Prevention of chemotherapy toxicity by agents that neutralize or degrade cell-free chromatin


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Background: Toxicity associated with chemotherapy is a major therapeutic challenge and is caused by chemotherapy-induced DNA damage and inflammation. We have recently reported that cell-free chromatin (cfCh) fragments released from dying cells can readily enter into healthy cells of the body to integrate into their genomes and induce DNA double-strand breaks, apoptosis and inflammation in them. We hypothesized that much of the toxicity of chemotherapy might be due to release of large quantities of cfCh from dying cells that could trigger an exaggerated DNA damage, apoptotic and inflammatory response in healthy cells over and above that caused by the drugs themselves.

Methods: We tested this hypothesis by administering cfCh neutralizing/degrading agents namely, anti-histone antibody complexed nanoparticles, DNase I and a novel DNA degrading agent—Resveratrol-Cu concurrently with five different chemotherapeutic agents to examine if chemotherapy-induced toxicity could be minimized.

Results: We observed (i) significant reduction in chemotherapy-induced surge of cfCh in blood; (ii) significant reduction in chemotherapy-induced surge of inflammatory cytokines CRP, IL-6, IFN and TNFα in blood; (iii) abolition of chemotherapy-induced tissue DNA damage (γH2AX), apoptosis (active caspase-3) and inflammation (NFκB and IL-6) in multiple organs and peripheral blood mononuclear cells; (iv) prevention of prolonged neutropenia following a single injection of adriamycin and (v) significant reduction in death following a lethal dose of adriamycin.

Conclusion: Our results suggest that toxicity of chemotherapy is caused to a large extent by cfCh released from dying cells and can be prevented by concurrent treatment with cfCh neutralizing/degrading agents.

Key words: chemotherapy toxicity, prevention of chemotherapy toxicity, dying cells, cell-free chromatin, neutralizing cell-free chromatin

Introduction

Fifteen million new cases of cancer are detected globally each year [1], the majority of whom receive some form of chemotherapy and suffer varying degrees of its toxic side-effects. Chemotherapy-induced toxicity results from systemic DNA damage and inflammation of healthy cells [2–4]. Symptoms of toxicity include bone marrow suppression, sore mouth, nausea and vomiting, diarrhoea, loss of appetite, fatigue, hair loss, sterility and nerve damage [5]. Several antidotes are usually prescribed to mitigate the toxic symptoms of chemotherapy with varying degrees of success [5]. We have recently reported that cell-free chromatin (cfCh) released from dying cells can readily enter into healthy cells of the body to integrate into their genomes to induce DNA double-strand breaks apoptosis and inflammation in them [6, 7]. We show here that chemotherapy-induced toxicity can be traced to these findings in that most of the toxic side-effects of chemotherapy are caused by cfCh released from dying cells. The initial round of chemotherapy-induced cell death triggers a cascading effect whereby the dead cells release more cfCh causing further rounds of DNA damage, apoptosis and inflammation thereby exaggerating or amplifying the toxic effects of chemotherapy. We also show that chemo-toxicity can be largely prevented by cfCh neutralizing/degrading agents administered concurrently with chemotherapy.
Methods

Institutional Animal Ethics Committee approval

The experimental protocol was approved by the Institutional Animal Ethics Committee of Advanced Centre for Treatment, Research and Education in Cancer, Tata Memorial Centre, Navi Mumbai, India and experiments were carried out according to the Committee’s animal safety guidelines.

Chemotherapy drugs and dosage

The chemotherapy drugs used in this study were: adriamycin (10 mg/kg), cyclophosphamide (100 mg/kg), cisplatin (20 mg/kg), methotrexate (25 mg/kg) and paclitaxel (10 mg/kg). All drugs were administered as a single i.p. dose.

Preparation of cfCh neutralizing/degrading agents

Pullulan-histone antibody nanoconjugates (CNPs) were synthesized as described by us earlier except that we exclusively used H4 IgG for preparing CNPs [6, 8]. We have shown that CNPs specifically bind to chromatin fragments and inactivate them both \textit{in vitro} and \textit{in vivo} [6, 8].

DNase I. Bovine pancreatic DNase I was obtained from Sigma-Aldrich (Catalogue No. DN25-1G).

Resveratrol-copper (R-Cu). Resveratrol (R) is a plant polyphenol which has been widely investigated for its antioxidant properties [9]. We and others have shown that R acts as a pro-oxidant in the presence of copper (Cu) by its ability to reduce Cu (II) to Cu (I) thereby generating a free radical [10, 11]. Because of this pro-oxidant property, R-Cu can degrade genomic DNA [12], and this pro-oxidant property is retained even when the molar concentration of Cu is reduced more than 10 000-fold with respect to that of R [12]. The detailed methodology for preparing R-Cu for \textit{in vitro} and \textit{in vivo} experiments is given in supplementary materials, available at Annals of Oncology online.

