that have a cross-sectional area less than a square millimeter (mm^2)[12,16,17]. The dimensionless parameter, called the Reynolds number (R_e), describes unique physical principles of the fluid in microchannels as a function of the channel geometry, fluid viscosity and flow rate (Figure 2)[16,17]. As described by the R_e, fluid flow in microfluidic channels is laminar and dominated by viscous forces (Figure 2). Importantly, in fluids under laminar flow, all fluid particles move in parallel to the flow direction in contrast to the 3D movement of particles in macroscale conditions (Figure 2)[16,17]. Under laminar conditions, the fluid flow has no inertia, enabling the precise dosing of drugs, both spatially and temporally[16,17]. Moreover, during laminar flow, the solute transport is dominated by a limited and local diffusion (Figure 2)[16,17]. As such, it can be effectively used for spatio-temporal stimulation of cells and drug delivery to restricted subcellular compartments (Figure 2)[18,19].

The enclosed and sterile formats of microfluidic LOC devices eliminate evaporative water loss from microsized channels[20]. Biocompatible and inexpensive polymers, such as polydimethylsiloxane (PDMS), are often materials of choice for the fabrication of disposable cell-based microfluidic devices[20,21]. These innovative polymers prevent cross-contamination of biological specimens that are subjected to parallel stimulation with a number of different drugs[20,21]. Innovative biopolymers used for the fabrication of microfluidic devices also provide secure biocontainment of infectious specimens, such as viral gene vectors or HIV^+ and blood samples[20,21]. When desirable, current microfabrication techniques also allow the creation of microfluidic circuitry in glass and quartz, which enhances the durability and application range in high-pressure applications.

Undoubtedly, the advent of microfluidics, and its integration into the design of micro-total analysis systems (μTAS), is leading to one of the most adventurous avenues to address the inherent complexity of cellular systems, with unprecedented experimental high-throughput at the single cell level (Figure 3)[10-17]. While the application of laminar fluid flow under low Reynolds numbers pro-

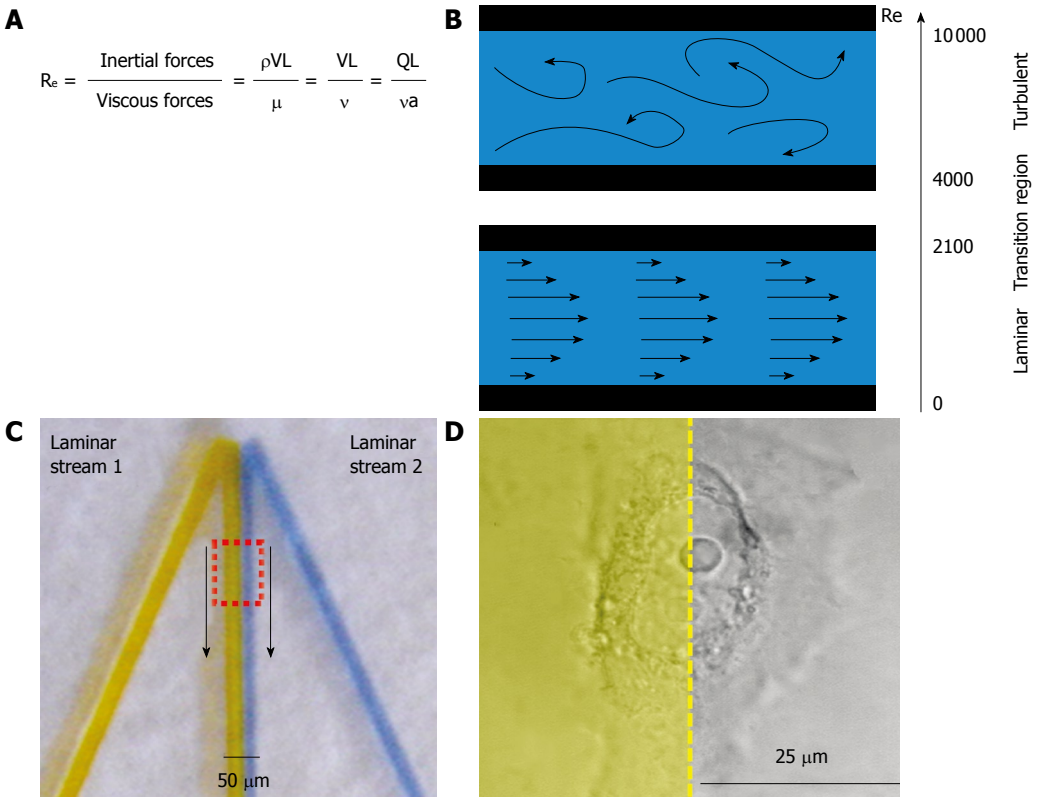

Figure 2 Principles of microfluidics. A: Microfluidics is aimed at manipulating liquids at ultralow volumes. The dimensionless parameter, the Reynolds number (R_e), describes unique physical principles of the fluid in channels within a cross-sectional area as a function of the channel geometry, fluid viscosity and flow rate. R_e is the measure of the ratio between the inertial to viscous forces where: ρ: density of the fluid (kg/m^3); V: Mean fluid velocity (m/s); L: Length of the channel (m); μ: Dynamic fluid viscosity (Pa•s); ν: Kinematic fluid viscosity (m^2/s); Q: Volumetric flow rate (m^3/s); a: Cross-sectional area of the channel (m^2); B: As described by the R_e, fluid flow in microfluidic channels is dominated by viscous rather than inertial forces. Laminar flow describes the conditions where all fluid particles move in parallel to the flow direction. Laminar flow is therefore represented by R_e values below 2100. In contrast, turbulent flow is characterized by movement of fluid particles in all three dimensions that do not correlate with the overall direction of the fluid flow. Turbulent flow is thus represented by larger R_e values (above 4000). R_e values between 2100 and 4000 describe the transition region where fluid flow may have the features of both laminar and turbulent flow; C: Laminar flow under low R_e can be effectively used for spatiotemporal stimulation of cells. Note that during fluid flow under low Reynolds numbers solute transport is dominated only by limited and local diffusion. Cell positioning is marked in red; D: An example of drug delivery to selected cell compartments using laminar flow streams. Phase-contrast image of HeLa cells stimulated using laminar flow under low Reynolds numbers. The yellow area (laminar stream 1) denotes the restricted cell compartment to which the drug is being delivered using laminar flow (as shown in panel C).

vides an attractive analytical avenue for the rapid delivery and exchange of reagents with exceptional accuracy, the transfer of traditional methods to a microfabricated format offers a means to increase both the resolution of analysis and sampling throughput while reducing the cost of a single assay (Figure 3)[10-17]. The disposable format of many LOC devices is particularly suitable for point-of-care diagnostics and future personalized therapy[22-25]. LOC devices also promise greatly reduced costs, increased sensitivity and ultra high throughput by implementing parallel sample processing and a vast miniaturization of integrated on-chip components (Figure 3)[14,26].

A number of emerging, microfluidic technologies for cell-based assays have recently been reported[13,14]. For instance, microfluidics offers an exceptional evolutionary route for flow cytometry, a technique known as microflow cytometry (μFCM)[27-32]. Micro fluorescently activated cell sorting (μFACS) and in-flow magnetically activated cell sorting (μMACS) are other rapidly up-and-coming examples of high-throughput on-chip cytometric technologies with substantial potential in anti-cancer drug discovery and personalized diagnostics[32-36]. Microfluidic flow cytometers and cell sorters require a greatly reduced number of cells per sample when compared with conventional FACS[27-36].

Spectral impendence using the Coulter principle has also been adapted for on chip devices to study the function of cell size, cytoplasmic resistance and membrane capacitance[37-39]. Precise differential white blood cell counts have already been demonstrated using this approach[40,41]. Recent reports suggest, however, that more high-throughput data can be obtained using in-flow dielectric spectroscopy on chips[42,43]. In this regard, innovative high throughput screening (HTS) technologies that are developed in a miniaturized format include capacitance and impedance cytometry[43-45]. Moreover, a number of unconventional technologies have recently been proposed for a non-invasive and real-time cell analysis on microfluidic chips. These include real-time studies on a single cell level, such as time-of-flight (TOF) optophoresis and scanning thermal lens microscopy (TLM)[46-50].

