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Exploring alternative cytotoxic strategies for cancer treatment

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Exploring Alternative Cytotoxic Strategies for Cancer Treatment

ZHU Yanting

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Philosophy

Principal Supervisor: Dr. SHI Jue

Hong Kong Baptist University

July 2014
Declaration

I hereby declare that this thesis represents my own work which has been done after registration for the degree of MPhil at Hong Kong Baptist University, and has not been previously included in a thesis, dissertation submitted to this or other institution for a degree, diploma or other qualification.

Signature: __________________

Date: July 2014
Abstract

Triggering direct cytotoxicity has been the most common strategy for developing cancer treatments. The cytotoxic regimens currently used in the clinic mainly include radiation therapy, classic chemotherapeutic drugs (e.g. DNA damaging drugs and anti-mitotic drugs) and selected new targeted drugs. Although these therapies are the standard of care for most cancer patients, they suffer significant limitations: responses to these therapies vary significantly between cancer types and patients; sensitive cancers tend to acquire resistance; and they cause serious toxicity, particularly to dividing cells in the bone marrow and gut, and to neurons. It is not clear whether major improvements in cytotoxic anticancer therapies are possible; if they are, progress is likely to come from either new methods for identifying sub-populations of patients that respond well to current drugs, or developing new therapies with novel cytotoxic mechanisms. To pursue the above two avenues towards potential improvement of cytotoxic therapies, this thesis investigates: biomarkers that determine the sensitivity of distinct cancer cell types to common anti-mitotic chemotherapeutics; and the mechanistic basis to employ alternating electric field and Natural Killer cells as alternative methods to trigger cancer cell death. The study uses time-lapse microscopy as the major technique to characterize and quantify response dynamics to the different cytotoxic treatments, and the results provide important new insight not only for understanding existing cytotoxic anticancer drugs but also for developing novel cytotoxic regimens.
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Finally, I take this opportunity to express the profound gratitude from my deep heart to my Mom. Thank you so much for everything you have done for me.
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilization</td>
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<td>PARP</td>
<td>Poly ADP-ribose polymerase</td>
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<td>K5I</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>MDC1</td>
<td>Mediator of DNA Damage Checkpoint 1</td>
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Chapter 1  Introduction

1.1. Cancer

Cancer is a broad class of diseases, involving multiple genetic mutations and alterations of a wide spectrum of cellular pathways and processes [1]. According to Ruddon's CANCER BIOLOGY, cancer is defined as abnormal cell growth caused by multiple changes in gene expression, which leads to imbalanced regulation of cell proliferation and cell death, and results in a population of cells with the capacity to invade surrounding tissues and metastasize to distant sites. These abnormal cells bring significant morbidity and, if untreated and uncontrolled, eventually death of the host [1].

Clinically there are more than one hundred distinct types of human cancer, which vary in their respective age of onset, rate of growth, state of cellular differentiation, cell antigenicity, invasiveness, metastasis, diagnostic detectability, and response to treatment [1]. At the molecular level, all types of cancer share three common characteristics, including multiple alterations of gene expression, dysregulation of the normal cellular program for cell division and cell differentiation, and the abilities of invasion and metastasis. These three abnormal characteristics are fundamentally all caused by alterations in gene expressions, e.g. due to multiple genetic mutation of proto-oncogenes and tumor-suppressor genes that are essential for regulating physiology of the cell [1, 2]. Cancer is thus believed to be a disease of abnormal gene expression [1].
Examples of common and well-known genetic mutation associated with cancer include mutations in Ras (a family of small GTPase), Retinoblastoma (Rb) and p53. Mutations of the above genes in general over-activate the growth-promoting signals, thus desensitizing cells to cell cycle checkpoint controls and apoptosis [2-8]. Moreover, alterations of gene expressions, such as expression of oncogenic Ras, can also promote angiogenesis [1, 9, 10]. The blood vessels inside the tumor mass provide nutrients and oxygen to the cancer cells and facilitate the removal of their metabolic wastes, both of which are essential for sustaining the cancer cell growth. To enhance its own growth, cancer cell releases pro-angiogenic factors that favor the growth of blood vessels. The expansion of tumor mass not only compresses its surrounding normal tissue but also triggers further genetic mutations that allow cancer cell to eventually invade through basal membranes and metastasizes to foreign sites [1].

1.2. Cancer therapy

Cancer is a leading cause of death worldwide, accounting for 8.2 million deaths in 2012 [11]. It is the No.1 killer disease in Hong Kong, accounting for 31.4% of all registered deaths in 2011 [12]. WHO statistics indicate that the worldwide deaths due to cancer are projected to continue to rise, with estimated 13.1 million deaths in 2030 [11]. The high incidence and mortality rate of cancer bring heavy burden to both the patients’ families and the society as a whole. Research on improving existing anticancer treatments and developing new treatment strategies are thus imperatively needed.
Since clinically cancer is a highly heterogeneous diseases, treatment methods vary depending on the specific cancer type, age of onset, location and grade of the tumor, state of cellular differentiation, ability of invasive and metastasis, as well as the patient conditions. Currently cancer patients are treated mainly by surgery, radiation therapy, chemotherapy and selected targeted therapy (such as various kinase inhibitors and angiogenesis inhibitors). Limited clinical treatment also involves immunotherapy (mainly T-cell therapy) and electromagnetic therapy. As new understanding of the biology of cancer emerges, new treatments will likely be developed to improve the effectiveness and specificity of the treatment, thus improving the long-term survival prospect and quality of life of the cancer patients.

**Surgery**

For cancer surgery, an operation is performed to remove the tumor entirely. In most cases, the surrounding healthy tissues would also be cut off to ensure the complete elimination of the cancer cells. Since its first record in ancient Egypt, cancer surgery has been the primary treatment choice for most solid tumors, especially for the localized tumors. However, if the cancer has already metastasized to other sites in the body, complete surgical excision is usually impossible. Thus, surgery is usually supplemented with other treatments, such as radiotherapy and chemotherapy [1, 12].

**Radiotherapy**

Radiotherapy, also called X-ray therapy or irradiation, uses high-energy waves to destroy cancer cells and induce tumor shrinkage. Development of radiotherapy started from the late nineteenth century. Radiotherapy has been used widely in
various types of cancers, including solid carcinomas in different tissues, such as breast, skin, lung, uterus, mesenchymal sarcomas, leukemia and lymphoma. The specific radiation doses used for treatment are cancer-type and tissue-type dependent, as the sensitivities of various cancer cells and also surrounding tissues to radiation are different. Although used extensively, radiotherapy is known to cause harmful side effects [11, 12].

**Chemotherapy**

Chemotherapy, also referred to as antineoplastic therapy and cytotoxic therapy, was first developed in the early 20th century. In chemotherapy, anticancer drugs are used to directly damage or destroy cancer cells [1, 13]. Different from surgery or radiation therapy, which are localized treatment, drugs used in chemotherapy circulate all around the body and thus act as systemic treatment. Chemotherapeutic drugs are particularly effective on damaging proliferating cells, thus causing harmful side-effects on healthy fast-dividing cells, such as neutrophils. The cytotoxicity of distinct chemotherapeutics is mediated by various cellular processes, such as interfering with DNA duplication or perturbing cell division. For chemotherapy, drugs are often given in combination instead of stand-alone treatment [11, 13, 14].

**Targeted therapy**

Targeted therapy has become an increasingly common treatment method, since its first appearance in the late 20th century. Targeted drugs are in general specific to particular cellular components, which often time are over-expressed in tumor cells and/or whose functions are more crucial for tumor cell survival as compared to normal cells. By more specifically targeting cancer-associated components, targeting
drugs are believed to have less side effects on normal cells than traditional chemotherapy. Among the common targeted drugs are small-molecule inhibitors of various tyrosine kinases (e.g., Sorafenib and Imatinib) and monoclonal antibody drug (e.g., Trastuzumab) [14, 15].

**Immunotherapy**

Immunotherapy refers to treatment that activates the patient’s own immune system to contain and eliminate cancer cells. Most of the currently available immunotherapy involves activating T cell activity, either by using specific cytokines/chemokines or neutralizing antibody (e.g., Ipilimumab) *in vivo* or by pre-activating T cell *in vitro* and then transferring the tumor-reactive T cells to cancer patients, which is called adopted T-cell transfer therapy [16, 17]. Unfortunately, the current immunotherapy showed similar limitation as traditional chemotherapy, such as strong variability in patient response and substantial treatment-associated side effects. Therefore, the central challenge for developing immunotherapy is to find more effective and specific methods, such as combinatorial strategies, to improve the therapeutic outcome across a broader patient population [1, 18].

**Hormonal therapy**

Hormonal therapy was first introduced and developed in the late 19th century, based on the observation that exogenous administration of specific hormones could inhibit growth of certain cancer types and even induce tumor cell death eventually. Hormonal therapy is currently used mainly for cancers originated from hormonally responsive tissues, such as breast and prostate [19]. The most well-known hormonal therapy is the use of tamoxifen for breast cancer treatment [20].
1.3. Aims of the Study

Most of the existing anticancer therapies work through activating direct cell death, in particular radiotherapy and chemotherapy. These cytotoxic regimens so far show limited success in the clinic, as their effectiveness varies greatly among cancer patients and many cancer types simply do not respond. Moreover, the significant side-effect of cytotoxic therapies on killing essential normal cells, such as the fast dividing neutrophils in the bone marrow, greatly compromises the patient health and works exactly opposite to the intended treatment outcome. To improve the specificity of cytotoxic therapies, we need to understand how the therapies work and identify molecular biomarkers that can be used to define patient populations that are likely to respond to the particular therapy. In addition, alternative cytotoxic strategies should be examined for developing new, potentially better anticancer treatments.

With the above two goals in mind, this thesis sets out to identify the molecular mechanisms underlying the variable cytotoxic efficacy of different anti-mitotic drugs, one of the most commonly used chemotherapeutics in treating solid tumors, as well as to elucidate the cytotoxic mechanisms of alternating electric fields and Natural Killer cells as potential cytotoxic regimens for cancer treatments. As these three topics cover distinct aspects of cancer biology and cellular processes, separate sections of introduction, materials and methods, results and discussions are elaborated in the following three chapters, respectively.
Chapter 2  Post-slipage multinucleation and DNA damage
determine cytotoxic variation of distinct anti-mitotic drugs

2.1. Introduction

Inhibiting cell proliferation by perturbing mitosis is a widely used therapeutic strategy for cancer treatment. Currently available anti-mitotic chemotherapeutics mainly include classic microtubule-targeting drugs, such as paclitaxel and vinca alkaloids, and the new spindle-specific drugs, such as inhibitors of Kinesin-5 (aka KSP, Eg5, KIF11), Polo kinase-1 and Aurora kinases. Anti-mitotic drugs work by activating prolonged mitotic arrest and subsequently triggering cancer cell death either during mitotic arrest or after mitotic slippage to an abnormal G1 state [21-25]. Most of the available data, including our own, show that although cell death induced by anti-mitotics is mediated by the intrinsic apoptotic pathway in both cell cycle states, i.e. during mitotic arrest and after slippage, these two types of cell death involve very different mechanisms and molecular pathways [26-28]. Death during mitotic arrest is mainly triggered by the loss of an anti-apoptotic protein, Mcl-1, due to an imbalance of synthesis and degradation [29-34], as transcription is silenced and translation is attenuated during mitosis [35-37]. We further identified that the strong variation between cell lines in their sensitivity to apoptosis during mitotic arrest is determined by the variation in the expression level of another anti-apoptotic protein, Bcl-xL [29]. Mcl-1 and Bcl-xL are thus the common, primary negative regulators of apoptosis during prolonged mitotic arrest induced by anti-mitotic drugs in general.

