Stable Water Clusters–Mediated Molecular Alterations in Human Melanoma Cell Lines

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ABSTRACT: Several reports have shown that the formation of stable water clusters (SWC) of different sizes underlie their physical and chemical properties, such as temperature-related density and anomalous melting at high temperature. Among the SWCs, one produces a double helix (DH), a structure that has been studied in a variety of biological systems. The major objective of this preliminary study was to investigate the effects of SWCs on several phenotypic and molecular characteristics of human cancer cell lines. Our previous findings have demonstrated that very high dilutions of chemical/biological agents, while having no direct observed effects on human cellular systems, can nevertheless induce molecular and genetic changes that contribute to signaling by other stimuli. These findings suggested that cells can respond to relatively fewer molecules than physiological levels comprising of high number of molecules. Accordingly, we hypothesized that treatment of cells with a water preparation containing SWCs would induce molecular and genetic changes in human cellular systems. This hypothesis was tested using human melanoma cell lines as a model for an in vitro analysis. We examined the effect of SWCs on cell viability, proliferation, expression of immune death receptors and response to death-ligand–induced apoptosis. In addition, we examined whether genetic changes might have also taken place. The preliminary findings demonstrate that treatment of melanoma cell lines with SWCs inhibits cell proliferation, upregulates the expression of death receptors, sensitizes the tumor cells to FasL-induced apoptosis, and selectively modifies gene products that regulate growth and the apoptotic pathways.

KEY WORDS: stable water clusters, melanoma, proliferation, Fas, Fas-ligand, apoptosis, PCR array, gene expression

ABBREVIATIONS: CDDP: cis-diamino-dichloro-platinum; FasL: Fas-ligand; mRNA: messenger RNA; SWC: stable water clusters

I. INTRODUCTION

Cancer is a disease that affects a significantly large human population, with loss of life and drastic economic consequences. Cancer is not a single disease but is manifested in hundreds of different types and subtypes that affect all organs and tissues of the human body. Because cancer cells, unlike the majority of human tissues, proliferate rapidly, this led to the development of several drugs were developed that inhibit cell proliferation and reducing tumor loads. Hence, several anti-proliferative chemotherapeutic drugs were been developed several decades ago and were found to be effective in the treatment of
various cancers with prolongation of survival but seldom with cure.\textsuperscript{1,2} Other treatment modalities have also been recently developed, such as radiotherapy, hormonal therapy, and immunotherapy, with significant clinical responses.\textsuperscript{3} However, a major drawback of these various treatment strategies has been the finding that a subset of patients initially does not respond and another subset that has initially responded becomes refractory to further treatments.\textsuperscript{4,5} Hence, tumor-cell resistance remains a major problem, and there are no current effective treatments. Therefore, the need to develop new therapeutic strategies is urgent; this requires the initial identification of the underlying mechanisms of resistance and subsequently the development of novel targeted therapies.

We and others have examined potential underlying mechanisms of resistance in several cancer cell lines used as models, and examined the effects of various chemo-immuno sensitizing agents that reverse resistance when combined with cytotoxic agents or cytotoxic cells have been investigated.\textsuperscript{6} Our studies have focused on the mechanisms of resistance in cancer cells regulated by the apoptotic pathways that, if triggered, may be responsible for cell death by chemo-immunotherapeutic drugs and radiation.\textsuperscript{7-10}

The rationale for our studies is based on the findings that most chemotherapeutic and immunotherapeutic drugs mediate cell death, primarily by the activation of the apoptotic pathways.\textsuperscript{11} Hence, cancer cells develop various mechanisms of resistance to the apoptotic pathways by dysregulating gene products that lead to cell death by apoptosis. Therefore, we hypothesized that agents that can target a dysregulated gene product(s) involved in resistance can facilitate the completion of the apoptotic signaling mediated by cytotoxic stimuli and resulting in apoptosis. We tested this hypothesis and validated it in a variety of tumor models.\textsuperscript{6}

Lo et al.\textsuperscript{12,13} have reported on the physical properties of the rigid association of water molecules called I\textsubscript{p} crystals. These I\textsubscript{p} crystals display a variety of distinct chemical and physical properties when compared to ordinary water preparations. In addition, Yo et al. have reported the existence of double-helix water (DHW).\textsuperscript{14} Several studies on the biological effects of water clusters were presented at the First International Symposium titled “Physical, chemical, and biological properties of stable water (I\textsubscript{p} ) clusters.”\textsuperscript{15}

The objective of the present study was to investigate the effect of SWC on the phenotypic, molecular, and genetic biologic manifestations induced on drug-immune resistant human melanoma cell lines. Two specific aims were examined: (1) the effect of SWC on tumor cell viability, proliferation, and response to an immune cytotoxic stimuli and, (2) if positive results were obtained, the molecular and genetic mechanisms that may underlie the observed phenomena.