The living cell microarrays and microfluidic cell arrays are yet other examples of emerging LOC technologies that provide important technological advances in the

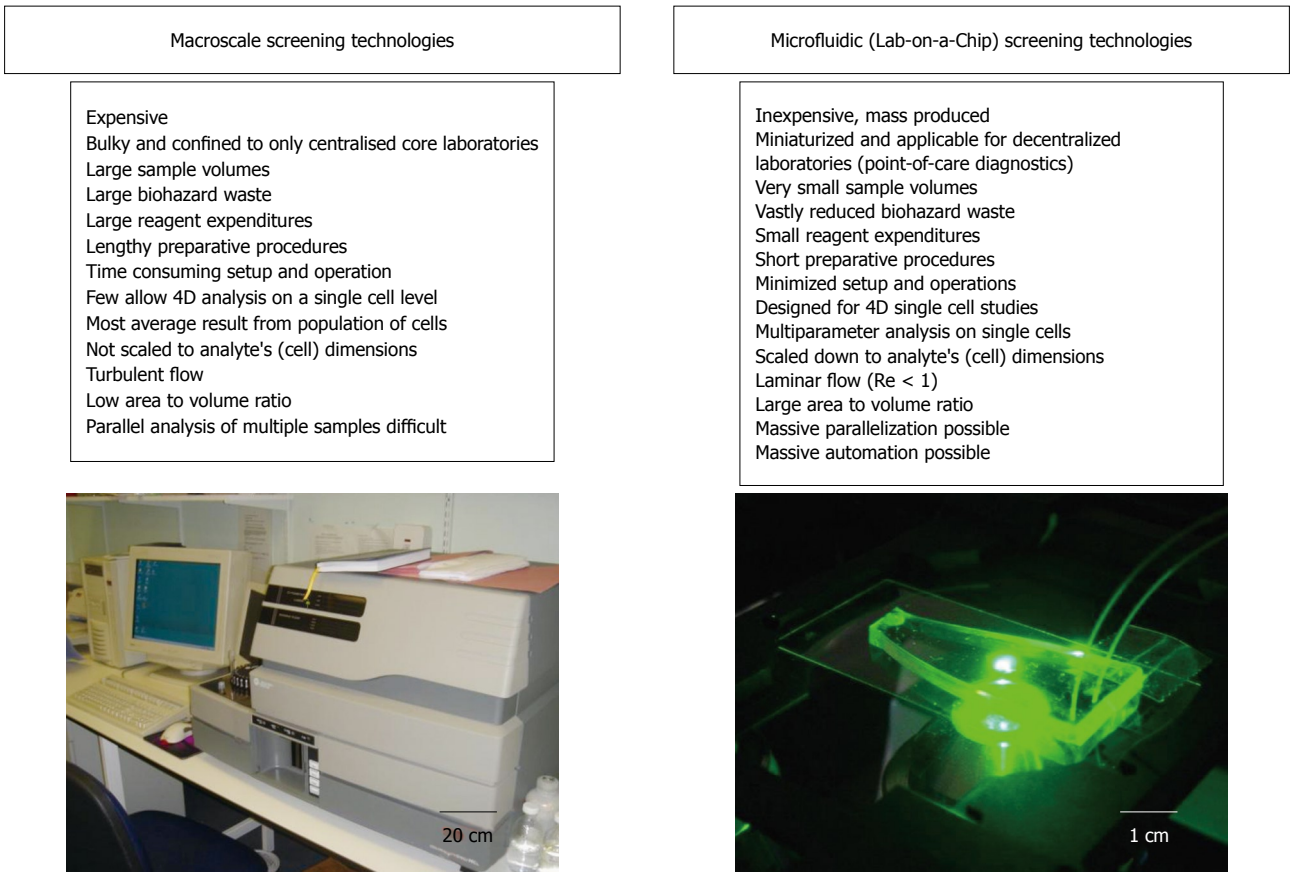

Figure 3 Comparison between conventional (macroscale) and microfluidic (Lab-on-a-Chip) technologies for cell-based assays. The advent of microfluidics and its integration into design micro-total analysis systems (μTAS) and Lab-on-a-Chip (LOC) devices is one of the most promising avenues to address the inherent complexity of cellular systems with massive experimental parallelization and analysis on a single cell level. LOC technologies promise greatly reduced equipment costs, simplified operation, increased sensitivity and throughput by implementing parallel processing principles and a vast miniaturized of on-chip components. Only low cell numbers and operational reagent volumes are required for LOC technology. It, in turn, opens up new prospects for high-throughput and high-content screening of anti-cancer drugs on patient derived specimens.

spatiotemporal control of biomolecules and cells[6,10,51-56]. Pioneering microfluidic cell arrays allow collection of real-time and multiparameter data obtained from functional cell-based assays[6,10,56]. These emerging technologies create living-cell arrays that are ideal for modeling cancer microenvironments and inherently scalable for constructing a high-throughput screening platform[52-55]. Its particular advantage lies in the ability to enable the kinetic and multivariate analysis of signaling events on a single cell level[6,10,55,56]. Cell microarray technology seems, thus, to be particularly suitable for uncovering intricacies in cell-to-cell variability and its relevance to cancer therapy. Recent studies in systems biology have recently shed new light on the underlying molecular mechanisms of cell-to-cell variability in cancer cell decision making[57,58]. To uncover the stochastic basis of cellular decision making, each cell has to be isolated from others to minimize the influence of extrinsic factors, such as cell-to-cell contacts and paracrine signaling. LOC systems provide innovative ways to simultaneously analyze large populations of cells whereby the position of every cell is encoded and spatially maintained over extended periods of time[6,54,56]. In this regard, microfluidic platforms that can track single cell responses on a large scale are, thus far, the only tool that can support the integrative mathematical oncology with systems biology efforts to yield new vistas for a generation of rationally designed anti-cancer drugs. Our recent studies have validated the application of live-cell microarrays for the kinetic analysis of investigational anti-cancer agents in hematopoietic cancer cells and hematopoietic cancer stem cells[6,56].

CONCLUSION

Understanding cell-to-cell variability in cancer is fundamental to the development of successful therapeutic regimens[57-59]. In this context, systems biology, cytomics and integrative mathematical oncology are new research arenas that can explain and simulate the cell-to-cell variability in cancer cell responses[57-59]. The experimental confirmation of mathematical models is difficult, however, mainly due to the inherent heterogeneity and complexity of cellular systems. Limitations of conventional cell-based techniques, such as flow cytometry and single cell imaging, make high-throughput dynamic analysis on cellular and subcellular processes tedious and exceedingly expensive[10]. Moreover, conventional assays do not incorporate physiological processes that are normally encountered by cells/tissues

in the human body, such as microperfusion, gas/drug diffusion rates and shear stress[12-15]. These design limitations of macroscale analytical systems have led to a biased understanding of many transient and intermittent physiological processes. This is often reflected by the failure of many therapeutic leads, selected after *in vitro* screening, to perform *in vivo* in animal models[60]. The microfluidic platforms that can track single cell responses, multiparametrically on a large scale are, so far, the only tools that can support mathematical oncology and systems biology efforts and provide new vistas for a new generation of rationally designed anti-cancer drugs. LOC systems provide innovative ways to simultaneously analyze large populations of cells whereby the position of every cell is encoded and spatially maintained over extended periods of time. The microfluidic environment closely mimics the physiological microenvironment, including gas and drug diffusion rates, shear stress and cell confinement. In the context of tumor biology, for example, and anti-cancer drug discovery, microfluidic technologies warrant a "quantum leap" for drug discovery and personalized diagnostics.

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TOPIC HIGHLIGHT

Joanna Skommer, PhD, *Series Editor*

How can we kill cancer cells: Insights from the computational models of apoptosis

Subhadip Raychaudhuri

Subhadip Raychaudhuri, Department of Biomedical Engineering, Biophysics Graduate Group, Graduate Group in Immunology, and Graduate Group in Applied Mathematics, University of California, Davis, CA 95616, United States
Author contributions: Raychaudhuri S wrote the manuscript.
Correspondence to: Subhadip Raychaudhuri, PhD, Department of Biomedical Engineering, Biophysics Graduate Group, Graduate Group in Immunology, and Graduate Group in Applied Mathematics, University of California, Davis, CA 95616, United States. raychaudhuri@ucdavis.edu
Telephone: +1-530-7546716 Fax: +1-530-7545739
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Abstract

Cancer cells are widely known to be protected from apoptosis, a phenomenon that is a major hurdle to successful anticancer therapy. Over-expression of several anti-apoptotic proteins, or mutations in pro-apoptotic factors, has been recognized to confer such resistance. Development of new experimental strategies, such as *in silico* modeling of biological pathways, can increase our understanding of how abnormal regulation of apoptotic pathway in cancer cells can lead to tumour chemoresistance. Monte Carlo simulations are in particular well suited to study inherent variability, such as spatial heterogeneity and cell-to-cell variations in signaling reactions. Using this approach, often in combination with experimental validation of the computational model, we observed that large cell-to-cell variability could explain the kinetics of apoptosis, which depends on the type of pathway and the strength of stress stimuli. Most importantly, Monte Carlo simulations of apoptotic signaling provides unexpected insights into the mechanisms of fractional cell killing induced by apoptosis-inducing agents, showing that not only variation in protein levels, but also inherent stochastic variability in signaling reactions, can lead to survival of a fraction of treated cancer cells.

Key words: Apoptosis; Cell death; Cancer; Computational modeling; Monte Carlo simulations

Peer reviewers: George G Chen, MD, PhD, Professor, Department of Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong, China; Guido Cavaletti, MD, Associate Professor, Department Neuroscienze e Tecnologie Biomediche - Università di Milano "Bicocca", v. Cadore 48 - 20052 Monza, Italy

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INTRODUCTION

Induction of apoptosis by chemotherapeutics is considered as one of the major anti-cancer effects leading to inhibition of tumour growth. Over the past years our understanding of signaling pathways associated with induction of apoptosis, and knowledge on executioners of apoptosis, has substantially increased. Recently, cell-to-cell stochastic variability has become central to apoptotic cell death signaling[1-6]. Computational models are well suited to provide mechanistic insight into the system level regulations of apoptosis signaling and its large cellular variability. Studies that are possible using *in silico* approaches might be inaccessible by other techniques. Recent novel findings in the area of apoptotic cell death signaling can have far-reaching implications in cancer biology and therapy. Our computational efforts, in synergy with parallel biological experiments, attempt to explore some of the fundamental issues in cancer biology within this new paradigm of apoptosis signaling.

