Novel Nanoscale Delivery Particles Encapsulated with Anticancer Drugs, All-trans Retinoic Acid or Curcumin, Enhance Apoptosis in Lymphoma Cells Predominantly Expressing CD20 Antigen

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Abstract. *Background: Mantle cell lymphoma (MCL), a B-cell lymphoma, pursues a relatively aggressive course, is resistant to long-term remission, and is associated with a poor prognosis. There is a pressing need for innovative treatment approaches against MCL. One such approach is targeted delivery of cytotoxic drugs to MCL cells. Materials and Methods: In the current investigation, we pursued a strategy to employ retinoid-based or curcumin-based nanoscale delivery particles, called nanodisks (NDs), for targeted drug delivery to MCL cells (Granta), and human follicular lymphoma (HF-1) cells. The cells were incubated with NDs made of CD20 single-chain variable antibody fragment (scFv)/apolipoprotein A-1 fusion protein, and loaded with either all-trans retinoic acid (ATRA) or curcumin, and cell apoptosis was measured using flow cytometry. Results and Conclusion: At 10 μM, curcumin-ND induced cell death more effectively than ATRA-ND. Combination of curcumin-ND and ATRA-ND significantly enhanced the biological activity of these drugs against lymphoma cells compared to individual treatments.*

Mantle cell lymphoma (MCL) is a distinct genetic subtype of B-cell non-Hodgkin's lymphoma. It is genetically characterized by the t(11:14)(q13:q32) translocation leading to a constitutive expression of cyclin D1, facilitating deregulation of the cell cycle at the G_1-S phase transition (1). There is currently no standard therapy for newly-diagnosed or relapsed disease. New treatment approaches are needed that target novel biologic pathways (2).

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Nanotechnology serves as a valuable tool for cancer therapies. The unique size of nanoparticles, their properties of self-assembly, stability, specificity, drug encapsulation, and biocompatibility offer the potential to radically change cancer therapies for the better. Our nanoparticles (called nanodisks, NDs) (3) for example, serve as targeted drug-delivery vehicles capable of carrying doses of chemotherapeutic agents into malignant cells while sparing healthy cells, thus increasing the amount of therapeutic agent within cancer cells while reducing toxic side-effects that accompany many current therapies.

All-*trans* retinoic acid (ATRA) and curcumin are useful drugs in cancer therapy as they possess diverse pharmacological properties (4-7). Despite their anticancer effects, their use is limited by the fact that they exhibit poor solubility in water, and low bioavailability (8). Our earlier studies have shown that these water insoluble drugs can be solubilized by stable integration into NDs (9, 10).

We therefore used a formulation of ATRA, and of curcumin in NDs which were scaffolded with a fusion protein comprising apolipoprotein A1 (APOA1), and a single chain variable antibody fragment (scFv) against CD20 to provide targeting to the MCL cells which predominantly express CD20 (3, 11, 12). Herein we assessed the relative ability of these novel targeted drug-delivery agents to induce apoptosis of lymphoma cells (MCL-Granta), as well as cells from a neoplasm of follicle center B (HF-1).

Materials and Methods

Materials. RPMI-1640 and fluorescein isothyocyanate (FITC)-annexin V apoptosis kit were obtained from Invitrogen (Carlsbad, CA, USA); propidium iodide (PI) was from Biosource (Camarillo, CA, USA).

Nanodisk preparation. NDs with CD20 targeted scFv-APOA1 were formulated as described previously (3). Drugs containing CD20 targeted scFv-APOA1 were prepared as described by Ghosh *et al.* (3, 12). The NDs were a kind gift from Dr. Robert O. Ryan, Children's Hospital Oakland Research Institute, CA, USA.