\textbf{II. RESULTS AND DISCUSSION}

Our preliminary studies used the human melanoma cell lines M233 and A375 as models for analysis. These two cell lines exhibit different phenotypic properties and different responses to cytotoxic drugs (M233 is more resistant than A375). Standard conditions of cell culture were used, and several laboratory methods were adapted for the study. The analyses were performed, in an unbiased way. Two unknown (blinded) water preparations received from Dr. Yin Lo: preparation
A and preparation B. One preparation contained SWCs, and one did not. In addition, we used our laboratory water as an internal control. The media by which the tumor cell lines were cultured were adjusted with different final percentages of preparation A or B to establish a titration. Several experimental designs were performed.

A. Analysis of Preparations A and B by Gas Chromatography and Mass Spectrometry

Preparations A and B were chemically analyzed by both gas chromatography (GC) and mass spectrometry (MS). These procedures were performed by Professor Selim Senkan at UCLA. Analysis of the two preparations revealed identical patterns and the absence of any contaminant organic molecules. These findings verify the identical constitution of the two preparations.

B. Metabolic Cell Assay (Viability/Proliferation) Assessed Using the XTT Assay

The XTT assay consists of measuring the metabolic activity of living cells and excluding dead cells. It measures the total viable cells and, indirectly, cell proliferation. The two melanoma cell lines were cultured in media containing different final percentages (0–10) of preparations A or B for 24 and 48 hours. The cultures were assessed for metabolically active, viable cells. The finding with the M233 cell line did not reveal any differences between treatment with either preparation A or preparation B at all concentrations used. In contrast, however, the A375 melanoma cell line showed a significant inhibition of cell proliferation, and the extent of inhibition was a function of the final concentration of preparation B. The inhibition was primarily observed following 48 hours of culture. No inhibition was observed following treatment with preparation A. These findings indicate that not all cell lines were sensitive to preparation B; the findings with M233 are consistent with their different threshold of resistance and phenotypic and genetic makeup compared to A375. Furthermore, these findings demonstrate that the inhibition observed in the A375 cell line by preparation B may reflect inhibition of cell signaling and gene products that regulate cell growth. Clearly, these preliminary findings need to be validated with several other cell lines of different histological types. In addition, biochemical and molecular analyses of the cell-cycle pathways that regulate cell proliferation need to be analyzed to identify gene products that were modulated by the preparation B. It will also be important to examine whether preparation B has any effect on the proliferation and viability of normal proliferating cells such as bone marrow-derived stem cells and other cells.

C. Sensitization of the Melanoma Cell Line A375 to FasL-Induced Apoptosis

Our findings demonstrating that preparation B inhibited cell growth proliferation suggest that it mimics many other compounds, used at very high dilutions, which have been shown to inhibit cell growth. In addition, these highly diluted compounds have also been shown to modulate the apoptotic pathways in several drug/immune resistant tumor cells and can reverse resistance to apoptosis when used in combination with cytotoxic stimuli. For instance, we have reported that treatment of drug- and/or immune-resistant cells with
very low concentrations of cytotoxic drugs sensitized the resistant tumor cells to both the same drug and to death ligand-induced apoptosis.\textsuperscript{7–10}

Human cytotoxic cells (CTL, NK, macrophages) exert their cytotoxic activity through several mechanisms, including the perforin/granzyme pathways as well as surface expression of death ligands (e.g., TNF-α, FasL, and TRAIL).\textsuperscript{17} Tumor cells express death receptors (e.g., TNFRI, TNFR II, Fas, DR4, and DR5), and if sensitive, can be killed by corresponding ligands on the surface of cytotoxic cells.

In this study, we investigated one pathway of apoptotic cell death, namely, via the FasL-induced apoptosis. We used human recombinant FasL, which mimics the activity of FasL expressed on cytotoxic cells. The A375 cell line was treated with different concentrations of preparation A or B and different concentration of FasL (0–50 ng/mL); the cells were incubated for 48 hours, then were harvested and tested for apoptosis by flow cytometry. We used the activation of the executioner caspase-3 as the marker for apoptosis as determined by the use of a specific fluorescent-labeled antibody specifically directed against activated cleaved caspase-3 and does not react with pro-caspase-3.\textsuperscript{18} The frequency of the apoptotic cells was then quantified. The findings revealed that there was no detectable apoptosis of tumor cells treated with preparation A at all final concentrations used and with all FasL concentrations used. In contrast, however, there was a significant potentiation of FasL-induced apoptosis in cells treated with preparation B, which was significant with a high FasL concentration (50 ng/mL). These findings suggest that treatment with preparation B significantly affected the apoptotic pathway that resulted in the potentiation of the level of apoptosis to FasL when compared to preparation A.