While the different anti-mitotic drugs induce largely similar degree of cell death during mitotic arrest, their activities in triggering cytotoxicity after mitotic slippage
appear to be highly variable. For instance, in cell culture we found that Kinesin-5 inhibitors (K5Is) were much less pro-apoptotic than paclitaxel in activating cell death after mitotic slippage, although they induced similar duration of mitotic arrest and similar extent of death during mitotic arrest [25, 29]. Moreover, clinical data showed that so far paclitaxel is still more effective than the spindle-specific anti-mitotics [38], but the molecular origins of the extra cytotoxicity of paclitaxel are unclear. Compared to death during mitotic arrest, the molecular pathway activated by anti-mitotics, which links mitotic arrest, abnormal slippage out of the arrest and death after slippage, is poorly understood. Previous studies showed that caspase activities, which were partially activated during prolonged mitotic arrest, would induce DNA fragmentation after mitotic slippage, subsequently leading to p53-mediated DNA damage response [39-41]. However, such DNA damage did not necessarily activate post-slippage apoptosis [39]. It also does not account for the significant variability in the extent of post-slippage cell death triggered by the different anti-mitotics. Identifying the mechanisms that govern post-slippage cell death is thus clearly needed so as to not only improve our understanding of the molecular basis underlying the variable efficacy of different anti-mitotic drugs but also provide new insight for elucidating the large variation between cancer cell types in their response to anti-mitotics, leading to better prediction of the variable clinical outcomes.

Here we investigate the cellular and molecular mechanism by which paclitaxel activates more extensive post-slippage apoptosis than the spindle-specific anti-mitotic, K5I. In order to distinguish cells dying after slippage from those dying during mitotic arrest and examine molecular changes specific to the post-slippage population, we
used time-lapse microscopy as the primary technique for measuring the extent and kinetics of apoptosis and other relevant cellular events at the single cell level. Our results revealed that formation of multiple small nuclei was a marked feature of cells that slip out of paclitaxel-induced mitotic arrest, while K5I typically resulted in one large nucleus after slippage. Moreover, the extent of DNA damage and apoptosis after mitotic slippage strongly depended on the degree of multinucleation. Together with data that showed post-slippage apoptosis was mediated by the p53-dependent DNA damage response pathway, our findings suggest that multinucleation induced by paclitaxel is a key trigger of post-slippage DNA damage and apoptosis, and likely renders the extra cytotoxicity of paclitaxel towards tumor cells as compared to the spindle-specific anti-mitotics.
2.2. Materials and methods

2.2.1 Cell lines

U-2 OS cells were purchased from American Type Culture Collection (ATCC) and cultured under 37°C and 5% CO₂ in McCoy’s 5A supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100U/mL penicillin and 100μg/mL streptomycin (Invitrogen). The fluorescent U-2 OS cell line that expresses the mitochondria reporter, IMS-RP (a generous gift from Dr. Peter Sorger, Harvard Medical School), was generated by infecting U-2 OS cells with retrovirus encoding a construct consists of a monomeric red fluorescent protein targeted to the inter-membrane space of mitochondria by fusion to the leader peptide of SMAC [42]. The fluorescent U-2 OS cell line that expresses the reporter of wild-type p53 fused with Venus was generated by lenti-viral infection of a p53-Venus construct (a generous gift from Dr. Galit Lahav, Harvard Medical School) [43].

For mitotic shake-off experiment, we grew large volume of U-2 OS cells in 25 cm dishes to 90% confluency, and then treated the cells with 150 nmol/L paclitaxel to induce mitotic arrest. After 4 hours of drug treatment, the mitotic fraction of cells was collected by gently shaking and washing the dish to detach the mitotic cells from the bottom.

2.2.2 Chemicals

Paclitaxel was purchased from Sigma and used at 150 nmol/L (the saturating concentration for cell death induction). The potent kinesin-5 inhibitor (EMD534085) was provided by Merck-Serono. Responses to EMD534085 have been shown to be
similar to s-trityl-cysteine, a commercially available Kinesin-5 inhibitor. zVAD-FMK was purchased from Calbiochem and used at 100 mol/L.

2.2.3 Gene knockdown by RNA interference (RNAi)

siRNA oligos were custom synthesized by Invitrogen. siRNA against Kinesin-5 (5’-AGGACAACUGCAGCUACUC-3’) was used at final concentration of 20 nmol/L for RNAi experiments. siRNA against p53 (5’-UGAACCAUUGUUCAUAUCGUCCGG-3’) was used at final concentration of 5 nmol/L. Dharmacon On-Target plus siControl (D-001810-01) was used as non-targeting siRNA control. All siRNA transfections were performed using HiPerFect (Qiagen) according to manufacturers’ instructions. Experiments were conducted after 36 hours (Kinesin-5 RNAi) or 24 hours (p53 RNAi) of gene silencing.

2.2.4 Western Blot Analysis

Cell lysates were obtained using LDS sample buffer (NuPAGE, Invitrogen). Proteins were resolved on 8-15% Tris-glycine gels and transferred onto PVDF membranes. Blots were probed with commercial primary antibodies and chemiluminescent detection using ECL-plus (Amersham). Antibodies: PARP1 (#9542), tubulin (#2148), Kinesin-5 (#7625), p21 (#2947) and Puma (#4976) were purchased from Cell Signaling; p53 (#sc-126) from Santa Cruz; \( \gamma \)H2A.X (#06-570) from Millipore. Anti-actin (#A5316) from Sigma was used as a loading control.
2.2.5 Immunofluorescence staining and analysis

Cells grown on glass coverslips were fixed in 3.7% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) for 15 minutes; permeabilized with 0.5% Triton-X100 for 30 minutes; washed 3 times in PBS; blocked in 5% BSA/0.1% Triton-X100/PBS for 1 hour; incubated with primary antibody for 2 hours; washed 3 times in PBS; incubated with fluorescently conjugated Alexa-Fluor secondary antibodies (Invitrogen) for 1 hour; washed 3 times in PBS; stained with 1 g/mL Hoechst (Invitrogen) for 2 minutes. All procedures were conducted at room temperature. The coverslips were then mounted on glass slides with ProLong Gold antifade reagent (Invitrogen). The stained cells were imaged using an Olympus FV1000 Laser Scanning Confocal microscope. Antibodies: tubulin (#2144) and p53 (#2527) were purchased from Cell Signaling; γH2A.X (#06-570) from Millipore; Actin (#A5316) from Sigma; and MDC1 (#ab11169) from abcam.

For quantification of the integrated γH2A.X fluorescence, nuclei of the cells were segmented using the DNA stain, and the total γH2A.X fluorescence intensity inside the nuclei was measured using the MetaMorph software.

2.2.6 Gene expression by transient transfection

To express MDC1-EGFP (a generous gift from Dr. Jiri Lukas at the Institute of Cancer Biology, Denmark), U-2 OS cells were seeded in 6-well plate and transiently transfected with the plasmid using X-tremeGENE (Roche) according to manufacturer’s instruction. Time-lapse imaging experiment of U-2 OS cells expressing the MDC1-EGFP construct were conducted 36 hours after the transfection.
2.2.7 Time-lapse microscopy and Data analysis

Cells were plated in 35 mm imaging dish (μ-dish, ibidi) and cultured in phenol red-free CO2-independent medium (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Cell images were acquired with the Nikon Ti-Eclipse-PFS inverted microscope enclosed in a humidified chamber maintained at 37°C. Cells were imaged every 10 minutes using a motorized stage and a 20x objective.

Images were viewed and analyzed using the MetaMorph software (Molecular Dynamics). Based on the phase-contrast images, we scored by morphological tracking: interphase (by flat morphology), entry into mitosis (by cell rounding), mitotic slippage (by re-spreading without cytokinesis) and cell death (by blebbing followed by cell lysis) (Figure 2.1). The time to death since mitotic slippage, which was plotted in Figure 2.2C and 2.4A, was calculated as the time of post-slippage death (scored by cell lysis) minus the time of mitotic slippage.
2.3. Results

2.3.1 Multinucleation induced by paclitaxel engender stronger post-slippage apoptosis than K5I

We previously profiled the anti-mitotic drug response of 11 mammalian cell lines, including cancer cell lines derived from the lung (A549, H460), colon (HCT116, HT29), breast (MCF7, MDAMB435S), ovary (OVCAR5), cervix (HeLa), prostate (PC3) and bone (U-2 OS) as well as a normal cell line, RPE [25]. Our results showed that across all cell lines that were profiled, paclitaxel was consistently more cytotoxic than K5I and triggered substantially more cell death particularly after mitotic slippage (refer to Figure 2.1 for the typical response dynamics to paclitaxel and K5I after mitotic slippage). The molecular pathway(s) that controls post-slippage apoptosis is evidently much more highly activated under paclitaxel. One striking post-slippage feature that we observed from the panel of mammalian cell lines was that cells that slip out of paclitaxel-induced mitotic arrest always have multiple small nuclei, while cells that slip out of K5I-induced arrest have mostly one and occasionally two large nuclei (Figure 2.2A).

To determine whether the degree of multinucleation affects the extent of apoptosis after mitotic slippage, we attenuated multinucleation in paclitaxel-treated cells by knocking down Kinesin-5 (K5) using RNA interference (RNAi), as loss of K5 activity (e.g. by K5Is) promotes formation of a single nucleus in post-slippage cells. We chose to use K5 RNAi instead of the chemical inhibitor, K5I, to attenuate multinucleation because in order to achieve the same attenuation effect as K5 RNAi, 5-10 μmol/L K5I, a concentration 10-fold higher than the saturating dosage for
inducing mitotic arrest, i.e. 0.5-1 μmol/L [25], had to be used in combination with paclitaxel; and at such high K5I concentration, significant non-specific cytotoxic effects that were independent of mitotic arrest were observed. Hence, we opted to use K5 RNAi to attenuate post-slipage multinucleation in this study, as K5 RNAi alone induced less than 5% cell death after 36 hrs of siRNA transfection (the time when we added paclitaxel for experiment). We selected U-2 OS as the model cell line from the panel of 11 mammalian cell lines that we profiled before [25], as U-2 OS cells were found to be sensitive to post-slipage apoptosis and representative of the general drug response behaviors across the different cell lines.