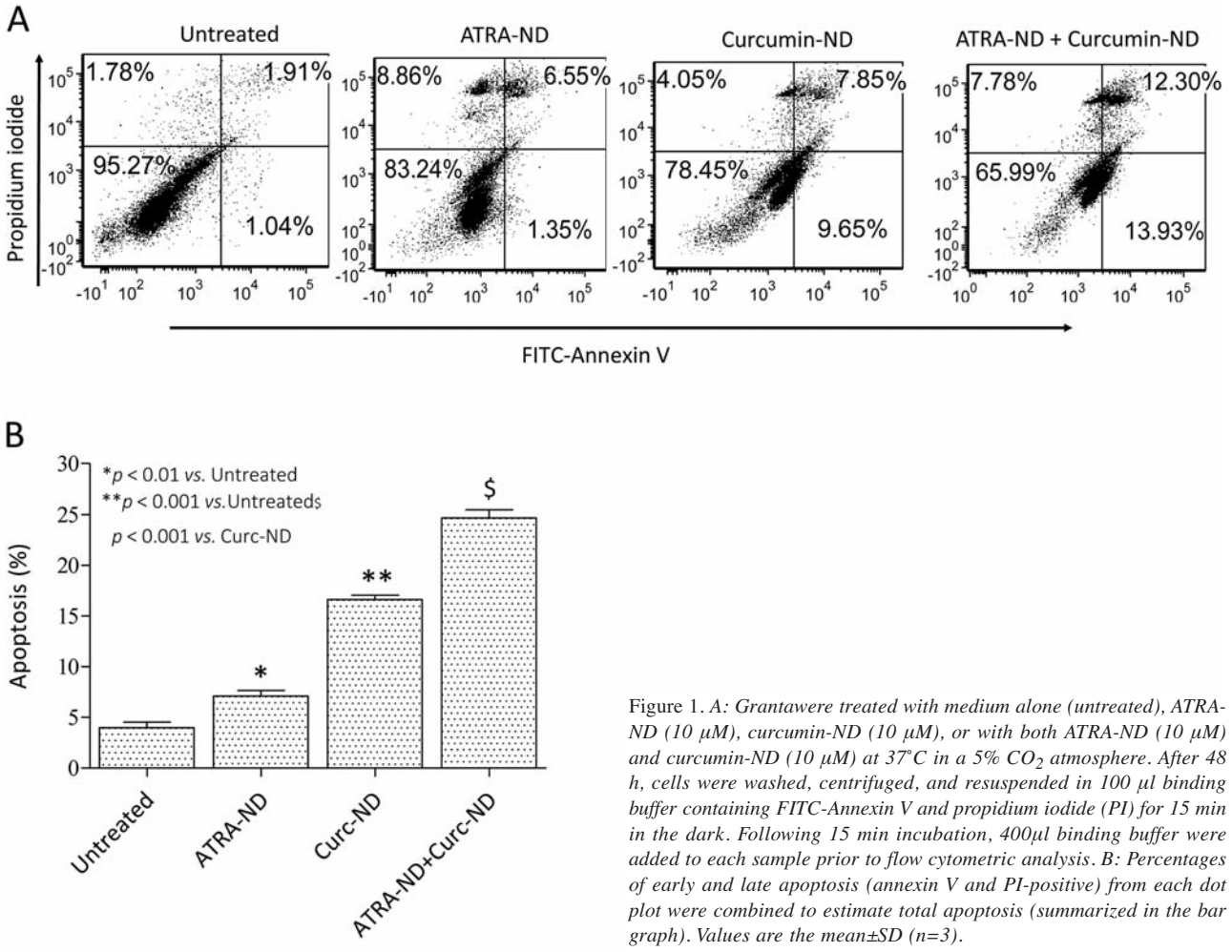

Figure 1. *A: Grantawere treated with medium alone (untreated), ATRA-ND (10 μM), curcumin-ND (10 μM), or with both ATRA-ND (10 μM) and curcumin-ND (10 μM) at 37˚C in a 5% CO_2 atmosphere. After 48 h, cells were washed, centrifuged, and resuspended in 100 μl binding buffer containing FITC-Annexin V and propidium iodide (PI) for 15 min in the dark. Following 15 min incubation, 400μl binding buffer were added to each sample prior to flow cytometric analysis. B: Percentages of early and late apoptosis (annexin V and PI-positive) from each dot plot were combined to estimate total apoptosis (summarized in the bar graph). Values are the mean±SD (n=3).*

Cell culture. Granta (MCL cells) and HF-1 (follicular lymphoma cells) (Dr. Steven H. Bernstein, University of Rochester, NY) were cultured in RPMI-1640 containing 10% fetal calf serum, and in the presence of penicillin, streptomycin, and glutamine at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Cell viability was measured using the trypan blue exclusion method.

Treatment. Cells were incubated with ATRA-CD20 scFv-APOA1 (10 μM) (ATRA-ND) or curcumin-CD20 scFv-APOA1 (10 μM) (curcumin-ND), and a combination of ATRA-ND or curcumin-ND for 48 h. In control groups the same amount of buffer in the medium was used, as was, for the treatment groups.

Apoptosis assay. Cellular apoptosis was measured by flow cytometry. In brief, Granta or HF-1 cells (1.0×10^6 cells/well) were incubated with either individual or combined treatments of ATRA-CD20 scFv APOA1 (10 μM) and curcumin-CD20 scFv-APOA1 (10 μM) for 48 h, washed with ice-cold phosphate-buffer solution and resuspended in binding buffer containing 2.5 μl FITC-annexin V and 5.0 μl PI for 15 min at 37˚C in a CO_2 incubator. Flow cytometric measurements were made on a Beckman Coulter EPICS XL-MCL Cytometer (Beckman Coulter, Inc., Brea, CA, USA). All experiments were performed in triplicate.