DEVELOPING COMPUTATIONAL MODELS OF CELL DEATH SIGNALING FOR NORMAL AND CANCER CELLS

Monte Carlo models are generally suitable for simulating inherent stochasticity of signaling reactions in complex signaling pathways[1,7]. Our recent work has elucidated that cell-to-cell stochastic variability is a fundamental characteristic of apoptosis signaling and a significant part of such variability can arise due to inherent stochastic fluctuations of chemical reactions[1,6,8,9]. Even when the intrinsic stochastic variability is not dominant, Monte Carlo models have the advantage over ordinary differential equation (ODE) based models, as they can simulate spatial heterogeneity in an explicit manner. Examples of such spatial localizations in apoptosis signaling include translocation of activated Bax molecules onto mitochondrial outer membrane, release of cytochrome *c* from mitochondria to cytosol and redistribution of Apaf-1 in the cytosol, all of which depend on the cell type and the level of Bcl-2 proteins[10,11]. In addition, we could easily incorporate realistic variations in (1) protein concentrations that may arise from stochastic gene regulations[3,12-15]; and (2) reaction rate constants, for example, due to variations in pH in the cytosolic environment. In our initial studies we grouped functionally redundant proteins so that a single representative protein simulates all proteins of similar function that are possibly expressed within a given cell type. For example, apoptotic inhibitor Bcl-2 captured the effect of all the inhibitory Bcl-2 like proteins[1,9]. In the future we plan to simulate a more cell-type-specific signaling network of apoptosis, the results of which can be readily compared with data obtained from parallel biological experiments for specific cell types. Such an expanded signaling network will often involve signaling species with low concentrations at initial time, or dynamically generate a few molecules due to specific inhibitory reactions, leading to inherent stochastic fluctuations that can be best studied using stochastic approaches.

DIFFERENTIAL SIGNALING THROUGH EXTRINSIC AND INTRINSIC PATHWAYS OF APOPTOSIS

Apoptosis is regulated through two distinct signaling pathways that are joined in a global loop structure as both pathways converge on activation of effector Caspase-3[10]. The extrinsic (also called the type 1) pathway directly activates Caspase-3 by enzymatic reactions catalyzed by activated Caspase-8 molecules. The intrinsic (also called the type 2) pathway is regulated by mitochondrial cytochrome *c* release and apoptosome formation. We can assume that three local signaling modules coordinate apoptosis in the type 2 pathway (Figure 1). Cell death can be induced by an apoptotic stimulus acting at any of the three different signaling modules (or right before them) of the apoptotic pathway (Figure 1): (1) death ligand binding and Caspase-8 activation; (2) Bax activation by BH3 only proteins; and (3) Ca^{2+} release that activates only the post-mitochondrial events. Our initial studies showed that, for the case of Caspase-8-mediated apoptosis, concentration of active Caspase-8 decides between the two pathways of apoptosis. Membrane reorganization, such as clustering of death receptors in raft signaling domains, determines the level of Caspase-8 activation in a cell type specific manner[16]. For large concentrations of Caspase-8, direct Caspase-3 activation occurs in a fast (minutes) deterministic manner. Such rapid activation of apoptosis has been observed in various cell types due to Fas ligand binding to Fas receptor[17]. Decrease in the strength of an apoptotic stimulus begins to activate the intrinsic pathway (Figure 2), as the rate constant for Caspase-8-Bid interaction is higher than that for Caspase-8-Capsase 3 association[1,18].

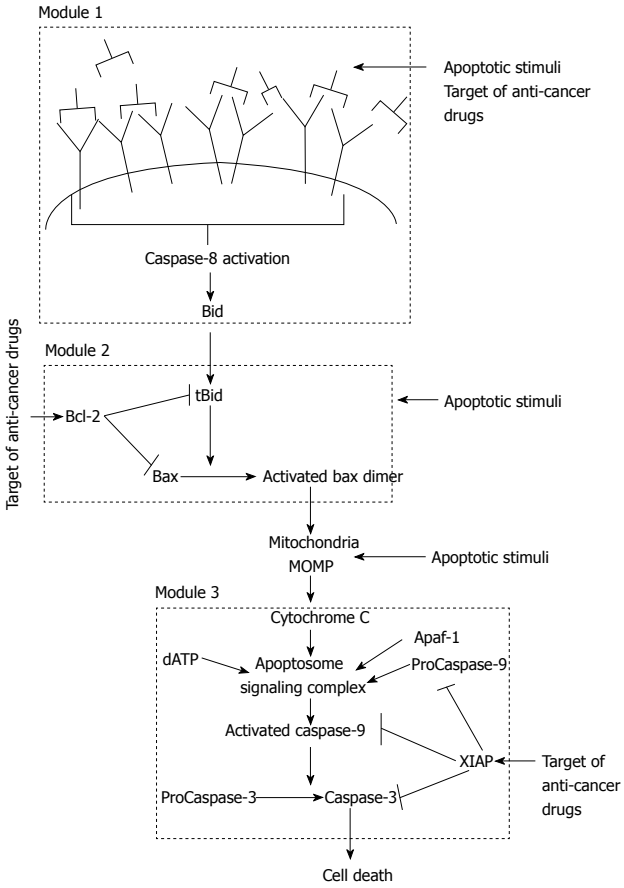

Figure 1 Schematic representation of the apoptosis signaling pathway that indicates existence of three distinct signaling modules in the apoptotic intrinsic pathway. Apoptosis can be activated at various locations in the intrinsic pathway. We also show some of the targets of cancer drugs.

LARGE CELL-TO-CELL VARIABILITY THROUGH THE INTRINSIC (MITOCHONDRIAL) PATHWAY CAN EXPLAIN SLOW APOPTOSIS

We observe slow apoptosis (hours) when low concentrations of Caspase-8 are used in our simulations. We also

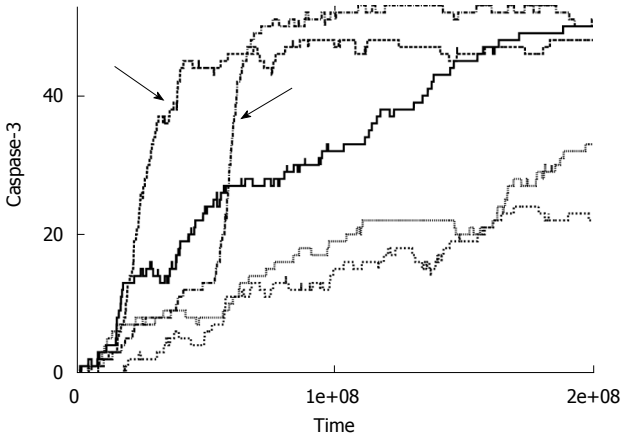

Figure 2 Time course of caspase-3 activation for low concentrations of caspase-8 (< 5 nmol). Data is shown for five individual cells. Arrows indicates switch to the intrinsic pathway of apoptosis at the level of single cells.

find large cell-to-cell stochastic variability in the case of slow apoptosis. Similar signaling behavior is observed, irrespective of the Caspase-8 concentration used, when we set the kinetic constants for the type 1 pathway to zero, confirming that slow apoptosis is a characteristic of the intrinsic pathway of apoptosis signaling. Such a study of pure type 2 apoptosis can be carried out *in silico* in a clean manner but will be difficult to achieve in biological experiments. When we perturbed the intrinsic apoptotic pathway downstream of Caspase-8 activation, we kept observing slow apoptosis with large cell-to-cell variability. Our results seem to explain very slow (1-100 h) apoptosis observed experimentally under a variety of conditions, for various types of cells and apoptotic stimuli, including under oxidative stress[2-6,9,10,19,20]. Additional variations in protein concentrations in our simulations act in tandem with intrinsic stochasticity of signaling reactions to enhance cell-to-cell variability in apoptosis. Caspase-3 activation occurs in an all-or-none (digital) manner for single cells implicating signaling amplification of a weak stimulus through the intrinsic pathway. However, the information of strength of the stimulus is contained in the time-to-death and its cell-to-cell variability. Large cell-to-cell variability with all-or-none type Caspase-3 activation, as observed in our simulations, resulted in bimodal probability distributions for Caspase-3 activation that are thought to be characteristic of apoptosis signaling through the intrinsic pathway[1]. Later experiments confirmed existence of such bimodal probability distributions in Caspase-3 activation[2,3,6,9].

MINIMAL MODEL OF A SIGNALING NETWORK DEMONSTRATES CELL-TO-CELL VARIABILITY IN APOPTOSIS IN A CELL-TYPE INDEPENDENT MANNER

In parallel, we derived a minimal model of a signaling network that is designed to sense an external stimulus and respond to it in an adaptive manner[8]. This minimal network is derived based on some simple assumptions on its signaling response without any prior knowledge of the apoptotic pathway. A three-step fast-slow-fast pathway in the minimal network was shown to be sufficient to generate large cell-to-cell variability as observed in our Monte Carlo simulations of the intrinsic pathway of apoptosis[1]. This minimal network also captures the change from rapid deterministic to slow stochastic signaling as the strength of the stimuli is varied, and a quantitative estimation of the threshold stimulus is obtained. This could be potentially important if one wants to engineer cancer cells to convert from type 2 to type 1 for fast apoptotic activation. The crucial slow reaction in the intermediate step of the minimal network can mimic the slow activation of Bax or the apoptosome formation in apoptosis signaling. Thus, we can infer that some of the pertinent qualitative features of apoptosis signaling, as observed in our Monte Carlo simulations, are cell type independent. Such a conclusion is significant given the fact that cellular protein levels and even the type of molecules present in the apoptotic pathways vary significantly among cell types[3,10,12]. Cancer cells are known to over-express a variety of apoptotic inhibitors, which confer them unusual resistance to apoptosis[21-24]. The level of over-expression varies significantly among cancer sub-types and even among patients having similar sub-types[25,26].