Isolation of cfCh from mouse serum and assessment of biological activity

The method for cfCh isolation from serum has been described by us in detail earlier [6]. Biological activity of the isolated cfCh [6] was ascertained by treating NIH3T3 mouse fibroblast cells with the cfCh isolates (10 ng of DNA) and analysing the activation of H2AX, active caspase-3, NFκB and IL-6 at 6 h by indirect immunofluorescence as described earlier [6, 7]. Detailed methodology for isolation of cfCh from mouse serum and assessment of biological activity is given in supplementary materials, available at Annals of Oncology online. The list of sources and catalogue numbers of the primary and secondary antibodies used is given in supplementary Table S1, available at Annals of Oncology online.

Estimation of serum cfCh and inflammatory cytokines following adriamycin treatment

Serum cfCh was estimated using the Cell Death Detection ELISA PLUS kit (Roche Diagnostics GmbH, Mannheim, Germany) [6] and

\begin{figure}
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Activation of H2AX, active caspase-3, NFκB and IL-6 in NIH3T3 cells treated with cfCh (10 ng DNA) isolated from mouse serum at 6 h with and without concurrent treatment with CNPs, DNase I and R-Cu. The bars represent mean ± SEM values of duplicate experiments in each group. Statistical comparison was carried out using unpaired Student’s \textit{t}-test. **** \textit{p} < 0.0001.}
\end{figure}
results expressed in arbitrary units in the form of absorbance value in spectrophotometer. Inflammatory cytokines viz., CRP, IL-6, TNF-α and IFN-γ were estimated by enzyme-linked immunosorbent assay (ELISA) according to vendor’s protocol (BD and R&D biosciences ELISA kits). Mean values (±SEM) were determined and compared between groups using unpaired Student’s t-test. Details of estimation of serum cfCh and inflammatory cytokines following adriamycin treatment are given in supplementary materials, available at Annals of Oncology online.

![Graphs showing absorption results](annalsofoncology-author-article-56.png)

**Figure 2.** Prevention of cfCh and inflammatory cytokine surge in blood at 18 h following a single i.p. injection of adriamycin by concurrent treatment with CNPs, DNase I and R-Cu. (A) Prevention of cfCh. Results are expressed in arbitrary units in the form of absorbance value in spectrophotometer. (B) Prevention of inflammatory cytokines. The bars represent mean values ± SEM of five animals in each group. Statistical comparison was carried out using unpaired Student’s t-test. * = p < 0.05; ** = p < 0.01; *** = p < 0.001; **** = p < 0.0001.

Detection of cellular DNA-damage, apoptosis and inflammation in various organs, tissues and peripheral blood mononuclear cells

Detailed methodology for detecting cellular DNA-damage, apoptosis and inflammation in various organs, tissues and peripheral blood mononuclear cells (PBMCs) is given in supplementary materials, available at Annals of Oncology online. Activation of the various parameters was examined by indirect immuno-fluorescence [6, 7].
Assessment of neutropenia

Detailed methodology for assessing neutropenia is given in supplementary materials, available at Annals of Oncology online.

Survival analysis

Forty animals were divided into 4 groups of 10 mice each and all groups received a lethal dose of adriamycin (20 mg/kg). CNPs, DNase I and R-Cu were administered in doses and frequencies as described above. Survival between groups was compared by Kaplan–Meier survival analysis using log-rank test.

Results

Since we had earlier demonstrated that cfCh isolated from serum of human subjects can phosphorylate H2AX and active caspase-3 [6], we first investigated whether mouse cfCh was also capable of activating DNA damage and apoptosis when added to cells in culture. We also examined whether mouse cfCh could, in addition, activate inflammatory cytokines. Figure 1 and supplementary Figure S1, available at Annals of Oncology online, show that addition of 10 ng DNA equivalent of cfCh isolated from mouse serum when added to NIH3T3 cells caused an approximately threefold increase in activities of γH2AX, active caspase-3, NFκB and IL-6 at 6 h. All these activities could be neutralized by simultaneous addition of cfCh neutralizing/degrading agents namely, CNPs, DNase I and R-Cu (P < 0.0001).