Statistical analysis. To measure the effects of drug-containing NDs on apoptosis, one-way analysis of variance and Newman-Keuls multiple comparison test were performed using GraphPad Software Inc. (San Diego, CA, USA). Data are reported as apoptotic cells (annexin V-positive and PI-positive) as a percentage of total cells. All experiments were performed in triplicate.

Results and Discussion

NDs increase apoptosis of Granta cells. Apoptosis of Granta cells was measured after treatment with medium alone (untreated), ATRA-NDs (10 μM), and curcumin-NDs (10 μM), alone and combined, using annexin V and PI cell labeling on a flow cytometer (Figure 1A). We found that ATRA-NDs combined with curcumin-NDs induced significant apoptosis ($p<0.001$) compared to apoptosis achieved with individual treatments ($p<0.01$ and $p<0.001$, respectively; Figure 1B).

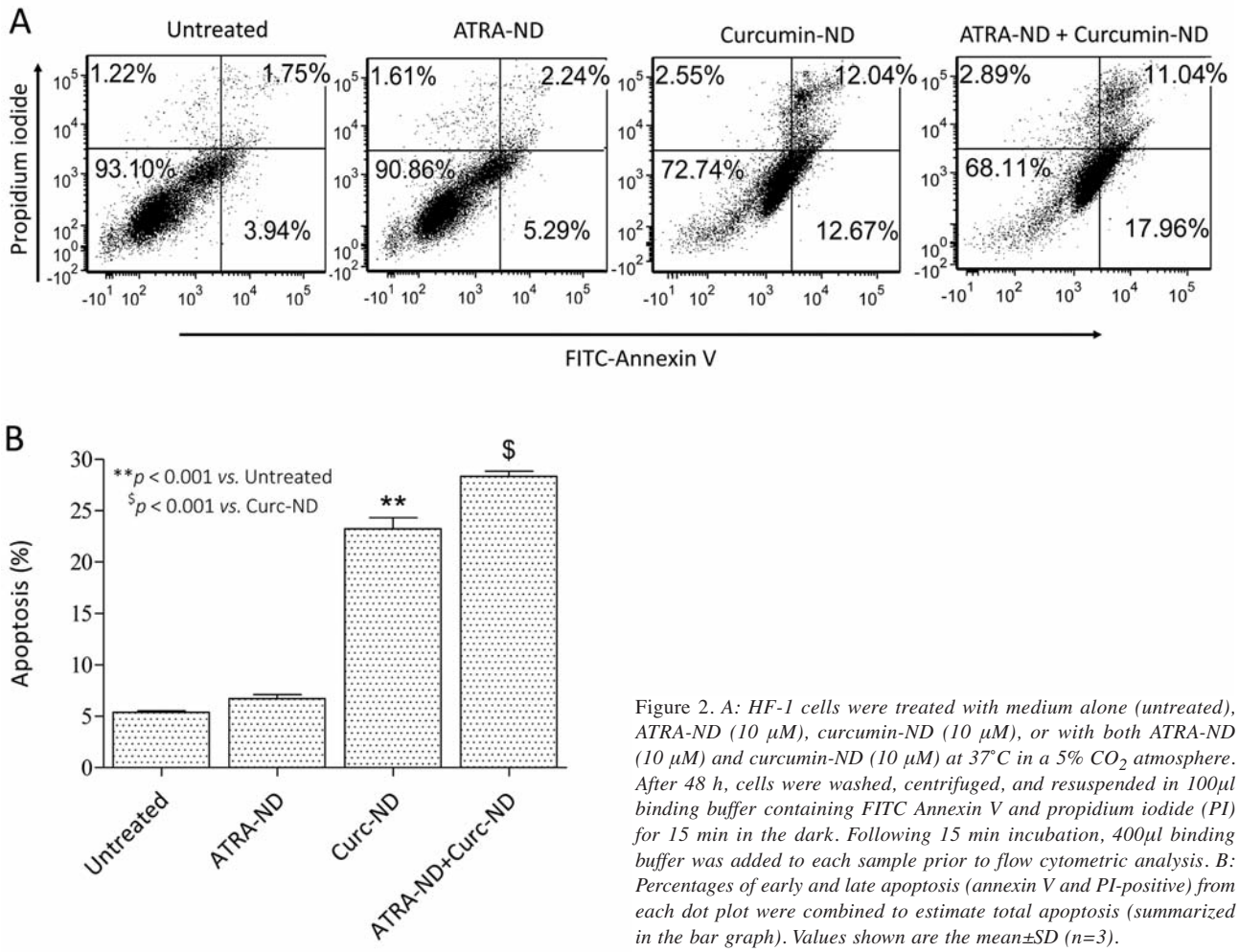

Figure 2. *A: HF-1 cells were treated with medium alone (untreated), ATRA-ND (10 μM), curcumin-ND (10 μM), or with both ATRA-ND (10 μM) and curcumin-ND (10 μM) at 37˚C in a 5% CO_2 atmosphere. After 48 h, cells were washed, centrifuged, and resuspended in 100μl binding buffer containing FITC Annexin V and propidium iodide (PI) for 15 min in the dark. Following 15 min incubation, 400μl binding buffer was added to each sample prior to flow cytometric analysis. B: Percentages of early and late apoptosis (annexin V and PI-positive) from each dot plot were combined to estimate total apoptosis (summarized in the bar graph). Values shown are the mean±SD (n=3).*