HOW APOPTOTIC INHIBITORS PROVIDE PROTECTION TO CANCER CELLS: IMPLICATIONS FOR CANCER THERAPY

In a recent study, we have shown that over-expression of Bcl-2 like proteins can slow down apoptosis and increase cell-to-cell stochastic variability[9]. A high Bcl-2 level allows activation of only a few Bax molecules under apoptotic stimuli and thus dynamically generates mechanisms for stochastic fluctuations caused by small number of molecules. Interestingly, cancer cells are often primed for death by increasing the levels of apoptotic BH3 proteins. However, in such cells, apoptosis is kept in check by continuous inhibition by anti-apoptotic Bcl-2-like proteins[22-24]. Bcl-2 binds with several pro-apoptotic molecules creating a local loop structure (signaling module 2) in the intrinsic pathway that leads to non-linear and stochastic effects in its inhibitory action. Our simulations demonstrate that, beyond a threshold level, Bcl-2 imparts a strong inhibitory effect on apoptosis and thus can explain apoptosis resistance of cancer cells. For normal cells, having over-expressed Bcl-2 proteins, prolonged time-to-death might provide an opportunity for a particularly slow cell to acquire tumor initiating features. Behavior similar to tBid-Bcl-2-Bax signaling (in module 2) might be observed downstream of mitochondria (in signaling module 3) where higher Apaf-1 level might make cancer cells prone to apoptotic death, although such an effect can be abolished by the dominant effect of increased X-linked inhibitor of apoptosis protein (XIAP) levels[27]. XIAP also has multiple binding partners in a local loop network structure and contributes to generation of highly non-linear and stochastic signaling. Hence, pre-

and post- mitochondrial events in the intrinsic pathway are heavily regulated by two different loop network structures in two distinct signaling modules (Figure 1). Computational models are well-suited to elucidate mechanisms of non-linear and stochastic signaling through those signaling modules. As a result, such models can help design optimal strategies to perturb those signaling modules by making use of the inherent apoptotic vulnerability of cancer cells. Initial simulations show increased cell death only for cancer cells over-expressing BH3 protein Bid (unpublished observations), under a single agent treatment scenario, such as under the action of Bcl-2 inhibitor HA14-1[28-30]. Our computational studies can clarify the basis of such inherent vulnerability of cancer cells for all three signaling modules (Figure 1). However, targeting only a single module (Figure 1), for example ligation of death receptors at the signaling module 1, will provide an opportunity for a significant number of cells to escape death. Such fractional killing of cancer cells occurs not only due to cellular variations in protein levels but also from inherent stochastic variability in signaling reactions[9]. Computational modeling was well-suited to establish that inherent stochastic variability by itself, even when all the other cellular parameters remain identical, can generate large cell-to-cell variability, comparable to that observed in apoptosis activation experiments[9]. Such large cell-to-cell variability in time-to-death provides an opportunity for opposing growth signals to up-regulate downstream apoptotic inhibitors such as XIAP. This is particularly relevant as apoptosis activation under chemotherapeutic treatment can be slow enough to allow synthesis of inhibitor proteins of varying concentrations through stochastic gene regulations. Targeting multiple signaling modules simultaneously, using combined treatments, can be effective in reducing stochastic effects and fractional killing of cancer cells. Computational studies can provide us with a range of concentrations for optimal induction of apoptosis in a combined treatment scenario and can guide the design of biological experiments. We are currently exploring the combined effect of HA14-1, an inhibitor of Bcl-2[28-30], and embelin[27], an inhibitor of XIAP, that can induce apoptotic collapse in cancer cells.

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REVIEW

Autograft mediated adoptive immunotherapy of cancer in the context of autologous stem cell transplantation

Luis F Porrata, Svetomir N Markovic

Luis F Porrata, Svetomir N Markovic, Division of Hematology, Department of Medicine, Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905, United States
Author contributions: Porrata LF wrote and reviewed the manuscript; Markovic SN reviewed the manuscript.
Correspondence to: Luis F Porrata, MD, Assistant Professor, Division of Hematology, Department of Medicine, Mayo Clinic College of Medicine, 200 First St. SW, Rochester, MN 55905, United State. porrata.luis@mayo.edu
Telephone: +1-507-2843158 Fax: +1-507-2664972
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Abstract

The infused stem cell autograft in autologous stem cell transplantation (ASCT) has been viewed mainly as hematologic rescue from the myelosuppressive side effect of conditioning regimens. However, recent reports have shown that the immune effector cells collected at the same time as the stem cells can produce an autologous graft-versus-tumor effect, similar to the graft-versus-tumor effect seen in allogeneic stem cell transplantation without the detrimental effects of graft-versus-host disease. In this article, we review the different immune effector cells collected and infused from the stem cell autograft and their association with clinical outcome post-ASCT, suggesting that ASCT can be viewed not only as a therapeutic maneuver to recover bone marrow function after deliver high-dose chemotherapy, but also as an adoptive immunotherapeutic intervention capable of eradicating residual tumor cells in patients with cancer.

Key words: Adoptive immunotherapy; Autologous graft versus tumor effect; Autograft

Peer reviewers: Maurizio Bendandi, MD, PhD, Associate Proessor, Laboratoire of Immunotherapy, Division of Oncology, Center for Applied Medical Research, University of Navarre, Cima Avda. Pio XII 55, 31008 Pamplona, Spain; Peter Hersey, FRACP, DPhil, Senior Staff Specialist in Immunology and Oncology, Conjoint Professor in Oncology, Room 443, David Maddison Building, Cnr King and Watt Streets, Newcastle, NSW 2300, Australia

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INTRODUCTION

The presumed lack of a graft-versus-tumor effect in autologous stem cell transplantation (ASCT) has been a long-standing argument against a higher curative potential of ASCT that relies mainly on high dose chemotherapy to eradicate tumor cells, compared with allogeneic stem cell transplantation (Allo-SCT)[1]. The clinical evidence of graft-versus-tumor (GVT) effect in Allo-SCT has been attributed to several indirect observations: (1) anecdotal reports demonstrating that abrupt withdrawal of immunosuppression in patients demonstrating tumor relapse post-Allo-SCT can re-establish complete remission; (2) higher risk of relapse in patients receiving syngeneic marrow grafts compared with recipients of allogeneic grafts; (3) patients developing graft-versus-host-disease (GVHD) experienced less relapse after Allo-SCT compared with those who did not; (4) T-cell depletion of an allogeneic graft increases the risk of relapse; and (5) the infusion of donor lymphocytes can induce complete remission after relapse following Allo-SCT[2]. The recognition of the adoptive GVT effect through the infusion of allo-reactive donor lymphocytes has changed our thinking of Allo-SCT from a therapeutic modality limited to high-dose chemotherapy to include immune-mediated GVT as an additional therapeutic intervention[3].

Despite the potential clinical benefit of the adoptive GVT in Allo-SCT, a fundamental problem is observed when using Allo-SCT as an immunotherapeutic modality. The infused allo-reactive donor immunocompetent cells that produce GVT can also target the host, producing GVHD. The transplant-related mortality (TRM) documented in myeloablative Allo-SCT has been reported to be between 40%-50%[3] compared with 3% TRM in ASCT. The rationale for non-myeloablative or reduced-intensity conditioning (RIC) is to decrease the high TRM observed after myeloablative Allo-SCT, though preserving the GVT effect. In lymphoid malignancies, the TRM after RIC has been reported from 5%-8% at day 100 to 19%-25% at 3 years[4]. In myeloid malignancies, the TRM after RIC has been reported around 20%[5]. Thus, the current research efforts in Allo-SCT center on ways of minimizing GVHD without compromising GVT.

The efficacy of ASCT as a treatment modality of cancer has relied mainly on the hope that high-dose chemotherapy will eradicate the resistant tumor clones that survived standard chemotherapy. Recently, we have reported the possibility of an autologous graft-versus-tumor effect without the detrimental effects of GVHD, based on the superior clinical outcomes observed in patients that achieved rapid post-ASCT recovery of their absolute lymphocyte count (ALC)[6]. Early ALC recovery, as a surrogate marker of host immunity in ASCT, is directly dependent on the absolute amount of infused lymphocytes (immune effector cells) harvested during CD34+ stem cell collection[7,8]. Herein, we review the different immune effector cells collected and infused from the stem cell autograft and their impact on immune recovery and survival post-ASCT.

RECOVERY OF ALC POST-ASCT

We reported superior overall survival (OS) and progression-free survival (PFS) in patients with multiple myeloma (MM) and non-Hodgkin lymphoma (NHL) that recovered higher ALC on day 15 (ALC-15) after ASCT[9]. We made similar observations in patients with other hematological malignancies[10-13] and metastatic breast cancer[14]. Several independent groups have confirmed our findings[13-20]. The superior survival observed for the first time post-ASCT based on ALC-15 in different malignancies suggests that patients' own (autologous) immunity has anti-tumor activity that is not disease specific[1,6]. More importantly, none these patients developed GVHD, favoring a more-specific immune response against the tumor and not the host in the ASCT setting[1,6]. We confirmed the prognostic ability of ALC-15 for survival post-ASCT prospectively[21].