We next examined whether cfCh neutralizing/degrading agents namely, CNPs, DNase I and R-Cu could prevent the systemic release of cfCh and inflammatory cytokines into the circulation following a single injection of adriamycin. We first determined the time-point when the maximum surge of cfCh and inflammatory cytokines occurred following adriamycin challenge (10 mg/kg) (supplementary Figure 2A and B, available at Annals of Oncology online). In case of cfCh, we observed two peaks occurring at 6 and 18 h, while in case of inflammatory cytokines (IL-6), we observed a single peak that occurred at 18 h. We chose the 18 h time-point for our experiments using the cfCh inhibitors. We injected mice with adriamycin (10 mg/kg) with and without concurrent treatment with CNPs, DNase I and R-Cu and collected blood samples by orbital puncture at 18 h. The marked rise in serum cfCh that occurred following adriamycin injection could be completely neutralized by concurrent treatment with the three cfCh neutralizing/degrading agents (Figure 2A) (P < 0.001). We also observed a marked rise in inflammatory cytokines CRP, IL-6, IFN-γ and TNF-α following adriamycin challenge. These could be significantly inhibited by CNPs, DNase I and R-Cu (Figure 2B) (P < 0.01–0.0001).

We next examined whether CNPs, DNase I and R-Cu could prevent tissue DNA damage and inflammation following administration of 5 different chemotherapy agents namely, adriamycin, cyclophosphamide, cisplatin, methotrexate and paclitaxel. A single dose of adriamycin induced remarkable cellular activation of H2AX, active Capsase-3, NFκB and IL-6 in lung, liver, heart, brain, ovary, skin and small intestine (Figure 3 and supplementary Figures S3A–D, available at Annals of Oncology online) (P < 0.01–0.0001). Concurrent administration of CNPs, DNase I and R-Cu was highly effective in preventing DNA damage, apoptosis and inflammation in all organs examined (P < 0.05 to <0.0001). In most cases the three cfCh neutralizing/degrading

Figure 3. Prevention of cellular DNA damage (γH2AX), apoptosis (active caspase 3) and inflammation (NFκB and IL-6) in various tissues following a single ip. injection of adriamycin by concurrent treatment with CNPs, DNase I and R-Cu. Estimation of γH2AX and active caspase-3 were done at 24 h whereas NFκB and IL-6 were estimated at 72 h. The bars represent mean values ± SEM of five animals in each group. Statistical comparison was carried out using unpaired Student’s t-test. **P < 0.01; ***P < 0.001; ****P < 0.0001.
agents could reduce the activated levels of the tissue parameters to those obtained in control animals (supplementary Table S2, available at Annals of Oncology online). These data show that cfCh neutralizing/degrading agents could inhibit Adriamycin-induced DNA damage, apoptosis and inflammation indicating that cfCh were responsible for inducing these cellular pathologies.

With respect to the other chemotherapy agents namely, cyclophosphamide, cisplatin, methotrexate and paclitaxel, we restricted our investigation to the study of activation of H2AX and NFκB in brain and PBMCs (Figure 4A–D and supplementary Figures S4A–D, available at Annals of Oncology online). All four chemotherapeutic agents induced DNA damage and inflammation in brain and PBMCs, albeit to varying degrees ($P < 0.05$ to $< 0.0001$). However, in each case, the administration of CNPs, DNase I and R-Cu concurrently with the respective chemotherapeutic agents caused significant reduction in the activation of these parameters ($P < 0.05$ to $< 0.0001$).

**Figure 4.** Prevention of cellular DNA damage ($\gamma$H2AX) and inflammation (NFκB) in brain and PBMCs of mice following a single i.p. injection of cyclophosphamide (A), cisplatin (B), methotrexate (C) and paclitaxel (D) by concurrent treatment with CNPs, DNase I and R-Cu. All animals were sacrificed at 72 h except in case of the $\gamma$H2AX-cisplatin experiment wherein animals were sacrificed at 24 h. The bars represent mean values $\pm$ SEM of five animals in each group. Statistical comparison was carried out using unpaired Student’s t-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. 
We next examined the effect of CNPs, DNase I and R-Cu in preventing neutropenia caused by adriamycin. A single injection of the drug (10 mg/kg) induced a significant reduction in total leukocyte count that reached a nadir at 24 h and remained low for several days (Figure 5A–C). Concurrent administration of CNPs, DNase I and R-Cu prevented neutropenia to a great extent throughout its course. Values at most time-points, with a few exceptions, were statistically significant ($P < 0.05$ to $<0.0001$).

Finally, we show that death from a lethal dose of adriamycin (20 mg/kg) could be prevented or delayed by concurrent administration of cfCh neutralizing/degrading agents (Figure 6). All animals in the control (adriamycin alone) group died by day 6, whereas 50% and 30% of animals treated with R-Cu and DNase I, respectively, survived ($P < 0.0001$ and $P < 0.05$, respectively). With respect to the group of mice treated with CNPs, although all animals ultimately died, their survival was significantly prolonged ($P < 0.01$).
**Discussion**