Based on phase-contrast images from time-lapse microscopy (refer to Materials and Methods for image analysis method), we counted the number of post-slipage nuclei. Our results showed that the average number of nuclei per post-slipage cell was 6 under treatment of 150 nmol/L paclitaxel plus K5 knockdown as compared to 14 under paclitaxel alone (Figure 2.2B). Cell death plotted as cumulative survival curves in Figure 2.2C demonstrated that both the kinetics and extent of post-slipage cell death were significantly attenuated in U-2 OS cells with K5 knockdown. Note that in Figure 2.2C we analyzed the time to death as the time from mitotic slippage to post-slipage cell death, as in this study we focused on understanding the mechanism of apoptosis activation specific to the post-slipage population. Under paclitaxel plus K5 knockdown, about 50% post-slipage cells died and the average time from mitotic slippage to death was 27 hours, in contrast to 91% post-slipage cell death and an average time to death of 20 hours under paclitaxel alone. Western blot analysis of an apoptosis marker, Parp1, confirmed the single-cell imaging data,
showing much higher percentage of Parp1 cleavage in cells treated with paclitaxel alone (Figure 2.2D). We noted that the level of K5 decreased as cells died in response to paclitaxel. We suspect this may possibly be due to leakage of cytoplasmic K5 to the medium upon cell lysis (a signature event of cell death in culture), given that the decrease of K5 signal occurred when cells died. Overall our data strongly suggest that multinucleation plays a crucial role in rendering the strong post-slippage apoptosis in response to paclitaxel.
**Figure 2.1.** Dynamics of U-2 OS cells in response to K5I/K5 RNAi (upper panel) or paclitaxel (lower panel). The still frames were acquired from time-lapse imaging. Timing is indicated in unit of hour at the upper corner of the phase-contrast images. Note that the time 0 for K5I and paclitaxel treatment is the time when drug was added, while the time 0 for K5 RNAi is 36 hours after transfection with K5 siRNA, as it takes 30-36 hours to sufficiently knock down the K5 protein. Based on the phase-contrast images, we scored by morphological tracking: interphase (by flat morphology), entry into mitosis (by cell rounding), mitotic slippage (by re-spreading without cytokinesis) and cell death (by blebbing followed by cell lysis). We then quantified the time of the respective cellular changes for further statistical analysis.
Figure 2.2. Characteristics of post-slippage multinucleation and apoptosis in response to anti-mitotic drugs. (A) Phase-contrast images of post-slippage U-2 OS cells treated with 150 nmol/L paclitaxel (Ptx), 150 nmol/L paclitaxel + K5 siRNA and 1 mol/L Kinesin-5 inhibitor (K5I), respectively. (B) Average number of post-slippage nuclei per cell (± SD) and (C) cumulative survival curves of U-2 OS cells under the indicated siRNA treatments in combination with 150 nmol/L paclitaxel or under treatment of K5I alone. Total number of cells analyzed for each condition ranges from 70 to 75. *, p < 0.05 vs. Ctrl (no siRNA). Individual cells were monitored by time-lapse microscopy, and time from mitotic slippage to morphological death was measured and plotted as cumulative survival curves. (D) Knock-down of Kinesin-5 (K5) attenuated post-slippage apoptosis (indicated by Parp1 cleavage) and DNA damage (indicated by γH2A.X) induced by paclitaxel.
2.3.2 Post-slippage multinucleation and apoptosis depend on the duration of mitotic arrest

We next investigate whether the duration of mitotic arrest affects the extent of multinucleation and apoptosis after mitotic slippage. To obtain cells with distinct mitotic arrest time, we first synchronized U-2 OS cells in paclitaxel-induced mitotic arrest by mitotic shake-off and then induced slippage using an Aurora kinase inhibitor, VX-680 [44, 45]. We found 2-hour treatment of 150 nmol/L VX-680 was sufficient to induce > 95% U-2 OS cells to slip out of mitotic arrest, and treatment of VX-680 for such a short period time did not induce significant DNA damage or cell death (Figure 2.3). Figure 2.4A shows cumulative survival curves of U-2 OS cells that were synchronized in mitotic arrest for 4 hours (i.e. short arrest) and 10 hours (i.e. long arrest) in comparison with cells that slip out of the normal paclitaxel-induced mitotic arrest (the average mitotic arrest time of unsynchronized U-2 OS cells is 13 hours). It is evident that longer mitotic arrest increased and accelerated apoptosis after mitotic slippage. Only 27% of U-2 OS cells that underwent 4 hours of mitotic arrest died 40 hours after mitotic slippage, in contrast to 64% and 91% of cell death subsequent to 10-hour and normal paclitaxel-induced arrest. This was further confirmed by western blot analysis of Parp1 cleavage (Figure 2.4B). Moreover, the average number of nuclei per post-slippage cell was 4 after 4-hour arrest and 8 after 10-hour arrest in comparison to 14 after normal arrest, which again demonstrated the strong correlation between the extent of multinucleation and post-slippage apoptosis (Figure 2.4C).
The mechanism by which duration of mitotic arrest affects the degree of post-slippage multinucleation is unclear. Paclitaxel is known to generate multipolar spindle during mitotic arrest with clusters of DNA attached to the multiple spindle asters [46, 47]. These well separated multipolar structures can be clearly observed by confocal microscopy (Figure 2.4D). In contrast, K5I induces the formation of only a single spindle pole. This raised the possibility that the post-slippage multinuclei induced by paclitaxel may originate from the multi-polar DNA clusters formed during mitotic arrest. Compared to cells that underwent normal and long mitotic arrest (i.e. 10 hours), the spindle asters of U-2 OS cells that experienced short mitotic arrest (i.e. 4 hours) aggregated closely and were not spatially well separated (Figure 2.4D). Knockdown of K5 also inhibited separation of the multiple spindle asters under paclitaxel, which correlated with the attenuation of subsequent post-slippage multinucleation. These observations suggest that formation of stable and well separated multipolar structures, around which nuclear envelop may reform upon mitotic slippage, may be necessary for generating the multiple post-slippage nuclei. Since short mitotic arrest may not provide sufficient time for the spindle asters to separate, it resulted in less post-slippage nuclei than the long arrest. Previous study that imaged α-tubulin-GFP showed that the paclitaxel-induced spindle asters indeed underwent dynamic separation as mitotic arrest prolonged [47], providing evidence that support the above hypothesis.
**Figure 2.3.** Comparison of cell death (measured by Parp1 cleavage) and DNA damage (measured by γH2A.X) induction under the indicated drug treatment. From left to right: Ctrl (no drug treatment); VX-680 (cells treated with 150 nmol/L VX-680 for 2 hours, 24 hours and 48 hours, respectively); Ptx arrest (cells were in paclitaxel-induced mitotic arrest for 4 hours or 10 hours and then induced to slip out of the arrest by VX-680. Cell lysates were collected 24 hours after the induced slippage); Ptx (cells treated with 150 nmol/L paclitaxel for 48 hours); K5I (cells treated with 1 mol/L K5I for 48 hours). VX-680 treatment alone did not appear to trigger observable cell death or DNA damage if the treatment time was short, i.e. less than 24 hours.
Figure 2.4. Post-slippage multinucleation and apoptosis depend on duration of mitotic arrest. (A) Cumulative survival curves of post-slippage U-2 OS cells that were synchronized in mitosis by 150 nmol/L paclitaxel for 4 hours or 10 hours in comparison with those that underwent normal arrest (no synchronization). Total number of cells analyzed for each condition ranges from 72 to 75. (B) Comparison of apoptosis induction (indicated by the percentage of Parp1 cleavage) at the indicated time points after slippage out of the 4-hour and 10-hour mitotic arrest induced by paclitaxel. (C) Average number of post-slippage nuclei (± SD) at the indicated condition of mitotic arrest. (D) Confocal images of representative U-2 OS cells during mitotic arrest under the indicated treatments. Blue: DNA; Green: microtubules.
2.3.3 Post-slippage apoptosis is mediated by p53-dependent DNA damage response

The multinucleated cells that slide out of paclitaxel-induced mitotic arrest were previously found to exhibit strong elevation of γH2A.X (a DNA damage marker) and p53 [25, 40]. This indicated that multinucleation may promote post-slippage cell death by inducing DNA damage and subsequently activating the p53-mediated DNA damage response pathway that triggers apoptosis. To examine the correlation between multinucleation and DNA damage, we used immunofluorescence of γH2A.X to quantify the extent of DNA damage in anti-mitotics treated cells. Figure 2.5A illustrates the confocal images of representative post-slippage U-2 OS cells under five treatment conditions, including 150 nmol/L paclitaxel, 1 μmol/L K5I, 150 nmol/L paclitaxel plus K5 knockdown, as well as induced slippage out of 4-hour and 10-hour mitotic arrest in 150 nmol/L paclitaxel. Paclitaxel evidently induced the highest level of γH2A.X (stained in green) in post-slippage cells. Quantification of the average integrated γH2A.X fluorescence showed a 5-fold higher damage signal in U-2 OS cells that slide out of paclitaxel-induced mitotic arrest than K5I-treated cells (Figure 2.5B). Knockdown of K5 as well as short mitotic arrest (i.e. 4-hour) both significantly reduced the level of DNA damage in post-slippage cells. Together with western blot results of ensemble γH2A.X level shown in Figure 2.2D, 2.4B and 2.5C, our data demonstrated a strong correlation between the level of post-slippage DNA damage and the extent of multinucleation and apoptosis.