ALC SOURCES POST-ASCT

The ALC-15 discovery as a prognostic factor for survival post-ASCT led to study of the sources affecting ALC-15 recovery post-ASCT. ALC-15 sources post-ASCT can be divided into two categories: (1) host lymphocytes; and (2) infused lymphocytes from the stem cell autograft[1]. From the host, lymphocyte sources include host stem cells and host lymphocytes surviving high dose chemotherapy. The host stem cells surviving high-dose chemotherapy most likely do not contribute to ALC-15 recovery post-ASCT because without stem cell rescue these patients remained myelosuppressed for a prolonged period of time. To identify host lymphocytes is more difficult in comparison with Allo-SCT where the development of mixed chimerism in Allo-SCT allows discrimination of host versus donor lymphocytes. Such discrimination is currently not possible in ASCT in the absence of marking studies for host lymphocytes.

The second possible source for ALC-15 recovery post-ASCT is the lymphocytes collected and infused from the stem cell autograft[1]. From the stem cell autograft ALC-15 recovery could come from: (1) infused stem cells ($CD34^+$); or (2) infused autograft lymphocytes collected during the apheresis process. In order to understand the impact of infused autografts on post-ASCT ALC-15 recovery, we evaluated the association of infused CD34 and/or lymphocytes from the autograft on the kinetics of post-ASCT lymphocyte recovery. No association was identified between infused CD34 stem cells and ALC-15. However, a strong correlation was found between the infused autograft lymphocyte numbers [autograft absolute lymphocyte count (A-ALC)] and ALC-15 recovery. Patients infused with an autograft containing higher A-ALC recovered greater numbers of ALC-15, resulting in better survival in NHL and MM post-ASCT[22,23]. An infused A-ALC $\geqslant 0.5 \times 10^9$ lymphocytes/kg was associated with a superior survival post-ASCT. This finding has been supported by other investigators[24]. These data suggest for the first time that the ASCT stem cell autograft should not be viewed only for the bone marrow rescue procedure to harvest CD34 stem cells necessary for hematologic engraftment, but also as an adoptive immunotherapeutic maneuver in which autograft lymphocyte content directly affects tumor-related clinical outcomes in multiple clinical settings.

The association between A-ALC and ALC-15 provides the first clinical evidence of an autologous GVT effect as the infusion of autograft lymphocytes has a direct impact on immune reconstitution and survival post-ASCT, similar to the GVT effect observed in Allo-SCT from the infused donor immune effector cells[6]. Therefore the identification of specific immune effector cell(s) infused from the autograft could be used as an immunotherapeutic strategy to improve immune recovery and survival post-ASCT.

Autograft $CD4^+$ T cells

Schmidmaier *et al*[25] reported better event-free survival in MM patients infused with higher numbers of $CD4^+$ helper T lymphocytes (HTL). Patients with a high percentage of HTL infused experienced a prolonged event-free survival (EFS) (2179 ± 170 d *vs* 1670 ± 212 d, $P < 0.003$). CD4 + T-cells with low HLA-DR expression produced a better EFS and overall survival compared with those that were HLA-DR+. Infusion of MM cells from the autograft did not affect survival, suggesting that the relapse post-ASCT

is due to the number of malignant cells surviving high-dose chemotherapy in the host and not due to the malignant cells infused from the autograft[26].

Autograft CD8$^+$ T cells

Infused autograft CD8$^+$ T cells have been associated with early lymphocyte recovery (ELR) post-ASCT. Defining ELR as an ALC $\geqslant$ 500 cells/μL at day 12 post-ASCT, Atta *et al*[27] reported a faster ELR in patients infused with a CD8$^+$ autograft lymphocyte dose of 0.1×10^9/kg. The authors stated that the autograft CD8$^+$ lymphocyte dose can be used as a marker of a faster ELR, thus translating to better clinical outcomes post-ASCT.

Autograft natural killer cells

Natural killer (NK) cells have shown to be the earliest lymphocyte subset that recovered early post-Allo-SCT and post-ASCT[1]. We reported that the dose of infused autograft NK cells directly correlated with day 15 absolute NK cells counts (NK-15) post-ASCT[28]. Patients with an NK-15 $\geqslant$ 80 cells/μL experienced superior OS and PFS compared with those who did not (median OS: not reached *vs* 5.4 mo, $P < 0.0001$; and median PFS: not reached *vs* 3.3 mo, $P < 0.0001$, respectively)[21].

Autograft dendritic cells

Dendritic cells (DC) are the antigen-presenting cells required for priming of naïve T-cells. DCs that express CD11c are classified as DC1 and they have a myeloid morphology and, when stimulated with tumor necrosis factor, produce high levels of interleukin-12 causing antigen naïve CD4$^+$CD45RA$^+$ T-cell differentiation to Th1 cells[29]. DC2, known as plasmacytoid DCs, have a CD11c-/CD123+ phenotype; they are the precursors of lymphoid DCs and serve to stimulate antigen naïve CD4$^+$CD45RA$^+$ T cells to differentiate into Th2 cells[29]. Dean *et al*[29] reported that the total number of collected and infused DCs affect survival post-ASCT. In patients infused with a DC dose $\geqslant 9.10 \times 10^6$/kg, the median OS was not reached compared with a median OS of 11.5 mo for patients infused with a DC dose $< 9.10 \times 10^6$/kg ($P < 0.022$)[29]. More interesting, in patients infused with DC1 $\geqslant 4.00 \times 10^6$/kg, the median OS was also not reached *vs* 11.3 mo for patients infused with a DC1 dose $< 4.00 \times 10^6$/kg. No association with survival was observed with infused DC2[29]. This finding suggests that the polarization of the host immunity towards an anti-tumor Th1 response (DC1) conveyed a superior survival than a Th2 anti-tumor down regulating immune response (DC2).

HIGH-DOSE CHEMOTHERAPY AND INFUSED AUTOGRAFT IMMUNE EFFECTOR CELLS ANTI-TUMOR ACTIVITY

In Allo-SCT, the role of the conditioning regimen (high-dose chemotherapy) has changed from mainly a direct anti-cancer therapy to prevent host versus graft rejection to a therapy which allows the donor immune effector cells to eradicate the tumor cells[3]. In addition, Allo-SCT conditioning regimens can be viewed as a therapeutic strategy to suppress tumor growth to allow the allogeneic graft-versus-tumor effect to develop, as resistance to allogeneic GVT has been observed in acute lymphoblastic lymphoma and high-grade lymphomas whereas the rapid tumor proliferation outgrows the establishment of GVT[30]. In ASCT, high-dose chemotherapy (HDC) has several important roles to allow the host graft (infused autograft immune effector cells) to create an autologous GVT effect. First the HDC in ASCT will reduce the tumor burden to help the host immunity eradicate minimal residual disease. The concept of tumor burden has been proven in animal models where animals inoculated with a tumor containing $\geqslant 10^6$ cells the immune system is unable to eradicate the tumor compared with animals inoculated with 10^6 cells[31]. In ASCT, delayed ALC recovery by day 30 post-ASCT is associated with worse OS and PFS compared with ALC recovery by day 15 post-ASCT, arguing that delayed host immunity recovery will allow rapid tumor growth post-ASCT counteracting the anti-tumor activity of the autologous GVT effect[32]. Second, by reducing the tumor burden, it is reasonable to assume that HDT will lower the immunosuppressive effect the tumor uses to evade immune recognition in the microenvironment. Third, by destroying the tumor, HDC might release tumor antigens that the infused DC1 can recognize and thus primed infused naïve CD4$^+$ T-cells that in turn will prime infused cytotoxic CD8$^+$ T cells from the autograft to eradicate minimal residual disease (the adaptive immune response from the autologous GVT effect). The innate immune response from the autologous GVT effect lies in the infused autograft NK cells that can recognize malignant cells without the need for antigen-presenting cells or with the help of antigen-presenting cells as recent reports have shown a bidirectional cross-talk between DCs and NK cells[33]. Thus, the combinations of the effects of HDC of reducing the tumor burden and re-setting the tumor microenvironment allow the infused autograft immune effector cells to create an autologous GVT effect, leading to the eradication of minimal residual disease and improved clinical outcomes post-ASCT (Figure 1).