It is the current belief that toxic side-effects of chemotherapy-induced systemic cellular DNA damage and inflammation are entirely caused by the drugs themselves. However, a puzzle relating to chemotherapy toxicity has remained unaddressed; this is, while the half-life of most chemotherapeutic agents is around 24 h [5], their toxic effects last for several days. For example, bone marrow suppression following chemotherapy reaches a nadir around 7–14 days and sore mouth is at its worst between 5 and 14 days [5]. For these reasons, chemotherapy cycles are usually spaced at 3-weekly intervals to allow for tissue recovery to occur. Our results suggest a different mechanism underlying chemotherapy toxicity. They suggest that the cellular damage and inflammation that are directly attributable to chemotherapy drugs are marginal and that most of the toxic side-effects of chemotherapy are induced by cfCh released from the initial

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**Figure 5.** Prevention of neutropenia over time following a single injection of adriamycin by concurrent treatment with CNPs, DNase I and R-Cu. TLC, total leukocyte count. Statistical analysis was carried out using unpaired Student’s t-test at each time point (n = 5 mice at each time-point).
round of drug-induced cell death triggering a cascading effect whereby dead cells release more cfCh causing further rounds of DNA damage, apoptosis and inflammation thereby exaggerating or amplifying the toxic effects. This proposal helps to explain why the duration of toxic effects of chemotherapy are disproportionally prolonged compared with the half-life of the drugs themselves.

In confirmation of our earlier report using cfCh isolated from serum of human subjects [6], we demonstrate that cfCh isolated from mouse serum can also activate γH2AX and active caspase 3 when added to cells in culture (Figure 1, upper two panels and supplementary Figure S1, available at Annals of Oncology online, upper two panels). Although inflammation is a major side-effect of chemotherapy, the mechanism by which chemotherapy-induced inflammation is triggered is poorly understood [2, 13]. We show that cfCh are directly responsible for inducing inflammation and that the activation of pro-inflammatory cytokines NFkB and IL-6 can be abrogated by concurrent treatment with cfCh degrading/neutralizing agents (Figure 1, lower two panels and supplementary Figure S1, available at Annals of Oncology online lower two panels).

We demonstrate that CNPs, DNase I and R-Cu can virtually abolish tissue DNA damage, apoptosis and inflammation resulting from chemotherapy. There was remarkable suppression of these toxic pathologies in all tissues examined namely, lung, liver, heart, brain, ovary, skin small intestine and PBMCs (Figures 3 and 4). cfCh release from dying cells following chemotherapy treatment appears also to be responsible for bone marrow suppression. The prolonged neutropenia following a single injection of adriamycin which lasted for 8–10 days supports our hypothesis that the initial round of cell death inflicted by adriamycin caused a cascading effect whereby cfCh released from the initial round of cell-death catalysed several further rounds of cfCh-induced cell death resulting in a prolonged neutropenic effect. In any event, we show that neutropenia could be prevented to a great extent by concurrent administration of CNPs, DNase I and R-Cu. Finally, we show that the above agents can prevent adriamycin induced lethality thereby implicating cfCh in causing death of mice following high-dose chemotherapy.

It is likely that suppression of DNA damage, apoptosis and inflammation by cfCh neutralizing/degrading agents is affected at two levels. The first is mediated via a reduction in the blood levels of cfCh (Figure 2A and B), while the second is mediated at a cellular level wherein cfCh released from dying cells are neutralized/degraded and prevented from inducing damaging effects on bystander living cells. In support of the latter proposal, we have recently shown that cfCh from dying cells can activate DNA damage and inflammation in surrounding cells and that these could be prevented by cfCh neutralizing/degrading agents both in vitro and in vivo [7].

It could be argued that the agents that we have used to neutralize/degrade cfCh may themselves be responsible for inactivating the cytotoxic drugs thereby suppressing their toxic side-effects. This is unlikely for two reasons: first, CNPs, DNase I and R-Cu have different chemical compositions and modes of action with respect to cfCh inactivation. It is unlikely that these three agents will have same drug inactivating action. Second, we have used five different cytotoxic drugs each one with a different mode of action. It is again improbable that CNPs, DNase I and R-Cu, which themselves have different modes of action, will interfere with the diverse pathways involved in induction of cytotoxic effects of five disparate drugs.

Our results have significant clinical implications. They suggest that cardiac, gastro-intestinal, neurological, cutaneous and reproductive side-effects of chemotherapy which include cardiac failure, nausea, vomiting, diarrhoea, neuro-toxicity, sterility and hair-loss can be potentially prevented by administration of cfCh neutralizing/degrading agents administered concurrently with chemotherapy. Similarly, bone marrow suppression and the resultant neutropenia as well as the occasional fatality resulting from chemotherapy may also appear to be preventable. If our results are successfully translated into the clinic, it might
help to bring relief to thousands of patients who are prescribed chemotherapy for cancer worldwide every day.

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**Disclosure**

The authors have declared no conflicts of interest.

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