One of the major signaling pathways that mediate DNA damage response is the p53 pathway, consisting of mainly the tumor suppressor protein p53 and its
downstream transcriptional targets [48]. In **Figure 2.5C**, we measured, by western blotting, the expression levels of p53 and two key p53 target genes, p21 and Puma, in paclitaxel- and K5I-treated cells. p53 as well as its target genes were all more highly induced under paclitaxel, correlating with the DNA damage level. Moreover, knocking down p53 by RNAi substantially attenuated post-slippage cell death in response to paclitaxel (**Figure 2.5D**). These data thus suggest that post-slippage apoptosis induced by paclitaxel is p53 dependent and likely regulated by the p53-mediated DNA damage response pathway.
Figure 2.5. The extent of post-slippage multinucleation and apoptosis correlated with DNA damage level. (A) Confocal images of representative post-slippage U-2 OS cells under the indicated treatments. Ctrl: no treatment; Ptx: 150 nmol/L paclitaxel for 60 hours; K5I: 1 μmol/L K5I for 60 hours; Ptx+K5 siRNA: 150 nmol/L paclitaxel plus K5 knockdown by RNAi for 60 hours; 4hr/10hr arrest: 40 hours after induced slippage out of 4-hour/10-hour mitotic arrest in paclitaxel. Blue: DNA; Green: γH2A.X; Red: Actin. (B) Average integrated fluorescence intensity of γH2A.X per cell (± SD) quantified from the confocal images. 20 cells from three independent experiments were analyzed for each condition. *, p < 0.05 vs. K5I; **, p < 0.05 vs. 4hr arrest in paclitaxel. (C) Expression of selected DNA damage response genes in U-2 OS cells treated with paclitaxel (150 nmol/L) vs. K5I (1 μmol/L) by western blot analysis. (D) Knockdown of p53 attenuated apoptosis induced by paclitaxel.
2.3.4 Both DNA damage and p53 induction occurred after mitotic slippage and in an asynchronous manner in individual nuclei

An important question regarding the observed DNA damage is how it occurs relative to mitotic arrest, slippage and apoptosis activation. To simultaneously monitor the real-time dynamics of DNA damage induction and activation of post-slippage apoptosis, we imaged, by time-lapse microscopy, paclitaxel-treated U-2 OS cells that expressed two fluorescent reporters, including a green fluorescent reporter (MDC1-EGFP) of wild-type MDC1 (Mediator of DNA Damage Checkpoint 1) fused with EGFP [49], and a red mitochondria reporter (IMS-RP) consisting of a monomeric red fluorescent protein targeted to the inter-membrane space of mitochondria by fusion to the leader peptide of SMAC [42]. Upon DNA damage, MDC1 is phosphorylated by ATM (Ataxia Telangiectasia Mutated) and then binds to γH2A.X, subsequently recruiting the downstream components involved in the damage response process [50]. As shown in the upper panel of Figure 2.6A, no increase in MDC1 signal was observable until U-2 OS cells slip out of the prolonged mitotic arrest. For the majority of cells that we analyzed, significant accumulation of MDC1 started to appear 4-6 hours after mitotic slippage. Interestingly, individual nuclei belonging to the same post-slippage cell showed highly heterogeneous MDC1 signal and distinct kinetics of the signal increase, suggesting that DNA damage in the multiple small nuclei occurred in an asynchronous manner. Moreover, we observed Mitochondrial Outer Membrane Permeabilization (MOMP), the defining event of apoptosis activation, in the post-slippage cells based on a switch-like change from punctuate to diffused distribution in IMS-RP fluorescence (Figure 2.6A, lower panel)
Since post-slippage MOMP always occurred hours after the onset of MDC1 accumulation, our data pointed to induction of DNA damage being an upstream event of apoptosis, i.e. not a consequence of apoptotic damage. To confirm that the two DNA damage markers that we used, MDC1 and γH2A.X, indeed correlate, we performed confocal imaging of U-2 OS cells with co-staining of MDC1 and γH2A.X antibody. As shown in Figure 3-4B, signal of both MDC1 and γH2A.X only significantly increased after mitotic slippage. And the immunofluorescence of MDC1 largely correlated with that of γH2A.X, i.e. most nuclei with strong γH2A.X staining generally also exhibited high MDC1 accumulation. Overall the confocal data indicated that MDC1 and γH2A.X were likely to report similarly on DNA damage. We noticed that U-2 OS cells also showed a very small number of γH2A.X foci during mitotic arrest, but this γH2A.X signal was not accompanied by increase in MDC1 (Figure 2.B). It appeared that the γH2A.X signal arising in mitosis may not relate to conventional DNA damage, which agreed with findings from several previous studies [51-53].

To determine the induction dynamics of p53 relative to post-slippage apoptosis, we used a clonal fluorescent U-2 OS reporter cell line, which stably expresses a p53-Venus construct as well as the MOMP reporter, IMS-RP [54]. Dynamics of the p53-Venus construct had been confirmed to behave similarly to the endogenous wild-type p53 in response to DNA damage by previous studies [43, 54]. From the time-lapse movies, we observed strong induction of p53 in ~90% of the post-slippage cells treated with paclitaxel. Similar to MDC1, p53 level was found to significantly increase only after mitotic slippage, and MOMP was observed hours after p53
induction (Figure 2.6C). The different small nuclei present in the same cell again showed distinct kinetics and level of p53 activation, which correlated with the variable dynamics of DNA damage induction that we observed by the MDC1-EGFP measurement, indicating that the different nuclei may activate DNA damage response in an asynchronous manner. Some of the small nuclei showed a decrease in p53 fluorescence after the strong, initial post-slippage induction (Figure 2.6C), which may be due to successful DNA damage repair. Confocal images of p53 and γH2A.X immunofluorescence confirmed the time-lapse data, i.e. increase in DNA damage and p53 level mainly occurred after mitotic slippage (Figure 2.6D). The p53 immunofluorescence did not show a very large difference between the different nuclei as in the time-lapse data, although the nuclei exhibited significantly different γH2A.X level. We suspect this may be due to saturating signal from immunostaining and confocal imaging.
Figure 2.6. Induction dynamics of MDC1 and p53 relative to the activation of post-slippage apoptosis in U-2 OS cells treated with 150 nmol/L paclitaxel. (A) MDC1 dynamics relative to induction of post-slippage MOMP. Still images of representative U-2 OS cell were from fluorescence time-lapse movies. Upper panel: MDC1-EGFP; lower panel: IMS-RP. Elapse time from paclitaxel addition was indicated at the bottom of the images (unit: hour). (B) Confocal images of γH2A.X (green), MDC1 (red) and DNA (blue) in interphase (pre-mitotic), mitotic, and post-slippage U-2 OS cells. (C) p53 dynamics relative to induction of post-slippage MOMP. Upper panel: p53-Venus; lower panel: IMS-RP. (D) Confocal images of γH2A.X (green), p53 (red) and DNA (blue) in interphase (pre-mitotic), mitotic, and post-slippage U-2 OS cells.
2.4. Discussion

Results from our study demonstrated that in addition to killing cancer cells during mitotic arrest, where different anti-mitotics exhibit largely similar efficacy, paclitaxel is much more potent in triggering cell death after mitotic slippage by engendering multiple small nuclei and subsequently strong DNA damage. Paclitaxel appears to exert dual cytotoxicity by two distinct mechanisms in the cell cycle state of mitosis and post-slippage interphase, capable of killing proliferating cancer cells by both triggering mitotic arrest and post-slippage DNA damage. For cancer cells that are resistant to apoptosis during mitotic arrest, such as those with high expression level of Bcl-xL [29], paclitaxel is particularly desirable among the different anti-mitotics, as it is able to also induce extensive apoptosis after mitotic slippage by activating a strong DNA damage response. Moreover, given the dual cytotoxic activities of paclitaxel, our results suggest that to predict tumor cell response to paclitaxel, two distinct sets of biomarkers may need to be considered, one involving genes that regulate cell death during prolonged mitotic arrest (e.g. Mcl-1 and Bcl-xL) and the other involving genes that control DNA damage-dependent post-slippage death (e.g. p53 and possibly other components in the p53 pathway).

Recent work by Crasta et al showed that DNA fragmentation and damage would arise as small micronuclei underwent defective and asynchronous replication [55]. To test whether this is also the mechanism by which extensive DNA damage was induced in the multinucleated cells that slip out of paclitaxel-induced mitotic arrest, we inhibited DNA replication in the post-slippage cells using thymidine or mimosine [56]. In order to generate post-slippage cells, we first synchronized cells in mitotic
arrest by mitotic shake-off in paclitaxel and then divided the cells equally into treatment of paclitaxel alone or paclitaxel plus thymidine/mimosine. Neither thymidine nor mimosine attenuated DNA damage (as indicated by $\gamma$H2A.X) or apoptosis in the post-slippage cells, as compared to paclitaxel treatment alone (Figure 2.7A). In addition, bromodeoxyuridine (BrdU) staining of the post-slippage cells showed little BrdU incorporation into the chromosome, indicating that the post-slippage cells were likely arrested in the G1 state before DNA synthesis (Figure 2.7B). It thus appeared unlikely that the post-slippage DNA damage observed in the paclitaxel-treated cells was originated from defected DNA replication of the multiple small nuclei.

Another possible mechanism by which DNA damage may be induced in the post-slippage cells was through partially activated caspase activities [39-41]. Orth et al [39] showed that in response to K5I, partially activated caspasases during prolong mitotic arrest may partly activate caspase-activated DNase (CAD), causing DNA damage and p53 induction after mitotic slippage. To examine whether this is the mechanism by which paclitaxel activated post-slippage DNA damage and cell death, we compared the extent of post-slippage DNA damage and apoptosis with and without a pan-caspase inhibitor, zVAD-FMK. Quantification of the integrated $\gamma$H2A.X fluorescence showed that treatment of zVAD-FMK significantly reduced the DNA damage level in response to paclitaxel by about 40%, indicating caspase activity is indeed involved. However, the reduced DNA damage level is still 3-fold higher than that induced by K5I treatment. So inhibition of caspase activity did not completely block the induction of post-slippage DNA damage under paclitaxel. More importantly, by
imaging the MOMP reporter (IMS-RP), we found that 68% of the post-slippage cells treated with paclitaxel plus zVAD-FMK still underwent MOMP (Figure 2.8A), although these cells did not die due to the lack of caspase activities to activate apoptotic events downstream of MOMP. Statistics of MOMP kinetics from the individual cells showed that the average time from mitotic slippage to MOMP was 18.7 hours under paclitaxel plus zVAD-FMK, very similar to 19.2 hours under paclitaxel alone (Figure 2.8B). These preliminary data suggest that besides caspase activities, additional cellular components/pathways also contribute to the induction of DNA damage and apoptosis in the post-slippage multinucleated cells. Further, more detailed mechanistic study is clearly needed to determine the molecular links between multinucleation, DNA damage and apoptosis.

