Poloxamer 188 enhances apoptosis in a human leukemia cell line

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Abstract. Poloxamer block copolymers have been studied in multiple applications as drug delivery systems (DDS). These A-B-A amphiphilic block copolymers up-regulate the expression of selected genes in cells and alter genetic responses to antineoplastic agents in cancer. One example is poloxamer 188, also known as pluronic F68, which may be promising as a carrier in DDS. To clarify the possible mechanistic role of pluronic F68 in several leukemia cell lines, we examined whether pluronic F68-inducible factors were capable of causing apoptosis. The influence of pluronic F68 on the cell lines was examined using a comprehensive analysis. It was found that treatment of K562 cells with 6% pluronic F68 resulted in G_2/M phase arrest of the cell cycle, followed by caspase activation and the accumulation of apoptotic cells. When used as a carrier in a DDS, pluronic F68 may provide a synergistic effect on the drug of interest. Although the mechanisms behind the function of pluronic F68 are not fully understood, the results suggests that pluronic F68 may act as a useful carrier in DDS for the purpose of leukemia therapy.

Introduction

The management of leukemia has become complex due to the availability of improved diagnostic procedures and life-prolonging or even curative treatment strategies. However, many technical difficulties (e.g., the prevention of adverse drug reactions) have to be overcome before effective leukemia therapy can be realized. One of the ways to enhance drug uptake and bioavailability is through the application of efficient drug delivery systems (DDS) (1,2). Although the concept of DDS is not new, significant progress has recently been made in the treatment of a variety of diseases (3-5). A recent therapeutic strategy in anti-cancer treatment is the application of DDS, such as micelles, liposomes and copolymers (6-8). To convey a sufficient dose of drug to the lesion, suitable carriers of drugs are also needed. Poloxamer (also known as pluronic®) surfactants are synthetic copolymers of ethylene oxides and propylene oxide. Pluronics are biocompatible and have been widely used for wound healing, drug and gene delivery and tissue engineering (9-11). Pluronic block copolymers have been recognized as pharmaceutical excipients by the US and British Pharmacopoeia. Poloxamer 188 (also known as pluronic® F68) is the most commonly used copolymer for drug delivery in clinical trials (12,13). Pluronic F68, a copolymer of poly(oxyethlene)-poly(oxypropylene) -poly(oxyethylene), has been used extensively in a variety of pharmaceutical formulations, including the delivery of low molecular mass drugs and polypeptides (14).

To understand how pluronic F68 acts *in vitro*, we cultured the human chronic myelogenous leukemia (CML) cell line K562 with pluronic F68 or polyethylene glycol (PEG). PEG (MW 7000-9000) is comparable to pluronic F68 (MW 8350) in terms of its molecular weight. Using flow cytometry, pluronic F68 was determined to be a specific inhibitor of cell proliferation in K562 cells. It is possible that it may be effective as a DDS for the treatment of leukemia via the synergistic effect of pluronic F68, as the carrier, and the drug.

Materials and methods

Cell culture. All solvents and reagents, except when otherwise specified, were purchased from Sigma-Aldrich (MO, USA) at the highest purity grade. Pluronic F68, a copolymer of poly(oxyethylene)-poly(oxypropylene)-poly(oxyethylene) with a POE/POP ratio of 20/80, was obtained from Wako Junyaku (Kyoto, Japan). PEG (molecular weight 7,000-9,000 kDa; mean 8,000 kDa) was obtained from Wako Junyaku. The K562, HL60, NALM-6 and Molt-4 cell lines (Table I) were grown at 37˚C in a humidified incubator with 5% CO_2/95% air in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, NY, USA), 1 mM of sodium pyruvate, 200 IU/ml of penicillin and 200 μg/ml of streptomycin. After subculture for 48 h, the cells were treated with 6% pluronic F68 or PEG for 48 h. Peripheral blood mononuclear cells (PBMC) derived from healthy volunteers were isolated by

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Abbreviations: DDS, drug delivery systems; CML, chronic myelogenous leukemia; PEG, polyethylene glycol; PBMC, peripheral blood mononuclear cells; PI, propidium iodide

Key words: poloxamer, pluronic F68, drug derivery system, apoptosis

Table I. Cell lines used in the study.