Enhanced effects of NDs on apoptosis of follicular lymphoma cells. To ensure that our findings were not simply a phenomenon specific or limited to MCL cells, an additional $CD20^+$ non-Hodgkin's lymphoma cell line, HF-1, was evaluated. The apoptotic response of cultured HF-1 cells to incubation with medium alone (untreated), and individual or combined treatments of NDs with ATRA-CD20 scFv-APOA1 and curcumin-CD20 scFv-APOA1 were analyzed by flow cytometry (Figure 2A). At 10 μM, curcumin-NDs induced a more than three-fold apoptotic response than did ATRA-NDs. When cells were treated with their combination, the effects of these drugs on apoptosis were even greater than those of curcumin-NDs alone, although not additive (p<0.001; Figure 2B).

The data presented in the present study show that compared to untreated cells, incorporation of cytotoxic drugs, ATRA or curcumin, into NDs enhanced their biological activity against Granta and in HF-1 cells. Importantly, our data with ATRA-NDs and curcumin-NDs demonstrated enhanced apoptosis in cell culture models of non-Hodgkin's lymphoma.

ATRA is a useful drug in cancer therapy as it has a central role in cell growth, differentiation, and apoptosis (13, 14). It is one of the first examples of targeted therapy in human cancer – acute promyelocytic leukemia (15). ATRA binds to Retinoic Acid Receptor and Retinoic X Receptor and transactivates target genes, leading to apoptosis or cell-cycle arrest (16). Since MCL cells also express retinoid receptors (16), we hypothesized ATRA would demonstrate apoptotic effects and may serve as a treatment option against MCL.

Curcumin has also demonstrated anticancer activity (17, 18). Its role in the treatment of hematological cancer has been well-documented (4-7). Several investigations in cancer cell models have demonstrated that curcumin prevents cell proliferation and induces apoptosis (19, 20) *via* transcription factors or cytokines (21).

Although ATRA and curcumin are documented anticancer drugs, disadvantages associated with them include their hydrophobicity and hence low availability to cells. ND technology takes advantage of the ability of members of the class of exchangeable apolipoprotein (*e.g.* APOA-I) to confer stability and water solubility by their binding interaction wherein amphipathic α-helix segments present their hydrophobic face to the lipid surface while their polar faces are directed toward the aqueous solvent (22). The discovery that hydrophobic drugs can be stably incorporated into the lipid milieu of NDs provides a vehicle for solubilization and transport of bioactive therapeutic agents (22). Following stable integration of ATRA or curcumin, NDs were formulated with an α-CD20 scFv/APOA-I fusion protein scaffold, to achieve targeted delivery of drugs to lymphoma cells (3).

In the present study, our Fluorescence Activated Cell Sorting analysis data show curcumin-NDs elicit pro-apoptotic responses in Granta and in HF-1 cells that are significantly greater than those seen in untreated cells. Our data on apoptotic response to curcumin are consistent with other reports on SP-53 (4), osteosarcoma (23), and lymphoma (24) cells. Likewise, NDs formulated with ATRA (ATRA-CD20 scFv-APOA1), induced apoptosis of HF-1 and Granta cells. Co-incubation with containing ATRA- and curcumin-NDs induced significant apoptosis in both cell lines compared to apoptosis seen with individual treatments. The observation that the two cell lines have different sensitivity to curcumin-CD20 scFv-APOA1 is possibly due to cell-specific effects of curcumin. Further studies are required to elucidate the effects of different incubation times as well as doses of curcumin and ATRA when presented to cells as components of NDs.

Conclusion

In summary, we examined cytotoxic effects of targeted NDs on lymphoma cell lines Granta and HF-1. These NDs induced a significantly stronger pro-apoptotic response than in untreated cells. CD20 targeting significantly enhanced the cytotoxic activity of the tested drugs. The enhanced apoptotic response observed with targeted NDs strongly suggests that combining the bioactive agent delivery capacity of ND with the targeting specificity of antibodies will translate into reduced toxicity and increased efficacy in the treatment of lymphoma.

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Conflicts of Interest

The Authors declare no competing financial interests.

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