The origin of the post-slippage multi-nuclei was alluded to by our results, but not completely resolved. We hypothesized that the DNA micro-clusters, which were attached to the multipolar mitotic spindles and formed during the prolonged mitotic arrest, may have given rise to the multiple post-slippage nuclei under paclitaxel. However, to determine whether they are indeed mechanistically related and identify the underlying molecular regulators, the cellular machinery that controls the slippage processes needs to be examined in detail, such as nuclear envelope reformation and chromosome unwinding. Results from such further study will also help to elucidate how duration of mitotic arrest impacts the extent of multinucleation and apoptosis in post-slippage cells.
Figure 2.7. Test of the possible mechanism I of extensive DNA damage induced in the multinucleated cells that slip out of paclitaxel-induced mitotic arrest. (A) Comparison of Parp1 cleavage in U-2 OS cells that were synchronized in paclitaxel-induced mitotic arrest and then treated with the indicated conditions for 40 hours: 150 nmol/L paclitaxel vs. paclitaxel + 2 mmol/L thymidine; 150 nmol/L paclitaxel vs. paclitaxel + 300 μmol/L mimosine. (B) Confocal images of control interphase U-2 OS cells vs. cells that slip out of paclitaxel-induced mitotic arrest. Blue: DNA; Green: bromodeoxyuridine (BrdU) (from Invitrogen).
Figure 2.8. Test of the possible mechanism II of extensive DNA damage induced in the multinucleated cells that slip out of paclitaxel-induced mitotic arrest. (A) Still images from time-lapse movies that showed MOMP kinetics of U-2 OS treated with 150 nmol/L paclitaxel or paclitaxel + 100 μmol/L zVAD-FMK. The MOMP fluorescent reporter, IMS-RP, consists of monomeric red fluorescent protein targeted to the inter-membrane space of mitochondria by fusion to the leader peptide of SMAC. The upper row are phase-contrast images, and the lower row are IMS-RP fluorescence. MOMP was scored by an abrupt change from punctate to smooth distribution in fluorescence in the cytoplasm. Elapse time is indicated in unit of hour at the lower corner of the IMS-RP image. (B). Comparison of MOMP kinetics in post-slippage U-2 OS cells treated with paclitaxel alone or paclitaxel + zVAD-FMK.
Chapter 3  Mammalian cell death induced by alternating (AC) electric fields

3.1. Introduction

Living cells and organisms are known to possess electromagnetic properties that enable them to interact with external electromagnetic fields [57-59]. The origin of cellular electrical properties is conceptually simple, as a large number of ions and charged molecules are major constituents of biological cells. Previous studies have demonstrated several distinct cellular alterations that resulted from interaction with either direct (DC) or alternating (AC) electric fields, such as electroporation [60], electrorotation [61], directed electrotaxis (i.e. cell migration) [62], and attenuation of tumor cell growth [63, 64], which varied mainly depending on the intensity of the applied field. The effect of AC fields on attenuating tumor cell growth was particularly intriguing, as the growth inhibition effect was replicated in both animal tumor model and pilot clinical trial, indicating that AC fields could be employed as an effective anti-cancer treatment to attenuate tumor growth [64].

Although Kirson et al. [64] hypothesized that AC fields likely trigger tumor growth arrest by disrupting microtubule polarization and subsequently abrogating cytokinesis into daughter cells, there lacks direct experimental data for both the dynamic process and the proposed mechanism, which in part hinders further development of AC field for clinical application. To elucidate the quantitative mechanisms underlying cell–AC field interactions, in this study we measured and analyzed the distinct cellular alterations induced by AC fields as a function of field frequency and intensity. Our primary technique for measuring cellular dynamics was
time-lapse microscopy, which allowed us to follow real-time dynamics of cellular alterations in single living cells. We chose HeLa, a cultured epithelial cell line derived from human cervical cancer, as the model cancer cell line. AC field in the form of sine wave was applied through a pair of stainless steel plates placed vertical to the imaging dish and in contact with the culture medium. We focused our study on the intermediate radio frequency range, from 100 kHz to 1000 kHz, and relatively low field intensity, from 1 V/cm to 10 V/cm, as attenuation in tumor cell growth was observed prominently in this frequency and intensity domain and the low field intensity minimized AC field induced heating.

Our live-cell imaging results revealed two distinct cell death processes induced by AC fields, i.e. death due to surface detachment or death resulting from prolonged mitotic arrest, depending mainly on frequency of the applied fields. We were able to effectively model these two processes by AC field-driven charge oscillation and field induced dielectric polarization, respectively, and calculate the frequency and intensity dependence of the cell death responses to AC fields. By comparing the modeling and experimental results, we identified the key electrical properties of proliferating adhering cell, including characteristic resonance frequency of surface charges and dielectric dispersion of cellular content in mitosis, which appear to govern cellular interaction with AC fields and the subsequent cell death process that led to attenuation of cell growth.
3.2. Materials and methods

3.2.1 Cell lines

HeLa, HeLa-H2B-GFP and HeLa-IMS-mRFP were cultured under 37 °C and 5% CO2 in Dulbecco's Modified Eagle Medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

3.2.2 Application of alternating electric field

Alternating electric field in the form of sine waves was generated by a signal generator and applied onto cells through a pair of stainless steel plates. Both plates are curved in a radian of 82.2° with an arc length of 1.53 ± 0.01 cm, and a vertical length of 1.40 ± 0.01 cm. The curvature significantly reduced the edge effect of uneven electric field strength. The two plates were mounted with the convex side facing outward and a distance of 0.89 ± 0.01 cm between the plates’ center (Figure 3.1). The metal plates were in an orientation vertical to the culture dish and in contact with culture medium, but not the bottom of the dish.

3.2.3 Time-lapse microscopy

Cells were plated in 35 mm imaging dish (μ-dish, ibidi, Germany) and cultured in Phenol Red-free CO2-independent medium (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 100 μl streptomycin. Phase-contrast and fluorescent cell images were acquired with the Nikon TE2000-PFS inverted microscope enclosed in a humidified chamber maintained at 37 °C. Cells were imaged every 10 min for 48 h using a motorized stage and a 20× objective (NA = 0.95).
3.2.4 Data analysis

Images were viewed and analyzed using the MetaMorph software (Molecular Dynamics). From the phase-contrast time-lapse movies, we scored by morphological tracking based on established criterion [25, 29]: interphase (by flat morphology), detachment from surface adhesion or entry into mitosis (by cell rounding), cell division (by re-spreading and splitting), cell-cycle arrest (by absence of cell division) and cell death (by cell lysis). To minimize variability due to uneven field strength, we only analyzed cells located near the center of the imaging dish. And more than 120 cells were scored for each experimental condition.
3.3. Results

3.3.1 Alternating electric fields activate frequency- and intensity-dependent cell death

Real-time dynamic response of HeLa cells to external AC fields was measured by time-lapse microscopy using the experimental set-up shown in Figure 3.1 (as discussed in Material and methods). AC fields were found to trigger distinct cellular alterations in an intensity- and frequency-dependent manner. At intensity below 6 V/cm, the majority of HeLa cells (>90%) progressed normally in cell cycle and divided with normal doubling time of ~20 h, for all investigated frequencies ranging from 100 kHz to 1000 kHz. However, at intensity equal to or larger than 6 V/cm, significant cell death started to be observable after exposure to AC field for 10 ~ 20 h. The characteristics and kinetics to cell death varied significantly, depending on the field frequency. At frequencies from 300 to 800 kHz, phase-contrast imaging showed HeLa cells gradually changed from flat to round-up morphology in a time scale of hours followed by cell lysis, a signature of cell death (Figure 3.2A referred to as type I cell death in the following discussions). Moreover, all cells in the field of view appeared to start rounding up nearly simultaneously with similar death kinetics (refer to grey curve in Figure 3.2B). In contrast, at frequency range of 100–300 kHz and 800–1000 kHz, although cells also changed to the round-up morphology, the onset of morphological change occurred at different times in individual cells, and the time to cell lysis (i.e. death) was much more variable (Figure 3.2A and black curves in Figure 3.2B, categorized as type II cell death). These observations indicated that AC
fields triggered cell death through two distinct cellular processes modulated by the field frequency.
**Figure 3.1.** Experimental set-up. (A) Alternating electric field was applied to cells through a pair of metal plates. The imaging dish was mounted on the microscope stage as shown. (B) Size and positions of the electrodes relative to the imaging dish.
Figure 3.2. AC fields triggered 2 types of distinct cellular alterations in an intensity- and frequency-dependent manner. (A) Still frames of phase-contrast images from time-lapse movies of HeLa cells under AC fields. Representative HeLa cells exhibited either normal cell-cycle progression and cell division (under $|E| < 6$ V/cm), or type I death following surface detachment (under $f = 500$ kHz, $|E| = 10$ V/cm), or type II death during mitotic arrest (under $f = 100$ kHz, $|E| = 9$ V/cm). Elapsed time was indicated on the top right corner of each image in unit hour. Typical cellular changes, such as detachment and morphological death, were indicated on the bottom of the images. (B) Cumulative survival curves showing the fraction of surviving cells, $F_s$, as a function of time, $t$, for the indicated AC field treatments. $F_s(t) = N(t)/N_0$, where $N(t)$ is the number of living cells at $t$ and $N_0$ is the initial number of cells at time 0. Individual cells were monitored by phase-contrast imaging, and time from the start of AC field application to cell lysis (i.e. cell death) was measured and plotted as cumulative survival curves. The total number of cells analyzed for each curve ranges from 125 to 161, and varies between conditions.
3.3.2 Cellular processes underlying the two distinct types of AC-field induced cell death

To pinpoint the cellular mechanisms that mediate the two AC field-induced cell death processes, we first determined the cell-cycle state when the two types of cell death occurred by using a live-cell fluorescent reporter, Histone-2B-GFP. Histone-2B is a nuclear protein that binds to the chromosome and by fusing Histone-2B to a green fluorescent protein (GFP), one can follow chromosome morphology in real time by time-lapse microscopy [65, 66]. As shown in Figure 3.3A, for type I cell death, Histone-2B exhibited de-condensed morphology that is a signature of cell-cycle state of interphase, indicating that the rounded up cells died from interphase and thus the gradual morphological change from flat to round-up likely corresponded to cell detachment due to loss of surface adhesion. In contrast, for type II cell death, Histone-2B was condensed and aligned at the metaphase plate, indicating cell death occurred during mitosis. Normal mitosis in general completes in 30-min to 1 h. However, under AC fields of type II frequencies (100 < f < 300 kHz and 800 < f < 1000 kHz), HeLa cells remained in mitosis for hours and eventually died from the prolonged mitotic arrest. Our results thus point to AC fields inducing type II cell death by activating mitotic arrest. AC fields likely triggered mitotic arrest by disrupting microtubule polarization, subsequently inhibiting bi-pole formation in mitosis. Failure of proper bi-pole formation, such as induced by antimitotic chemotherapeutics [21], is known to activate spindle assembly checkpoint (SAC) that arrests cells in the metaphase to anaphase transition of mitosis.
Another important mechanistic question is whether the type I and type II cell death were mediated by the common intrinsic (or mitochondrial) apoptosis pathway or other forms of programmed/nonprogrammed cell death. A key step of intrinsic apoptosis is mitochondrial outer membrane permeabilization (MOMP). To visualize MOMP by live-cell imaging, we infected HeLa cells with retrovirus encoding a reporter construct (IMS-mRFP), consisting of monomeric red fluorescent protein targeted to the inter-membrane space of mitochondria by fusion to the leader peptide of SMAC [67]. As shown in Figure 3.3B, for both type I and type II cell death, MOMP was evident preceding cell lysis (i.e. death), as an abrupt transition from punctate to smooth localization of IMS-mRFP fluorescence. Note that although mitochondrial localization was harder to visualize in rounded up mitotic cells compared to interphase cells, the punctate-to-smooth transition was easy to score unambiguously in both cell-cycle states. The observation of MOMP led us to conclude that both type I and type II death induced by the AC fields are mediated by mitochondria-dependent apoptosis, although the triggers as well as processes of these two types of cell death are mechanistically distinct, as discussed above.
Figure 3.3. Cellular processes underlying the two distinct types of AC-field induced cell death. (A) Still frames of fluorescence images of Histone-2B reporter for HeLa cells under distinct AC fields. (B) Still frames of fluorescence images of IMS-mRFP reporter.
3.3.3 Intensity and frequency dependence of type I cell death due to AC field-induced surface detachment

To determine the quantitative mechanisms that govern the AC field induced cell death processes, we measured in detail the intensity and frequency dependence of kinetics to cell death and analyzed their correlation with the electrical properties of HeLa cells based on computational models. Time to death was scored as the time elapsed from the start of AC field application to the time of cell lysis, providing a metric for the rate of cell death induction by AC fields. The inverse of average time to death, $1/T_d$, plotted versus frequency showed a bell-like shape indicative of resonance behavior, with maximum, i.e. shortest time to death, occurring around 500 kHz. Moreover, kinetics to cell death exhibited a relatively linear dependence on field intensity, with higher intensity activating faster cell death.