Name	Tissue	Morphology	Genetics
K562	Peripheral blood	Lymphocyte-like	Chronic myelogenenous leukemia
HL60	Peripheral blood	Lymphocyte-like	Premyeloblastic cell
NALM-6	Peripheral blood	Lymphocyte-like	B cell leukemia
Molt-4	Peripheral blood	Lymphocyte-like	T cell leukemia

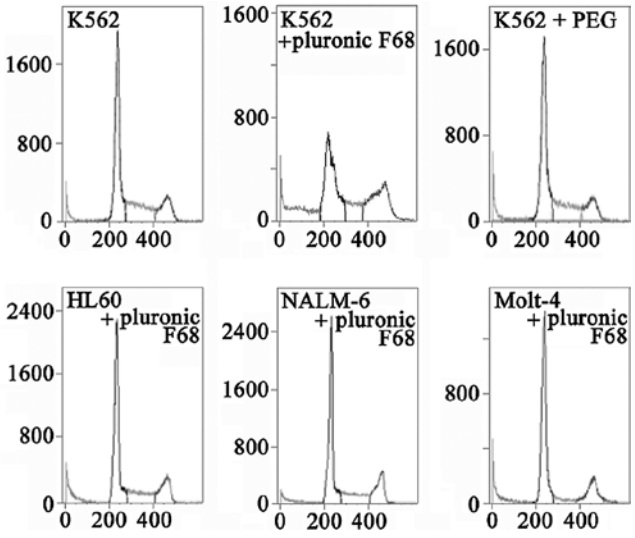

Figure 1. DNA histograms of cell lines in the absence or presence of 6% pluronic F68 or 6% PEG. The K562 cell line with pluronic F68 exhibited a detectable apoptosis signal (sub-G_1) and was synchronized in the G_2/M phase of the cell cycle. Other cell lines incubated with 6% pluronic F68 experienced no changes in either the apoptosis signal (sub-G_1) or the state of the cell cycle (G_0/G_1, S and G_2/M).

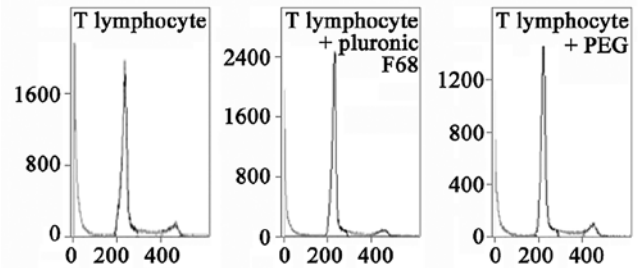

Figure 2. DNA histograms of T lymphocytes derived from healthy volunteers. T lymphocytes incubated with 6% pluronic F68 or 6% PEG experienced no changes in the apoptosis signal or the state of the cell cycle.

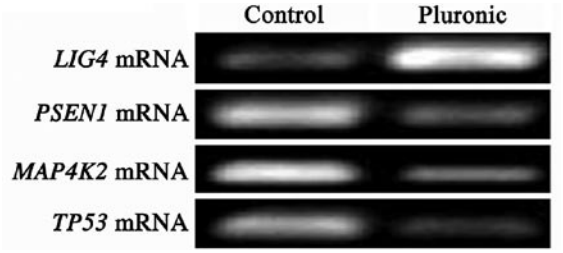

Figure 3. Detection of pluronic F68-inducible mRNA in the K562 cell line in the absence or presence of 6% pluronic F68. Control, the K562 cell line treated without pluronic F68; Pluronic, the K562 cell line treated with 6% pluronic F68.

Ficoll-based lymphosepar I (Immuno-Biological Laboratories, Gunma, Japan) centrifugation. Monocytes were eliminated from the PBMC and the PBMC were incubated at 37˚C for 1 h on a collagen-coated dish. Any non-adherent cells were recovered. The cells were cultured for 24 h with 100 ng/ml of anti-CD3 mbs (Beckman Coulter, CA, USA) and 40 μg/ml of phytohemagglutinin-P (Gibco, CA, USA) in RPMI1640 medium. After subculture for 48 h, the cells were treated with 6% pluronic F68 or PEG for 48 h.

Cell growth inhibition. Cell cycle distribution was determined by flow cytometry using a Quanta SC (Beckman Coulter, CA, USA). At 48 h after incubation, apoptosis was detected by Annexin V and propidium iodide (PI) staining. Cell cycle arrest was detected by PI staining.

DNA microarray. mRNA were hybridized on a DNA chip as described previously (15). Total RNA was extracted using QuickGene mini-80 (Fujifilm, Odawara, Japan) according to the manufacturer's instructions. The final RNA preparations were quantified by their absorbances at 260 nm using a NanoDrop ND-1000 (Biomedical Science, Tokyo, Japan). cDNA was prepared by incubating 22 μg of total RNA with cyanine 5- or 3-labeled dUTP (Applied Biosystems, CA, USA) in the presence of an oligo dT primer. cDNA probes were hybridized on a Human a la Carte array (Novus Gene, Tokyo, Japan) with a GeneTAC Hybstation (Genomis solutions, Ann Arbor, MI, USA). After hybridization, DNA chips were scanned using a GenePix 4000B (Axon Instruments, CA, USA). The factor that had been obtained by the DNA chip assay was confirmed by RT-PCR. cDNA was prepared by incubating DNase-treated total RNA (0.1 μg) with M-MLV reverse transcriptase (Invitrogen, CA, USA) in the presence of random primers (Invitrogen). The reaction parameters were 95˚C for 30 sec, 60˚C for 40 sec, and 72˚C for 30 sec for 40 cycles, followed by a 5-min extension at 72˚C using Paq 5000 DNA polymerase (Stratagene, CA, USA). Half of the PCR product was separated using electrophoresis in TBE buffer on a 2.0% agarose gel, then stained with ethidium bromide and detected under ultraviolet light.