STRATEGIES TO ENHANCE AUTOGRAFT IMMUNE EFFECTOR CELL COLLECTION

The same concept of using a stem cell mobilization regimen to mobilize enough CD34 stem cells from the bone marrow into the peripheral blood for stem cell collection for ASCT applies to lymphocyte harvesting. As the ALC-15 directly depends on the amount of infused A-ALC, it is logical to assume that the collection of A-ALC will depend on the peripheral blood ALC at the time of collection (PC-ALC). We identified a positive correlation between PC-ALC and A-ALC[23,24]. Thus, any interventions that might result in pre-collection lympho-

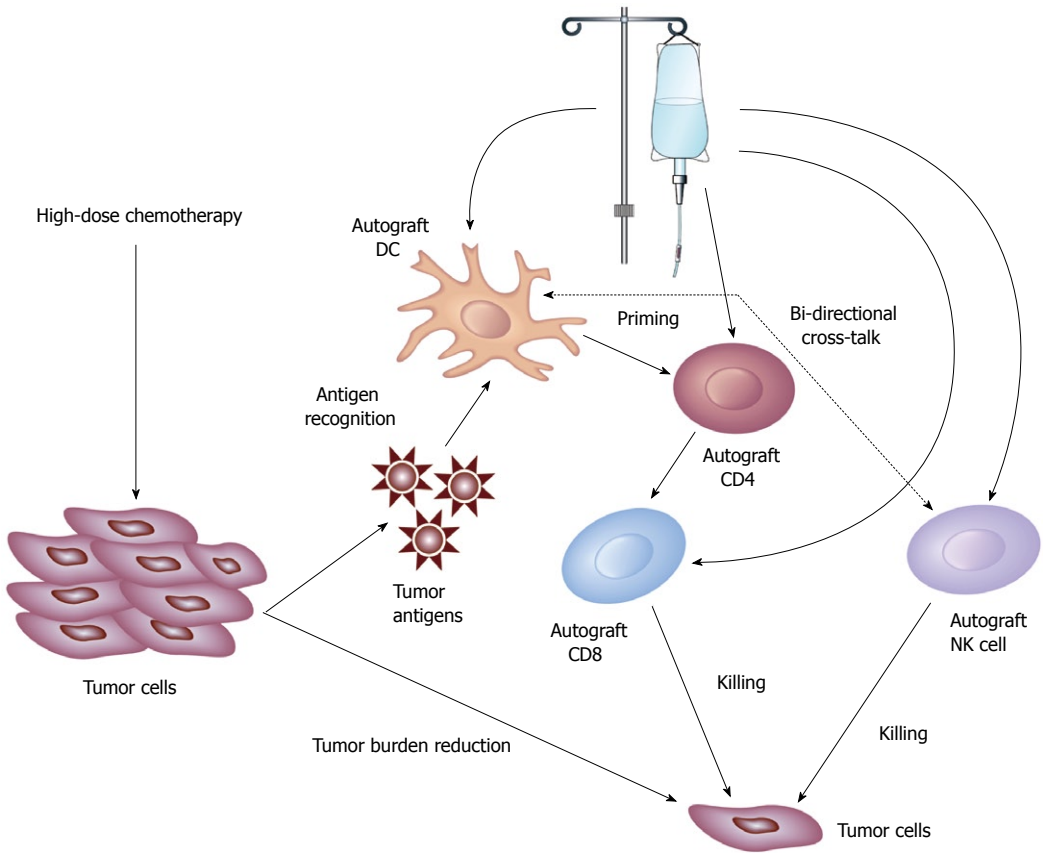

Figure 1 Schematic representation of the role of high-dose chemotherapy and infused autograft immune effector cells in the eradication of tumor cells post-autologous stem cell transplantation. The effects of high-dose chemotherapy (HDC) include tumor burden reduction and the release of tumor antigens. The infused autograft immune effectors cells include dendritic cells (DCs) that can recognize tumor antigens to priming infused autograft CD4+ T-cells, which in turn, activate infused autograft cytotoxic CD8+ T-cells to target tumor cells. The innate immune response component from the autograft is performed by the infused natural killer (NK) cells that can recognize tumor cells and NK cells anti-tumor function can be enhance with the help of DCs.

penia may negatively impact on A-ALC collection and ultimately lead to poor clinical outcomes in ASCT. This has been shown in MM patients. MM patients mobilized with granulocyte-colony stimulating-factor (G-CSF) and cyclophosphamide collected fewer A-ALC compared with MM patients that were mobilized with G-CSF alone[34].

However, the development of strategies to mobilize more peripheral blood lymphocytes and maximize lymphocyte harvesting should translate into higher A-ALC numbers, leading to faster immune recovery and improved clinical outcomes post-ASCT.

Interleukin-2 (IL-2) has been used in combination with G-CSF to mobilize NK cells for collection in the autograft. Sosman *et al*[35] found higher NK cell recovery by day 14 post-ASCT in patients in the IL-2 + G-CSF group. Other combinations of NK cells specific cytokines such as IL-15[36] and IL-21[37] could be studied to assess their ability to mobilize NK cells for harvesting in the autograft. Plerixafor is a CXCR4 inhibitor that has been approved for stem cell mobilization. We reported that plerixafor can also enhance lymphocyte mobilization for harvesting with the hope to improve immune recovery post-ASCT[38]. The number of apheresis collections is determined by the target dose of collected CD34 stem cells. Similarly, patients that had $\geqslant$ 4 apheresis collections harvested more lymphocytes compared with those who had < 4 apheresis collections[34]. Thus, the number of collections could be used as a strategy to achieve a target dose of A-ALC to maximize immune recovery and survival post-ASCT.

Another maneuver to enhance lymphocyte collection during apheresis is to reset the apheresis machine to not only collect enough $CD34^+$ stem cells but also high numbers of A-ALC. Three apheresis machines have been used for stem cell collection in the ASCT setting, including the COBE Spectra, the Fenwal CS 3000, and the Baxter Amicus. We identified that the COBE Spectra collected more A-ALC than the other two machines and better survival post-ASCT was observed in patients whose cells were collected by the Spectra machine compared to the others[39]. The survival benefit observed by the Spectra machine was not due to the machine itself; instead it was due to the fact that the Spectra machine collected more A-ALC. Because of this finding, we are currently doing a double blind randomized study where stem cells will be collected from patients the standard way versus a modified version of stem cell collection to maximize lymphocyte collection to assess if the new modified apheresis machine settings not only collect enough CD34 stem cells, but also more A-ALC, to affect survival post-ASCT.

CONCLUSION

The discovery of A-ALC affecting ALC-15, which in turn improved clinical outcome post-ASCT, created a new concept of viewing the stem cell autograft as an adoptive immunotherapeutic strategy with direct impact on survival post-ASCT. Further studies are warranted to understand how host immunity improves survival post-ASCT.

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REVIEW

Procathepsin D and cancer: From molecular biology to clinical applications

Vaclav Vetvicka, Aruna Vashishta, Sujata Saraswat-Ohri, Jana Vetvickova

Vaclav Vetvicka, Jana Vetvickova, Department of Pathology, University of Louisville, Louisville, KY 40202, United States
Aruna Vashishta, Sujata Saraswat-Ohri, Kentucky Spinal Cord Injury Centre, Department of Neurological Surgery, University of Louisville, Louisville, KY 40202, United States
Author contributions: All authors contributed equally to this work.
Correspondence to: Vaclav Vetvicka, PhD, Professor, Department of Pathology, University of Louisville, 511 S. Floyd, Louisville, KY 40202,
United States. vaclav.vetvicka@louisville.edu
Telephone: +1-502-8521612 Fax: +1-502-8521177
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Abstract

Procathepsin D (pCD) is overexpressed and secreted by cells of various tumor types including breast and lung carcinomas. pCD affects multiple features of tumor cells including proliferation, invasion, metastases and apoptosis. Several laboratories have previously shown that the mitogenic effect of pCD on cancer cells is mediated *via* its propeptide part (APpCD). However, the exact mechanism of how pCD affects cancer cells has not been identified. Recent observations have also revealed the possible use of pCD/APpcD as a marker of cancer progression. The purpose of this review is to summarize the three major potentials of pCD-tumor marker, potential drug, and screening agent.

Key words: Procathepsin D; Cancer; Screening; Enzyme; Cancer cells; Stimulation

Peer reviewer: Stephen H Safe, DPhil, Distinguished and Regent's Professor of Toxicology, Department of Veterinary Physiology and Pharmacology, Texas A and M University, 4466 TAMU, College Station, TX 77843-4466, United States

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MATURE ENZYME CATHEPSIN D

Cathepsin D (CD) is a soluble lysosomal aspartic endopeptidase (EC 3.4.23.5) synthesized in rough endoplasmic reticulum as pre-procathepsin D (Table 1). After removal of signal peptide, the 52 kDa pCD is targeted to intracellular vesicular structures (lysosomes, endosomes, phagosomes)[1,2]. pCD is a glycoprotein with two N-linked oligosaccharides modified with mannose 6-phosphate (M6P) residues and asparagine residues 70 and 199[3,4]. Lysosomal targeting is mediated by two M6P-receptors (cation-dependent 46 kDa and cation-independent 300 kDa M6PR)[2,5]. An alternate method to target pCD to lysosomes is independent of the M6P tag and is not yet fully understood. However, the role of sphingolipid activator precursor protein prosaposin has been suggested[6-10].

Upon entering the acidic endosomal and lysosomal compartment, the cleavage of the 44 amino acid N-terminal propeptide results in a 48 kDa single chain intermediate active enzyme form. In addition, proteolytic cleavage that does not result in dissociation of CD globular structure yields the mature active lysosomal protease which is composed of heavy (34 kDa) and light (14 kDa) chains linked by non-covalent interactions[11-13]. In addition, it was proposed that pCD can be converted to enzymatically active pseudo-cathepsin D by autocatalysis. Cysteine proteases and autocatalytic activity of CD is sure to be involved in pCD/CD processing[14-17]. Several factors were found to affect CD activation including a lipid second messenger ceramide and prosaposin[10,18].