One likely mechanism by which AC fields triggered epithelial cell detachment is that the interaction between charged molecules on cell surface and the AC field generates electrostatic force that overcomes the adhesion force. Such gradual loss of cell adhesion eventually led to cell death. The cell had started to round up before the selected time point (i.e. 6 h after AC field application) and continued to shrink in morphology afterwards. To estimate the electrostatic force, we modeled the interaction as AC fields driving oscillation of surface charged molecules. We hypothesized that the magnitude of this driven oscillation by AC field was proportional to and thus could describe the resulting electrostatic force [68]. In another word, the larger the oscillation, the larger the electrostatic force and shorter the time to detachment as well as subsequent cell death. Based on the above
assumptions and our experimental observations that stronger AC field induced faster cell death, we proposed a model that the time to death, $T_d$, is reciprocally correlated with the magnitude of oscillation, conducted simulation with the model. And we found a fairly good fit between the theoretical prediction and the experimental data for frequency dependence. The modeling and simulation were conducted by a fellow student in the lab. As the modeling and simulation were not conducted by me, details of the modeling are not provided here. Information regarding the models and simulation results can be found in our publication (refer to [69]).

### 3.3.4 Intensity and frequency dependence of type II cell death due to AC field-induced mitotic arrest

At frequency range of 100 - 300 kHz and 800 - 1000 kHz, cell death triggered by the AC fields was found to be activated as a result of field-induced prolonged mitotic arrest. Here time to death was scored as time elapsed from the time of entry into mitosis to the time of cell lysis, as the effect of AC field on triggering cell death through disrupting bi-pole formation is mitosis specific and is thus not active during time elapsed in interphase. Note that this is different from (i.e. shorter than) the time to death plotted in Figure 3.2B, which was scored from the start of AC field application to cell lysis. Quantification of time to type II mitotic death showed that it varied depending on field frequency as well as intensity, with higher intensity and lower frequency leading to faster cell death. Moreover, the inverse of mean time to death exhibited a largely linear functional dependence on both frequency and intensity of the external AC fields.
For type II death that occurred as a result of mitotic arrest, one of the likely interacting targets of AC fields is microtubules, whose proper orientations are known to be crucial for bi-pole formation and progression of mitosis. We hypothesized that these mitotic microtubules and potentially additional cellular content could be viewed as linear dielectric material with electric susceptibility $\chi_e(\omega)$, and thus were induced to polarize in the presence of an external electric field $E(\omega)$ of angular frequency $\omega$. Due to such polarization, normal dynamics of microtubules and possibly other dielectric materials in the mitotic cell were disrupted, leading to mitotic arrest followed by cell death. Intuitively, the stronger the induced polarization, the stronger the effect of the AC field $E(\omega)$ on triggering arrest and cell death. Based on this assumption, the time to mitotic death, $Td$, is modeled to be linearly related to the degree of polarization of the dielectric material. Again, as the modeling and simulation were not conducted by me, details of the modeling are not included here. Information regarding the models and simulation results can be found in our publication (refer to [69]).
3.4. Discussion

Death due to prolonged mitotic arrest has been extensively studied as a crucial mechanism by which anti-mitotic chemotherapeutics, such as paclitaxel, exert cytotoxicity on tumor cells [21]. Our results revealed that AC fields can also effectively activate prolonged mitotic arrest followed by extensive cell death. Considering AC fields can be administered locally, affecting only the tumor and its surrounding tissues, it has the potential to achieve a similar degree of cancer cell killing but less toxicity to healthy tissues, as compared to anti-mitotic drugs. Regarding the molecular mechanism of mitotic death, our previous results and others' have shown that depletion of an important anti-apoptotic protein, Mcl-1, is a major trigger of apoptosis during prolonged mitotic arrest [29, 70, 71]. This is likely also the mechanism by which type II cell death was activated by AC fields. Moreover, responses to anti-mitotic induced cell death were found to exhibit large variation between different epithelial cell lines [25, 29]. Drug treated cells may die in mitotic arrest, or slip out of mitotic arrest into an abnormal G1 state, in which they may die, or arrest in a tetraploid G1 state. Therefore, depending on the cell line investigated, the cell death phenotypes as well as the quantitative dependence of cell death kinetics on AC field intensity and frequency would likely vary significantly due to cell-line variability in the sensitivity to apoptosis during mitotic arrest. The model cell line that we studied, HeLa, is a cell line highly sensitive to mitotic death [29] so it died extensively during mitotic arrest induced by AC fields. However, for cell lines that are resistant to mitotic death, they would slip out of the mitotic arrest to an abnormal G1 state, exhibiting phenotypes different from what we reported in this study.
Compared to AC field-induced mitotic death, the mechanism by which the cell died of surface detachment is less resolved. Loss of adhesion or proper interaction with extracellular matrix (ECM) is known to cause a particular cell death called anoikis [72], which was proposed to be mediated by both intrinsic and extrinsic apoptosis. Our observation of mitochondrial outer membrane permeabilization (MOMP), a key step of apoptosis, before type I death suggests that it is likely to be mediated by mechanism similar to anoikis. However, it is not clear how the loss of adhesion activates the apoptotic machinery. Integrins, multiple growth factor receptors and signaling kinases are known to regulate proper cellular attachment to ECM. Exactly which molecular targets AC fields interact with and how the interactions cross-activate the cell death machinery are important questions that require further investigation. We suspect AC fields disrupt a broad range of adhesion proteins.

Our study is among the very few that provides quantitative metrics for examining the electrical properties of living cells, in particular proliferating adhering cells that are common in solid tumors. Our results suggest that interaction between the surface charged molecules of adhering cells and AC fields leads to surface detachment, with a characteristic resonance frequency. Resonance behavior in living cells were previously only observed in the rotational movement of suspension cells under AC fields [73, 74]. The resonance frequency of AC field-induced rotation varied from 20 kHz to 180 kHz, depending on the cell types [73]. Major cellular factors that affect the resonance frequency include characteristics of the surface charges and the degree of multilayer compartmentalization of a cell [74]. It is likely that different cancer cell
types would also have distinct resonance frequencies in response to AC fields. Regarding the dielectric property of proliferating adhering cells, our results revealed that induced polarization significantly disrupted cellular physiology during mitosis. The degree of induced polarization as measured by kinetics to type II cell death decreased with increasing frequency (Fig. 3A), which is similar to dielectric dispersion of biological tissues [75, 76]. Relaxation times of the cellular dielectric responses to AC fields were estimated to be around 0.3–0.5 μs for mitotic HeLa cell, shorter than those measured for large tissues [76]. Although a simple Debye-type model may not completely describe the complex dielectric spectrum of biological cells and tissues [77], our simulation results demonstrated that it generated results in good agreement with our experimental observations, suggesting that the dielectric materials in mitotic cells, e.g. microtubules, may be approximated as homogeneous, non-interacting dipoles.

Although the biophysical models largely accounted for the cell death processes that we observed, there was deficiency in our estimation of the AC field strength. We used voltage data recorded from the signal generator as the field strength. However, the field strength appearing on the cells might be different from that of the applied field. Even though the inaccuracy in estimating the field strength did not affect our main conclusion, i.e. AC fields induced two mechanistically distinct cell deaths by triggering surface detachment or mitotic arrest, it brought uncertainty to the quantitative features that we simulated using the models, especially regarding the intensity dependence. In order to construct more accurate biophysical models, new tools to measure in situ electric field strength appearing on the cell together with
mechanistic understanding of how electric field is magnified or attenuated in and around the cell are clearly needed in further study.

In conclusion, by profiling real-time cellular responses to alternating electric fields as a function of frequency and intensity, we uncovered two distinct mechanisms by which AC fields activate mammalian epithelial cell death. At intermediate radio-frequency, cell death occurred as a result of loss of surface adhesion, which we were able to describe and model by oscillation of surface charged molecules driven by the AC fields. In contrast, at low and high radio-frequency, we observed cell death as a result of prolonged mitotic arrest triggered by the AC fields. The quantitative and biophysical nature of this mitotic death process was effectively simulated by AC-field induced dielectric polarization of cellular content during mitosis. Our results thus provided not only new mechanistic insight for understanding the process of AC field-induced tumor cell death [63, 64] but also useful metrics for evaluating the electrical properties of proliferating adhering cells for cellular engineering application.
Chapter 4  Cytotoxic Dynamics of Natural Killer (NK) Cells towards distinct target cancer cells

4.1. Introduction

NK cells are large granular lymphocytes belonging to the innate immune system, which can spontaneously lyse target cells, such as virus-infected cells and cancer cells, without prior stimulation [18, 78]. NK cells comprise about 10% to 15% of human peripheral blood lymphocytes (PBL) and are also distributed in peripheral tissues, including the liver, peritoneal cavity and placenta [78-80]. NK cells generally participate in the early step against viral infection and abnormal cells [80-82]. NK cells can kill virus-infected cells or cancer cells directly through perforin/granzyme-mediated pathway and/or death receptor pathway [18, 80, 83]. Perforin/granzyme-mediated apoptosis is considered the principal pathway used by NK cells to kill and eliminate virus-infected and/or cancer cells. At the NK-cancer cell junction, cytotoxic granules, which contain perforin and granzymes (a type of protease), are released from NK cell into the cancer cell in an oriented manner. Perforin functions by disturbing the cancer cell membrane and allowing granzymes to enter the target cell. These granzymes subsequently induce apoptosis of the target cell through both caspase-dependent and caspase–independent pathways [80, 84]. Alternatively, NK cells also express FAS ligand, tumor necrosis factor (TNF) and TNF-related apoptosis inducing ligand (TRAIL). Through the ligation of these ligands on the NK cells surface with their cognate death receptors expressed on the target cells, the death-receptor pathway is activated, which triggers the classic extrinsic apoptosis [80].

Besides triggering direct cytotoxicity, NK cell can also activate other immune cell
types by secreting effector cytokines and chemokines that influence the host’s immune response. Interferon gamma (IFN-γ) is considered a particularly important NK cell cytokine. IFN-γ produced by NK cell can induce T helper cells immune response, activate antigen-presenting cells (APCs) to further up-regulate class I major histocompatibility complex (MHCI) expression, and activate macrophage to kill intracellular pathogens [83, 85-87].