Results

Cell growth inhibition. The K562 cell line with pluronic F68 exhibited a detectable apoptosis signal with Annexin V. The other cell lines, HL60, NALM-6 and Molt-4, when incubated with pluronic F68, had undetectable apoptosis signals. In addition, when the K562 cell line was incubated with pluronic F68, it was synchronized in the G_2/M phase of the cell cycle

(Fig. 1). None of the cell lines incubated with PEG exhibited a detectable apoptosis signal or experienced changes in the state of their cell cycle. T lymphocytes derived from healthy volunteers incubated with pluronic F68 or PEG exhibited no changes in the apoptosis signal or the state of the cell cycle (Fig. 2).

DNA microarray. After the mRNA expression was normalized to the *GAPDH* housekeeping gene expression, we identified pluronic F68-inducible target mRNAs. *LIG4* mRNA was up-regulated by >5-fold, while *PSEN1*, *MAP4K2* and *TP53* mRNA were down-regulated by >5-fold. These mRNAs were confirmed to be increased or decreased using both RT-PCR (Fig.3) and the DNA chip assay.

Discussion

In the present study, induction by pluronic F68 arrested the G_2/M phase of the cell cycle in the K562 BCR-ABL-mediated CML cell line. Furthermore, the G_2/M arrest consequentially induced the apoptosis. This cell-specific death was only observed in the K562 cell, and not observed in the other cell lines, HL60, NALM-6 and Molt-4. Moreover, this K562-specific apoptosis was induced only by pluronic F68, and not by PEG. PEG did not influence the cell proliferation of any of the cell lines. In addition, the influence of cell proliferation on T lymphocytes with pluronic F68 was not confirmed. Pluronic F68 is not only a K562-specific carrier of a DDS, but also acts as an anti-cancer drug in CML. No previous reports have found a relationship between pluronic F68 and K562 or its function as an apoptotic enhancer; therefore, we have no explanation for the K562-specific G_2/M arrest observed in the present study. However, the increase or decrease in mRNA clearly shows some features of apoptosis.

Using cDNA microarray analysis, we identified four pluronic F68-inducible target mRNAs, *LIG4*, *PSEN1*, *MAP4K2* and *TP53*. The up-regulated mRNA was *LIG4*, while the down-regulated mRNAs were *PSEN1*, *MAP4K2* and *TP53*. *LIG4* is involved in DNA repair and *PSEN1*, *MAP4K2* and *TP53* are apoptosis-related genes. LIG4, ligase IV forms a complex with the X-ray repair cross complementing protein 4, and further interacts with the DNA-dependent protein kinase (16). Increases in this gene result in apoptosis. PSEN1, presenilins-1, is postulated to regulate amyloid precursor protein processing through its effects on γ-secretase, an enzyme that cleaves the amyloid precursor protein (17,18). MAP4K2, mitogen-activated protein kinase 2, is a member of the serine/threonine protein kinase family. This kinase can be activated by TNF-α, and has been shown to specifically activate mitogen-activated protein kinases (19,20). The *TP53* gene encodes tumor protein p53, which responds to diverse cellular stresses to regulate the target genes that induce cell cycle arrest, apoptosis, senescence, DNA repair or changes in metabolism (21,22). Decreases in *PSEN1*, *MAP4K2* and *TP53* mRNA result in apoptosis.

Many anticancer drugs (e.g., paclitaxel, docetaxel) are generally G_2/M-phase-specific and show an antiproliferative effect on actively dividing cancer cells. These drugs also affect proteins or nucleic acids that are present in normal as well as malignant tissues. Targeted therapy (23,24) using pluronic F68 as a carrier may be more effective than current treatments and less harmful to normal cells. Pluronic F68 appears to possess unique drug-targeted cancer cell interactions in the K5262 cell line. Therefore, we propose a drug delivery strategy for BCR-ABL(+) CML cells that would target not only the fusion transcript, but also downstream signaling.

The current study demonstrates that treatment of K562 cells with 6% pluronic F68 results in G_2/M phase arrest of the cell cycle, followed by caspase activation and the accumulation of apoptotic cells. However, the mechanism behind the involvement of the K562-specific DDS carrier in apoptosis remans unclear. Before being applied in clinical studies, numerous validation studies for DDS using pluronic F68 are required. Further studies are critically important for the identification of new carriers to act in DDS for leukemia therapy.

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