Under normal physiological conditions, pCD is sorted to the lysosomes and found intracellularly which is unlike other members of the aspartic endopeptidase family that are mostly secretory proteins[19]. In some physiological and pathological conditions, pCD/CD escapes normal targeting mechanism and is secreted from the cells. pCD was found in human, bovine and rat milk[20-22], serum[23] and the presence of both pCD and CD (34+ 14 kDa) was demonstrated in

human eccrine sweat and urine[23,24]. pCD is a major secreted protein of numerous types of cancer cells[25]. It has been also shown that secreted pCD can be endocytosed *via* M6PR, or another yet unknown receptor, by both cancer cells and fibroblasts, and undergoes further maturation[26,27]. CD expression and activity was also detected in the extracellular matrix and synovial fluid of cartilage during physiological and pathological conditions[28-31]. pCD and mature CD was also found in macrophage-conditioned media and extracellularly in macrophage-rich regions of atherosclerotic lesions[32].

CATHEPSIN D/PROCATHEPSIN D AND CANCER

Increased levels of CD were first reported in several human neoplastic tissues, more than 20 years ago[33]. Several years later, the first clinical studies found pCD/CD related to metastasis-free survival and disease-free survival in breast cancer patients[34,35]. Since then, numerous clinical studies reported an association between pCD/CD level and tumor size, tumor grade, tumor aggressiveness, incidence of metastasis, prognosis, and a degree of chemoresistance in variety of solid tumors[25,36,37]. Studies dealing with pCD/CD diagnostic and prognostic value in cancer are complicated by the fact that, simultaneously, there are several forms of CD in a cell-inactive precursor pCD, enzymatically active intermediate (single chain) CD and mature (two chains) CD. Moreover, different forms of CD are also present in stromal cells and may result in pCD/CD quantification in tumor tissues and consequently its prognostic significance. Therefore, a standardization of techniques is needed to further evaluate the therapeutic and prognostic significance of pCD/CD expression in solid tumors.

Major studies and one meta-analysis found that pCD/CD level in tumor homogenate measured by either ELISA or IRMA represents an independent prognostic factor[38-40]. In these studies, antibodies that can detect both pCD (52 kDa) and CD (48 and 34+ 14 kDa) were used. Conversely, results of immunohistological (IHC) studies using antibodies specific to either pCD, CD or both are less consistent. This could possibly be due to different tissue fixation techniques, antibodies and semi-quantitative nature of IHC.

The mechanism of pCD mitogenic effect on cancer cells remains unclear. Numerous clinical studies have revealed that the level of pCD/CD represents an independent prognostic factor in a variety of cancers. These include breast and lung carcinomas[41]. It has been demonstrated that pCD/CD affects multiple stages of tumor progression including proliferation, invasion, metastasis, angiogenesis and apoptosis[41-43]. Clearly, prognosis of many types of cancer is significantly worse in cases of high pCD/CD expression and release.

We, addition to others, have shown that secreted pCD binds to surface of breast cancer cells[26,44]. We therefore hypothesize that pCD binds to a cell surface receptor with signaling properties. Despite a significant effort, the suggested pCD receptor has not been identified as yet and its molecular characterization remains elusive. Until now, the only receptors with known pCD/CD binding capacity are M6P receptors that recognize M6P tag on numerous glycoproteins. It has been shown that pCD secreted by cancer cells is highly glycosylated and is able to bind to M6P/IGF-Ⅱ receptor (cation-independent M6PR) on the breast cancer cell surface[45-48]. Numerous studies have demonstrated that neither binding nor pCD mitogenic potential is blocked by M6P, anti-M6PR antibodies or pCD deglycosylation[44,48-51]. Moreover, we recently showed that mutation in one or both glycosylation sites of pCD only slightly lower pCD mitogenic and pro-invasive activity *in vivo* and *in vitro*[52]. These results indicate that the sugar moieties are not important in the tumor-promoting effect of pCD and that M6P receptors are not involved in mediating pCD mitogenicity. However, the binding of pCD to M6P/IGF-Ⅱ receptor may decrease its binding capacity to other M6P/IGF-Ⅱ receptor natural ligands (e.g. IGF-Ⅱ, latent TGF-β) and thus perturb their biological functions[48].

Table 1 Cellular localization of procathepsin D/cathepsin D

Form	Localization	Process
Pre-procathepsin	Rough endoplasmic reticulum	Synthesis
Procathepsin	Golgi apparatus	Modification of oligosaccharides
Procathepsin	Prelysosomal compartments	Cleavage of the activation peptide
Single chain cathepsin	Lysosomes	Proteolytic processing, triming of oligosaccharides
Mature cathepsin	Lysosomes	

We determined that binding to cancer cells, as well as pCD mitogenic potential, is blocked by antibodies specific for propeptide part of pCD[49,53,54]. The propeptide (also called activation peptide-AP) of pCD serves at neutral or basic pH to block the access of substrates to the active site. The active site of CD forms (as is the case with other mammalian aspartic proteases) a deep cleft between the two lobes of the active enzyme[55,56]. According to the 3D model of the pCD structure constructed by *in silico* homology molecular modeling using known coordinates of pCD and pepsinogen. The AP forms a loop where most of the N-terminal half is making electrostatic bonds with the active site aspartates and most of the C-terminal part of AP is on the surface of the molecule of pCD suggesting that the C-terminal part can interact with other molecules[57,58].

Utilizing synthetic peptides that correspond to different parts of AP, we showed that the region responsible for binding of pCD to cancer cell surface is localized between amino acids 33-44 of the AP[44,54]. In numerous experiments using synthetic AP, anti-AP antibodies or mutant pCD with deleted AP, we demonstrated that AP itself stimulates growth of breast, prostate and lung cancer cells *in vitro* and *in vivo*[44,49,51-54,59-61]. Although the mitogenic effect of AP was not confirmed by Glondu *et al*[50] under their experimental conditions, Bazzett *et al*[62] independently demonstrated mitogenicity of AP in ovarian cancer cells.

Tumorigenesis is a complex process involving not only growth of the primary tumor cells or tumor stem cells, but also communication with surrounding tissues and cells. In this process, different parts of stromal tissue, including the vasculature, adipocytes, resident immune cells, and fibroblasts, play a role. All these cells are secreting numerous cellular products, including various growth factors and extra-

cellular matrix components. It is likely that tumor fibroblasts originate from normal fibroblasts and that they are very similar to fibroblasts involved in wound healing processes. There is clear evidence that fibroblasts communicate with the primary tumor cells and this communication is critical for development of the disease. There is an extensive *in vitro* and *in vivo* research demonstrating that this communication can promote the growth of cancer cells. For more information about pCD/CD and tumor environment[63].

Secretory proteins of different families play a role in primary tumor growth and metastasis formation and angiogenesis. In addition to this autocrine mitogenic effect, pCD was also found to possess paracrine proliferative properties. Berchem's group found that pCD stimulates not only parent cancer cell proliferation but also tumor angiogenesis by a paracrine mechanism[42]. This possibility was further potentiated by the work of the Liaudet-Coopman group who demonstrated that pCD was able to stimulate proliferation, survival, motility and invasion of fibroblasts[43]. The detailed mechanisms of the proliferative function of pCD remain unknown. In experiments testing the influence of IL-4, IL-10 and IL-13 on growth of several cancer cell lines, we have found that these cytokines had a similar proliferative effect as pCD. The difference between the pCD- and cytokine-induced proliferation lies in fact that the stimulation of proliferation has been observed only in the case of ER+ cell lines. Supposed mechanism of action is pCD-dependent triggering of IL-4, IL-8, IL-10, and IL-13, which subsequently further stimulate cancer cell growth.

In an attempt to better understand the autocrine and paracrine effects of pCD, we tested the possibility of secretion of cytokines upon pCD addition. We demonstrated substantial secretion of cytokines, especially IL-4, IL-8, IL-10, IL-13 and MIP-1β from both cancer cell lines and fibroblasts upon addition of pCD. This secretion was shown to promote the growth of both cancer cells and fibroblasts. As a result of our experiments, we can conclude that pCD secretion observed in many cancer derived cell lines leads to a secretion of cytokines which, in turn, promote the growth of both types of cells. Therefore, a selective inhibition of pCD interaction with a cellular receptor could decrease or halt this process. These data underline pCD as a potential target for cancer therapy.

PCD IN SCREENING

Research performed in our laboratory, in addition to others, has demonstrated the presence of anti-pCD autoantibodies[64]. As these antibodies are specific to pCD only, and do not recognize mature CD[65-67], they represent an ideal target for comparison of the pCD presence and cancer progression.

We hypothesized that the level of anti-pCD autoantibodies correlates with the stage of breast, lung, and prostate cancer and offers development of a cost-effective, non-invasive screening test. We prepared an ELISA assay for evaluation of the presence of anti-AP/pCD antibodies. Attributable due to the low affinity of the antibodies, activation peptide alone is not optimal for evaluation in ELISA or RIA assays. We decided to overcome this potential setback by using a specifically modified synthetic activation peptide as an antigen assay. Employing Multiple Antigen Peptide (MAP), we were able to measure the level of anti-pCD autoantibodies in patient serum.