Activation of the NK cell function is regulated by the competing signals transmitted by the activating and inhibitory receptors on NK cell surface. Every NK cell expresses a series of activating receptors, which recognize ligands on virus-infected and cancer cells, and inhibitory receptors, which recognize MHCI molecules expressed by all nucleated cells. Under non-stimulated conditions, the inhibitory receptors on the NK cell surface prevent NK cells from killing the normal cells by interacting with their MHCI molecules. Upon binding to the activating ligands on the virus-infected cells and cancer cells, signaling from the activating receptors on NK cell is dominant and outweighs the inhibitory signaling, thus activating the cytotoxic activity of NK cell towards the target cell [80, 88]. Cancer cell could be particularly sensitive to NK cell killing, as many cancers have down-regulated MHCI expression. In general, the overall balance of activating and inhibitory signaling determines the extent of NK cell response and the outcome of NK-target interaction.

In addition to regulation by the different surface receptors, activity of the NK cells is also regulated by various cytokines. Recent data showed that NK cells require priming by distinct cytokines in order to achieve full effector potential, highlighting the intricate regulatory interactions between NK cells and other components of the
immune system. In particular, NK cells are known to respond to interleukin (IL)-2, IL-12, IL-15 and IFNs (α, β and γ), which increase NK cell’s cytolytic, secretory, proliferative and anticancer functions [80, 89, 90].

Although NK-cancer cell interaction is known to play an important role in regulating tumor physiology, question remains how characteristics of their interaction dynamics affect the killing efficacy of NK cell towards distinct cancer targets. In the past decade, a number of research groups have examined the interaction dynamics between NK and cancer cells at the single cell level, using live-cell imaging and microfluidic platform [84, 91-93]. By monitoring behaviors of individual cells and individual interaction intermediates, they identified interesting dynamic features involved in NK cell killing, such as population heterogeneity and serial killing [91-93]. For instance, by utilizing a microchip-based time-lapse imaging approach, Yamanaka et al found that NK cells operate independently when lysing the cancer targets, and target cell lysis is most likely to occur when an NK cell encounter a target the first time [91]. Moreover, Vanherberghen et al showed that approximately half of the NK cells do not kill any cancer target, with a minority of NK cells being responsible for the majority of cancer cell killing [93]. In addition, the interaction between NK and cancer cells appeared to involve the secretion of distinct cytokines, such as interferon- γ (IFN- γ), but these cytokines may not have direct effect on the cytolytic activity of NK cells [91]. Overall, these findings not only demonstrate the importance and advantage of studying the dynamics of NK-cancer cell interactions at the single cell level but also point to the need of more further studies to identify the rate-limiting kinetics in the killing process and dynamic determinants of the cytolytic
activity of NK cells towards distinct cancer targets.

In order to employ NK cell as an alternative cytotoxic regimen to kill cancer cells, one first needs to understand the cytotoxic mechanisms underlying NK-cancer cell interactions and identify, if there is any, cell-type variation associated with the interaction process. Therefore, in this study we will quantify the real-time cytotoxic dynamics of primary NK cells purified from human blood against distinct target cancer cell lines. By imaging live-cell fluorescent reporters, we will correlate the observed dynamics with specific molecular alteration and explore the underlying cytotoxic mechanisms. We will also characterize how cytotoxic dynamics vary between primary human NK cells and a stable NK cell line, NK-92MI, as well as between different cancer targets.
4.2. Materials and methods

4.2.1 Cell lines

HeLa cells were grown in Dulbecco’s modified Eagles medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100U/mL penicillin and 100μg/mL streptomycin (Invitrogen). U-2 OS cells were grown in McCoy’s medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 100U/mL penicillin and 100μg/mL streptomycin (Invitrogen). Primary NK cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 0.2 ng/mL IL-2 (Invitrogen), 10% FBS (Invitrogen), 100U/mL penicillin and 100μg/mL streptomycin (Invitrogen). NK-92MI cells (ATCC; CRL-2408) were cultured in MyeloCult™ H5100 Medium (STEMCELL Technologies). All cell lines were maintained with 5% CO₂ at 37°C in the humidified incubator.

4.2.2 Generation of fluorescent reporter cell lines

Clonal HeLa and U-2 OS reporter cells were generated by viral infection to stably express double fluorescent reporters of either IMS-RP plus GzmB-CFP-YFP or IMS-RP plus caspase8-CFP-YFP. The IMS-RP retroviral construct is a reporter of mitochondrial outer membrane permeabilization (MOMP), as discussed in chapter 2 and 3. The GzmB-CFP-YFP is a FRET reporter of granzyme B activity, consisting of cyan (CFP, donor) and yellow (YFP, receptor) fluorescent protein linked by a peptide substrate specific for granzyme B, i.e. VGPDFGR [84]. Similarly, the caspase8-CFP-YFP construct reports on caspase 8 activity by linking CFP and YFP by the tandem copies of IETD, which is a substrate specific for caspase 8 [94].
Both the GzmB-CFP-YFP reporter and the caspase8-CFP-YFP reporter report on the respective protease activity by Förster resonance energy transfer (FRET). When the linker peptide is intact (i.e. without protease), CFP emission is transferred to YFP, resulting in YFP fluorescence. Upon linker cleavage by the specific protease, YFP is no longer in close proximity to CFP to allow energy transfer and thus CFP fluorescence is observed. By monitoring the characteristic change in the fluorescence from YFP to CFP (i.e. FRET), we can determine whether granzyme B and caspase 8 activities precede target cell death upon interaction with NK cells. This provides the crucial information regarding the cytotoxic mechanism of primary NK cells towards cultured cancer targets.

4.2.3 Primary NK cells isolation and purification

Isolation of primary NK cells from human blood consists of two main steps: isolation of Peripheral Blood Mononuclear Cells (PBMCs) from human blood; and subsequent isolation of NK cells from the PMBCs.

Peripheral Blood Mononuclear Cells (PBMCs), which mainly consist of lymphocytes, monocytes and also a small amount of platelets, were isolated from human buffy coat by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare) [95, 96]. Specifically, fresh human buffy coat from healthy donor (obtained from Hong Kong Red Cross) was diluted and gently added onto the top of the Ficoll-Paque Plus solution, followed by low speed centrifugation. After the centrifugation, four layers would appear as a result of the density gradient and differential migration. The upper layer was plasma, which was free of cells. The
interface between the plasma and the Ficoll-Paque PLUS solution was the PBMCs layer consisting of lymphocytes (NK cells, T cells and B cells), monocytes and platelets. The bottom layer below the Ficoll-Paque PLUS solution was aggregated erythrocytes and granulocyte.

From the PBMCs, Dynabeads® UntouchedTM Human NK Cells Kit (invitrogen) was used to isolate fresh primary NK cells. Briefly, an antibody mixture that binds to non-NK cells was added and incubated with PBMCs. Dynabeads were then added and incubated with the mixture at room temperature. As the antibody-labeled cells would be bounded to Dynabeads and be removed by a magnetic force, pure and viable untouched NK cells were obtained through this negative selection method.

4.2.4 Chemicals

Interleukin (IL)-2 was purchased from Invitrogen and used at the concentration of 0.2 ng/mL (the minimal concentration for maintaining primary NK cells in culture) and 50 ng/mL (the saturating concentration for polarization and activation of primary NK cells).

4.2.5 Time-lapse microscopy and Data analysis

Cells were plated in 12-well glass-bottom imaging plate (MatTek, USA) and cultured in phenol red-free CO₂-independent medium (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin and 100μg/mL streptomycin. Cell images were acquired with the Nikon Ti-Eclipse-PFS inverted microscope enclosed in a humidified chamber maintained at 37°C. Cells were imaged every 3-10 minutes using
a motorized stage and a 20x objective. Images were viewed and analyzed using the MetaMorph software (Molecular Dynamics).
4.3. Results

4.3.1 Cytotoxic characteristics of freshly isolated primary human NK cells in comparison with the highly activated NK-92MI

To characterize the cytotoxic activity of primary human NK cells towards different cancer targets, we first quantified the killing dynamics of primary NK cell in comparison with a highly activated NK cell line, NK-92MI, using bright-field time-lapse microscopy. In our experiments, we treated the primary NK cells with a minimal concentration of Interleukine 2 (IL-2, 0.2 ng/mL) to maintain its survival, as (IL-2) sustains survival of primary NK cells both in vitro and in vivo [78, 90, 94]. We then compared the cytotoxic dynamics of primary NK cells under low IL-2 with those highly activated with a high concentration of IL-2 (50 ng/mL, saturating concentration). High level of IL-2 is known to enhance the cytolytic and killing activity of primary NK cells. In addition to primary NK cells, we also quantified the cytotoxic dynamics of a cultured human NK cell line, NK-92MI. NK92-MI is a NK cell line engineered to stably express and secret IL-2. NK-92MI cells are thus highly activated and presumably elicit the most efficient killing of target cells. We used NK-92MI as a positive control so as to determine the similarity as well as difference between the killing dynamics and mechanisms of primary NK cells and those well-known for NK-92MI. We chose HeLa (derived from cervical cancer) and U-2 OS cells (derive from bone cancer) as the model target cancer cell lines for the initial characterization. The target cells, i.e. HeLa cells or U-2 OS cells, were co-cultured with the freshly isolated primary human NK cells at target-to-NK (Effector cell) (T:E) ratios of 1:2 and 1:5, respectively. For the co-culture of target cancer cells with NK-
92MI cells, the T:E ratio was chosen to be 1:2. Time-lapse experiments based on phase-contrast imaging were carried out on the co-cultured cells upon addition of IL-2.

Figure 4.1 shows phase-contrast images of primary NK cells or NK-92MI in co-culture with U-2 OS. The small, suspending NK cells are easily distinguishable morphologically from the large, adherent target cancer cell, U-2 OS. Three dynamic features of primary NK cells are immediately noticeable when interacting with U-2 OS: (1) Primary NK cells changed from ball-like shape to an elongated, polarized morphology in the timescale of 6-10 hrs after co-culture. The polarized NK cells exhibited the characteristic structure of a leading edge followed by the cell body and the tail or uropod, known to be important for both NK cell activation and migration. (2) Contacts between primary NK and U-2 OS cells (scored by co-localization of the two cell types) were in general short, about 5-10 minutes. More than 90% of the contacts were not followed by target cell lysis. (3) The cytotoxic kinetics of primary NK cells was much slower than that observed in NK-92MI. At the primary NK-target cell ratio of 5:1, little target cell death occurred until 24 hours after the co-culture. Pre-activating primary NK cells with IL-2 significantly increased the killing (Figure 4.2).