Reports in the literature have suggested that, in several instances, the overexpression of surface Fas expression on tumor cells that are resistant to FasL apoptosis can reverse their resistance.\textsuperscript{19} Therefore, we examined whether the observed potentiation of FasL-induced apoptosis in the A375 cells by preparation B may have resulted in the upregulation of Fas expression on the tumor cells. This was tested following treatment of A375 cells with various concentrations of preparation A or B for 48 hours, and cell lysates were prepared and subjected to western blot analysis for total Fas protein. The results revealed significant induction of Fas protein in the cells treated with preparation B at final concentrations of (5–7.5%) in the absence of any induction following treatment with preparation A. These findings support the contention of a correlation between the observed potentiation of FasL-apoptosis by preparation B and the enhanced expression of the Fas receptor protein. The findings observed with FasL-induced apoptosis suggest that treatment with preparation B may also sensitize tumor cells to other recombinant ligands (e.g., TNF-α, TRAIL), to agonist TRAIL receptors mAbs, and to cytotoxic effector cells expressing various death ligands. These findings also suggest that the enhanced FasL cytotoxicity observed by preparation B may be of physiological relevance.

D. Differential Gene Analysis of Cells Treated with Preparation A or B

The results of our study demonstrate the upregulation of Fas expression in A375 cells treated with preparation B. Therefore, prepa-
reration B might have exerted its activity on the basis of genetic modulation, and other gene products may have also been affected in the regulation of apoptosis. To examine this possibility, we used the RT Profiler PCR assay to identify any genes that had been either downregulated or upregulated following treatment of A375 with preparation B (compared to preparation A) (Qiagen, Valencia, CA). The RT Profiler PCR assay exhibits sensitivity, reproducibility, specificity, and reliability to accurately profile multiple genes simultaneously. The tumor cells were treated with either preparation A or B and were cultured for 48 hours, then lysates were prepared for RNA extraction. The RNA was then converted to cDNA, and aliquots were added to the RT Profiler assay in a 96-well plate containing 84 different oligonucleotides for specific genes, the majority of which were chosen as regulators of the apoptotic pathways. The plate also contained several internal controls. The plates were then run for real-time PCR using a PCR equipment. The data were analyzed for fold increases or decreases for each gene. In comparison with untreated cells, in cells treated with the preparation B, only a small number of genes were modified, and those modified genes were primarily involved in the regulation of apoptosis (Table 1). These findings suggest that the observed sensitivity of A375 to FasL apoptosis by preparation B was the result of various modified gene products that are implicated in the regulation of apoptosis. These findings also suggest that preparation B exerts its effects at the gene level by regulating the transcription of selective genes.

### III. CONCLUSIONS

Upon termination of these preliminary findings, we learned from Doctor Lo that the preparation B contained the SWCs and that the preparation A was the control without SWCs. These unbiased findings validate our motivation to pursue more intensive investigations on the biological and molecular effects of SWCs in melanoma model studies both in vitro an in vivo as well as in other model systems. Our current findings are summarized

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**TABLE 1: Differential Gene Expression**

<table>
<thead>
<tr>
<th>Identity of gene</th>
<th>Fold increase</th>
<th>Fold decrease</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL-B/Boo</td>
<td>-</td>
<td>2.2</td>
<td>Novel human marker in the Bcl-2 family. Suppresses apoptosis by binding to Bcl-2 and BcIXL</td>
</tr>
<tr>
<td>NIP1/SEC20*</td>
<td>1.52</td>
<td>-</td>
<td>Interacts with Bcl-2 and regulates apoptosis</td>
</tr>
<tr>
<td>ICE/IL1BC</td>
<td>1.73</td>
<td>-</td>
<td>Cleaves IL-1β and IL-18 – Induces cell necrosis</td>
</tr>
<tr>
<td>DP5/HARAKIRI</td>
<td>2.7</td>
<td>-</td>
<td>Interacts with Bcl-2 and BcIXL and activates apoptosis</td>
</tr>
<tr>
<td>APO2L/Apo-2L</td>
<td>-</td>
<td>2.2</td>
<td>Inhibits TRAIL-induced invasion</td>
</tr>
<tr>
<td>ASC/CARD5</td>
<td>-</td>
<td>1.7</td>
<td>Adapter protein that mediates assembly of large signaling complexes in inflammation and apoptosis via activations of caspases</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>1.6</td>
<td>-</td>
<td>Regulator of apoptosis and survival signaling complexes</td>
</tr>
<tr>
<td>DDIT1/GADD45</td>
<td>1.74</td>
<td>-</td>
<td>Stress sensors that modulate genotoxic stress and tumor formation. Growth arrest and DNA damage (Gadd45)</td>
</tr>
</tbody>
</table>
in Table 2.

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