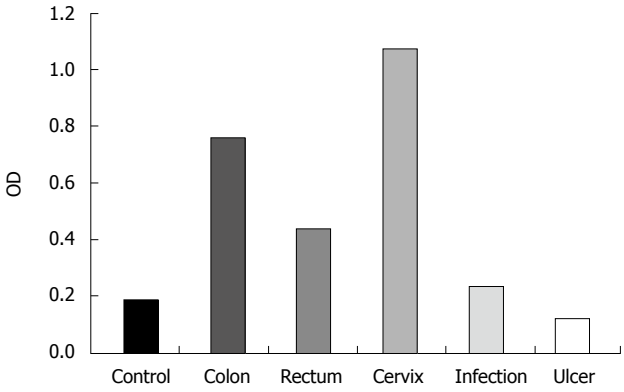

Figure 1 Level of anti-procathepsin D autoantibodies in patients with three types of cancer and two cancer-unrelated diseases evaluated by enzyme-linked immunosorbent assay. Data represent mean value from 5 patients/group.

Since pCD has been found to be involved in numerous types of cancer, one can assume that the specific autoantibodies will also be formed in additional types of cancer. Using a small number of samples, we found the elevated levels of these autoantibodies in lung, prostate, and stomach cancer (unpublished data). Serum samples were mostly from the commercial source ProMedDx, which provides serum samples from over 60 different diseases. Compared to the cancer samples, sera of patients suffering from additional diseases were negative (Figure 1).

We hypothesize that the amount of the APpCD/pCD in the patient's serum will change with the progress of the cancer disease, thus corresponding with the increased number of pCD-releasing cancer cells. This hypothesis configures well with our preliminary findings on breast cancer (Figure 2) and clearly shows higher levels of antibodies in more advanced stages. These preliminary data define the high clinical potential in the evaluation of specific anti-AP autoantibodies.

Based on these data, we prepared a model of a proposed mechanism of pCD action (Figure 3). The overexpressed pCD escapes normal intracellular targeting pathways and is secreted out of the cancer cells. Subsequently, pCD interacts with surrounding proteins and is recognized *via* its AP part by a specific cell surface receptor. This interaction releases a signal resulting in differential expression of cancer-promoting genes including various cytokines that, in turn, stimulate tumor growth. pCD secreted by cancer cells is also captured by stromal cells and promote fibroblasts proliferation, motility and invasion that results in cancer progression. In addition, stress affects keratinocytes resulting in increased both cytokine and pCD synthesis and secretion leading to the elevated proliferation of keratinocytes (Figure 4).

OTHER POTENTIAL ROLE OF PCD

In recent decades, there has been focused on pCD's additional contribution toward wound healing, tissue remodeling[68] and programmed cell death-apoptosis[69,70]. Epidermis, the barrier between the body and external environment, is constantly exposed to various environmental and physical stresses. Keratinocytes are elemental cells forming the epidermis and are crucial

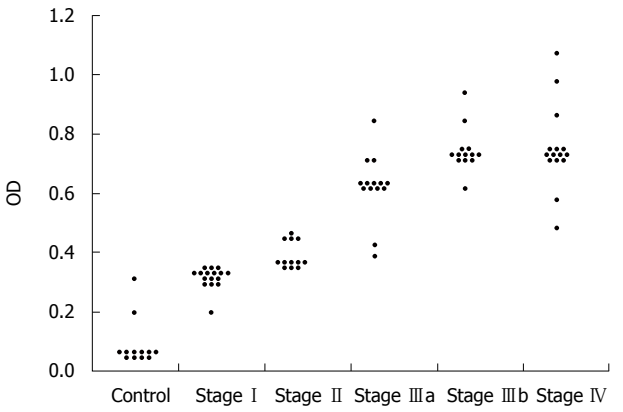

Figure 2 Level of anti-procathepsin D autoantibodies in patients with various stages of breast cancer evaluated by enzyme-linked immunosorbent assay. Average age of patients in each group varied from 31 to 69.

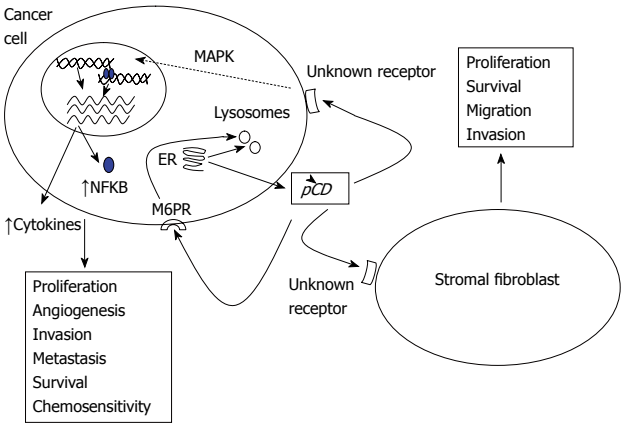

Figure 3 Proposed mechanism of procathepsin D/cathepsin D function in cancer progression. The over-expressed procathepsin D (pCD) escapes the physiological intracellular targeting pathways and is secreted by the cancer cells. Small part of secreted pCD is bound and internalized *via* M6PR pathway, the rest by a yet unidentified receptor. The receptor-pCD interaction activates mitogen-activated protein kinases (MAPK) pathway with subsequent changes in expression of numerous cancer-promoting genes including NFKB2 and some cytokines. Interaction of pCD with endothelial and stromal cells is also involved.

for normal regeneration and healing. Skin healing is dependent upon several processes that comprise inflammation, protein synthesis, matrix deposition, migration and subsequent proliferation of keratinocytes[71,72]. Keratinocytes are known to secrete numerous proteins that include proteolytic enzymes such as matrix metalloproteinases[73], interstitial collagenase[74] and cathepsin B[75]. During the wound healing process, these proteolytic enzymes might play a role in motility of keratinocytes by remodeling of extracellular matrix for migration of keratinocytes to peripheral layers of epidermis. When Katz *et al*[76] studied proteins secreted by cultured human epidermal keratinocytes, they found that CD was one of them.

In skin, increased levels of the mature form of CD has been shown in basal keratinocytes during hyperproliferative skin disorders such as psoriasis[77]. In addition, the involvement of different isoforms of CD in the epidermal cell differentiation was also suggested. The presence of pCD was shown in the spinous layer. These forms were present in stratum corneum, where they played a role in epidermal desquamation[78,79]. Although, the role of CD in epidermal differentiation has been defined, the presence of pCD at different stages of differentiation is still unclear. Moreover, many of these studies were performed using cell lysates where all the isoforms are present, making clear definition of the roles played by individual isoforms virtually impossible.

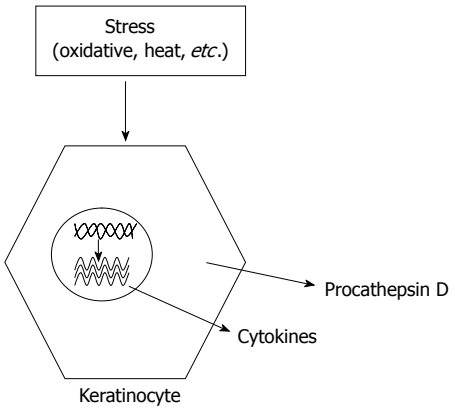

Figure 4 Stress affects keratinocytes resulting in increased both cytokine and procathepsin D synthesis and secretion leading to the elevated proliferation of keratinocytes.

Therefore, we tested the hypothesis that the secretion of pCD from cells is one of the normal physiological features in the skin. Initially, we began by demonstrating the secretion of pCD *via* the human keratinocytes cell line HaCaT. Subsequent experiments showed that the exogenous addition of purified pCD enhanced the proliferation of HaCaT cells. The proliferative effect of pCD was inhibited by monoclonal antibody against the activation peptide (AP) of pCD. Treatment of HaCaT cells with pCD or AP led to the secretion of a set of cytokines that may promote the growth of cells in a paracrine manner. The role of secreted pCD and its mechanism of action were further studied in a scratch wound model. The presence of pCD and AP enhanced the regeneration of monolayer. Simultaneously, this effect was reversed by the addition of anti-AP antibodies. Expression and secretion of pCD was upregulated in HaCaT cells exposed to various stress conditions. Taken together, our results strongly suggest that the secretion of pCD is not only linked to cancer cells but also plays an essential role in the normal physiological conditions such as wound healing and tissue remodeling[80]. However, current knowledge does not support the possible connection between pCD function in cancer and wound healing. This proteinase (both in enzymatically active and inactive state) just has several biological functions, including both pathological and physiological ones.

CONCLUSION

While many functions of pCD/CD in the physiological and pathological processes could be attributed to its enzymatic activity, it is clearly established that some of its functions in the organism are independent of its protease activity and rely on the ability of pCD to interact with other important molecules. It appears to be inevitable that the search for pCD-interacting partners should be conducted to explore the mechanism of pCD actions.

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Peter Hersey, FRACP, DPhil, Senior Staff Specialist in Immunology and Oncology, Conjoint Professor in Oncology, Room 443, David Maddison Building, Cnr King and Watt Streets, Newcastle, NSW 2300, Australia

E YK Ng, PhD, PGDTHE, Associate Professor, School of Mechanical and Aerospace Engineering, College of Engineering, Nanyang Technological University, 50, Nanyang Avenue, Singapore 639798, Singapore

Des R Richardson, BSc, MSc, PhD, DSc, Professor of Cancer Cell Biology, NHMRC Senior Principal Research Fellow, Director, Iron Metabolism and Chelation Program, Department of Pathology, University of Sydney, Sydney, NSW 2006, Australia

Domenico Rubello, MD, Professor, Director of the Department of Nuclear Medicine, PET/CT Centre, Radiology, Medical Physics, Santa Maria della Misericordia Hospital, Via Tre Martiri 140, ZIP 45100, Rovigo, Italy

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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