NK-92MI cells induced potent and rapid cell death in both HeLa and U-2 OS cells. Almost all the target cells were killed by NK-92MI within about 10 hrs, which was consistent with previous results (Figure 4.2). The cell size of NK-92MI appeared to be much larger than that of primary NK cells, and NK-92MI cells killed in a non-polarized state. These observations may indicate that NK-92MI contains a large
amount of cytotoxic granules and therefore is capable of eliciting much more effective killing of target cells. From the cumulative survival curves of primary NK cells, an initial plateau could be observed before the curves started to drop (i.e. when target cells started to die due to NK cell killing), which was not observed for NK-92MI. This indicated that the freshly isolated primary NK cells may require a period of time to active its killing machinery, while NK-92MI cells are already fully primed to kill immediately upon recognition of target cancer cells. This initial activation of primary NK cells appear to correlate with their polarization, as discussed below.
Figure 4.1. Still frames of co-culture of effector NK cells and target cancer cells. Upper panel is the still frames of U-2 OS cells co-cultured with primary NK cells in high level of IL-2 (hIL-2, 50ng/mL). Lower panel is the still frames of U-2 OS cells co-cultured with NK-92MI.
Figure 4.2. Cumulative Survival curve of target cancer cells in co-culture with NK cells. Individual cells were monitored by phase-contrast imaging, and time from the start of NK-cancer cells co-culture to cell death was measured and plotted as cumulative survival curves. The total number of cells analyzed for each curve ranges from 75 to 92. (A) Survival curves of HeLa cells with primary NK cells (pNK) in low level of IL-2 (lIL-2, 0.5ng/mL) or in high level of IL-2 (hIL-2, 20ng/mL), or NK-92 MI (NK92) and (B) Survival curves of U-2 OS cells with primary NK cells (pNK) in low level of IL-2 (lIL-2, 0.5ng/mL) or in high level of IL-2 (hIL-2, 20ng/mL), or NK-92 MI (NK92).
4.3.2 Target cell-type dependence of primary NK cell killing

One important cytotoxic difference that we observed is in the killing efficacy of primary NK cells towards distinct epithelial cancer targets. As shown in Figure 2, HeLa is much more resistant to primary NK cell killing than U-2 OS. And pre-activation of primary NK cells by IL-2 enhanced cytotoxicity to U-2 OS cells much more than HeLa cells. The striking target variation raises the important question over mechanisms that govern the target specificity of NK cell activation and killing. The mechanistic origins of variability in NK cell killing with respect to the genetic heterogeneity of cancer targets are clearly in need of further investigation. Elucidating how pathways that govern NK-cancer cell interaction are differentially regulated and how these differences alter NK cell cytotoxicity towards distinct cancer targets will provide crucial new insight for understanding both NK cell regulation and cancer immunogenicity.

4.3.3 Primary NK cells kill mostly by granzym-B independent mechanism

To explore the killing mechanisms of primary NK cells, we generated fluorescent HeLa and U-2 OS cells that stably express FRET (Förster Resonance Energy Transfer) construct reporting on either granzyme B or caspase 8 activity. The FRET reporter consists of cyan (CFP, donor) and yellow (YFP, receptor) fluorescent protein linked by peptide substrate of the respective protease, i.e. VGPDFGR for granzyme B [84] and tandem copies of IETD for caspase 8 [94]. Cell death activated by NK-92MI were all preceded by FRET of the granzyme B reporter (i.e. decrease of YFP fluorescence and increase of CFP upon linker cleavage), indicating that the
cytotoxic process is mediated by granzyme B (Figure 4.3). This observation agreed with results reported previously by Choi et al (figure 1 of ref [84]). However, primary NK cells did not activate granzyme B-dependent FRET, but only FRET signal from the caspase 8 reporter (Figure 4.3), upon the killing of both U-2 OS cells and HeLa cells. This suggests that primary NK cells in culture did not kill epithelial cancer cell only by the granzyme B-dependent pathway, but likely also by other cytotoxic mechanism, for example, extrinsic apoptosis mediated by death ligands (e.g. Fas ligand). One intriguing new question, which we will investigate in further study, is what molecular components and cellular processes engender the above difference in rate-limiting kinetics and cytotoxic mechanisms between primary NK cell and the highly activated NK-92MI.
Figure 4.3. Fluorescent images of Granzyme B FRET reporter and Caspase-8 FRET reporter in target cancer cell. Fluorescent images of the Granzyme B FRET reporter (upper panel) and Caspase-8 FRET reporter (lower panel) from U-2 OS cells alone (1st column), U-2 OS in co-culture with primary NK cells for 24 hrs (2nd column) and U-2 OS in co-culture with NK-92MI for 10 hrs (3rd column). The images are overlay of the CFP (indicated by blue) emission and YFP (indicated by green) emission.
4.3.4 Polarization kinetics of the freshly isolated primary human NK cells

As discussed above, the freshly isolated primary NK cells showed round morphology and gradually became polarized (Figure 4.1). Based on the phase-contrast movies, we identified two distinct polarization states of primary NK cells, one with a round head and short tail (denoted as “semi-polarized”) and the other showing an elongated head and tail (denoted as “fully-polarized”) (Figure 4.4A). By counting the number of polarized vs. non-polarized cells as a function of time based on the time-lapse movies, we quantified the extent and kinetics of polarization of primary NK cells. The ratio of fully polarized primary NK cells and semi- plus fully polarized NK cells to the total number of NK cells in the field of view was shown in Figure 4.4B. The polarization curves illustrated that polarization of the primary NK cells was enhanced and accelerated by addition of IL-2 in the culture medium. The polarization dynamics again showed target cell type dependence. Although the extent of NK cell polarization (semi- plus fully polarized cells) was largely similarly under co-culture with HeLa and U-2 OS cells, the polarization kinetics was evidently faster under U-2 OS. Moreover, interaction with U-2 OS cells led to a higher level of fully polarized NK cells, which may contribute to the higher cytotoxicity of primary NK cells towards U-2 OS cells.
**Figure 4.4.** Polarization kinetics of the freshly isolated primary human NK cells. (A) The typical images of non-polarized (right), semi-polarized (middle) and fully-polarized (left) primary NK cells. (B) Fraction of polarized primary NK cells in the co-culture with HeLa. (C) Fraction of polarized primary NK cells in the co-culture with U-2 OS.
4.3.5 Enhanced cytotoxicity of cultured and pre-activated primary NK cells

To examine the effect of priming and activating primary NK cells before the coculture experiments, we cultured the freshly isolated primary human NK cells for 3 days with low level of IL-2 (0.2 ng/ml). The 3-day culture induced full polarization of more than 95% of the primary NK cells. These fully polarized and presumably activated primary NK cells elicited very efficient killing of both U-2 OS and HeLa cells, with cytotoxic dynamics comparable to those observed for NK-92MI (Figure 4.5). HeLa cells again showed slower and less cell death as compared to U-2 OS. Interestingly, the fast killing by the cultured primary NK cells still did not show FRET signal from the granzyme B reporter, as discussed above. This suggests that primary NK cells indeed utilized cytotoxic mechanism distinct from that used by NK-92MI at least in cell culture.
Figure 4.5. Cumulative Survival curve of target cancer cells in co-culture with cultured, pre-activated primary NK cells. The total number of cells analyzed for each curve ranges from 55 to 85. (A) HeLa cells in co-culture with cultured primary NK cells (pNK) in low level of IL-2 (lIL-2, 0.5ng/mL) or in high level of IL-2 (hIL-2, 20ng/mL), or NK-92 MI (NK92) and (B) U-2 OS cells in co-culture with cultured primary NK cells (pNK) in low level of IL-2 (lIL-2, 0.5ng/mL) or in high level of IL-2 (hIL-2, 20ng/mL), or NK-92 MI (NK92).
4.4. Future study

The above preliminary data on cytotoxic dynamics of primary NK cells pointed to multiple new angles for elucidating its mechanistic basis, which will provide important insight to potentially use NK cell as an alternative cytotoxic therapy for cancer treatment. Firstly, as the majority of target cells died after multiple interactions with the NK cells, it raised the question of how contact frequency and contact duration between NK and its target affect the killing dynamics. To acquire data on contact frequency and duration truly at the single cell level, the current bulk assay, i.e. co-cultures of a large number of NK cells with target cells, is deemed not suitable, as it is extremely difficult to track single NK cells when a large number of them move quickly in the field of view. Therefore, we are currently developing microfluidic chips to monitor single cell dynamics and accurately control NK-target cell ratio for live-cell imaging analysis. Specifically, in collaboration with Prof. S. Pang at the City University of Hong Kong, we are engineering prototypes of microfluidic chips, i.e. arrays of polydimethylsiloxane (PDMS) nanowell columns of 50-100 µm (Figure 4.6), and plan to use these microfluidic chips to encage single target cancer cell with different number of NK cells (e.g. 1 to 5) and measure the interaction dynamics at the single cell level. Moreover, we also plan to fabricate microfluidic channels, whose width is similar to the cancer cells, to measure NK cell chemotatic response to the cancer target. As the narrow channels will trap cancer target and constrain primary NK cells to one-dimension (1D) trajectories, it will allow us to track and quantify the motion of NK cells more easily by automated image analysis and conduct comprehensive analysis of the chemotatic dynamics of NK cells in distinct chemical
At the mechanistic level, we will explore molecular pathways that regulate the cytotoxic activity of primary NK cells. Given its independence of granzyme B activity, the killing by primary NK cells in culture could be mediated by two alternative mechanisms, one involving distinct types of granzymes (e.g., granzyme M), and the other involving extrinsic apoptosis activated through Fas receptor. To test the first possibility, we will first examine whether pore formation on the target cell membrane, which is required for delivery of the granzymes into target cell, is involved in the cytotoxic process. To monitor pore formation, we will add Propidium iodide (PI) into the culture medium. If accumulation of PI fluorescence is observed in the cytoplasm of the target cell prior to cell death, it will indicate that pore formation and cytolytic granule are likely involved in mediating the killing process [94, 97]. We will then use techniques, such as western blotting and gene knockdown, to identify the specific type of granzyme(s) involved in the process. If PI fluorescence is not observed prior to cell death, it will indicate that the cytotoxic process is not dependent on delivery of cytolytic granule and granzyme. In this case, we will use neutralizing antibody to Fas receptor to examine the role of extrinsic apoptosis in activating the observed NK cell killing. If Fas receptor is also found not to be involved in mediating the process, we will perform microarray and cytokine/chemokine array analysis to profile the gene expression and cytokine/chemokine secretion of primary NK cells as a function of time, so as to identify molecular changes that correlate with the NK cell cytotoxicity for further mechanistic study.
**Figure 4.6.** NK-cancer cell interaction dynamics assay with microfluidic chips and time-lapse imaging experimental platform. Upper panel: prototype of microfluidic chip with arrays of 100 μm nanowells. Lower panel: representative images of NK92-MI in co-culture with U-2 OS cells at ratio of 1:1, 2:1 and 3:1 (left to right).
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