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Trafficking Microenvironmental pHs of Polycationic Gene Vectors in Drug-Sensitive and Multidrug-Resistant MCF7 Breast Cancer Cells

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Abstract

While multidrug resistance (MDR) has been a significant issue in cancer chemotherapy, delivery resistance to various anticancer biotherapeutics, including genes, has not been widely recognized as a property of MDR. This study aims to provide a better understanding of the transfection characteristics of drug-sensitive and drug-resistant cells by tracing microenvironmental pHs of two representative polymer vectors: poly(L-lysine) and polyethyleneimine. Drug-sensitive breast MCF7 cells had four- to seven-times higher polymeric transfection efficiencies than their counterpart drug-resistant MCF7/ADR-RES cells. Polyplexes in MCF7/ADR-RES cells after endocytosis were exposed to a more acidic microenvironment than those in MCF7 cells; the MDR cells show faster acidification rates in endosomes/lysosomes than the drug-sensitive cells after endocytosis (in the case of PLL/pDNA complexes, ~ pH 5.1 for MCF7/ADR-RES cells vs. ~ pH 6.8 for MCF7 cells at 0.5 hr post-transfection). More polyplexes were identified trapped in acidic subcellular compartments of MCF7/ADR-RES cells than in MCF7 cells, suggesting that they lack endosomal escaping activity. These findings demonstrate that the design of polymer-based gene delivery therapeutics should take into account the pH of subcellular compartments.

1. Introduction

Chemotherapy, a potential treatment for cancers, has been limited in many cases by multidrug resistance (MDR). MDR mechanisms in tumor cells are known to be multifaceted, and many features distinguish MDR cells from their counterpart drug-sensitive cells [1,2]. Among their distinctive characteristics are intracellular pH profiles and specific subcellular compartment recycling activities (including those of endolysosomes), which are both related to MDR mechanisms of sequestration [1,3] and exocytosis [1,4–6].

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Compared to drug-sensitive cells, MDR cells have pHs consistent with more acidic endosomal and lysosomal compartments [1]. These features may be linked to a vacuolar H⁺ - ATPase (V-ATPase) that regulates endosomal acidification and intracellular pH gradients [7,8]. Overexpressed V-ATPases in MDR cells [9,10] induce more acidic endolysosomal compartments. For example, the late endosomal and lysosomal pHs of drug-resistant MCF7 cells were approximately pH 6.0 and pH < 5.8, respectively, whereas the pHs of drug-sensitive MCF7 cells were pH 6.5 and pH > 5.8, respectively [1]. Furthermore, V-ATPase overexpression caused the cytosolic pHs of MDR cells to become more basic than those of drug-sensitive cells (pH 6.8 for MCF7 cells vs. pH 7.1 for drug-resistant MCF7 cells [1]), the protons for endolysosomal acidification coming from the cytosol. Consistent with these results, increases in intracellular pH gradients generated in MDR cells caused an increase in the accumulation of anti-cancer chemicals (mostly weak bases) in acidic intracellular compartments. The reduced ability of ionized drugs to traverse membranes may contribute to this effect [1,3,5].

Additionally, drugs can be expelled by ATP-dependent efflux pumps (*e.g.*, P-glycoproteins) overexpressed in MDR cells [5]. Drugs can bypass these efflux pumps through endocytosis, but endosomal recycling still causes exocytosis of drugs sequestered in endolysosomal compartments [1]. Endocytic recycling is a natural process for maintaining essential components of the plasma membrane [11], but drug-resistant tumor cells have demonstrated faster membrane turnover (recycling) than their drug-sensitive counterparts [1,12,13]. Aggressive endosomal recycling mechanisms of MDR cells are not clear, but a possible cause might be that low cytosolic pHs inhibit more exocytic pathways compared to high cytosolic pH [3,14].

The two aforementioned MDR characteristics not only apply to free chemical drugs but also to nanosized drug carriers [1,6,15]. To avoid being expelled by exocytosis or being sequestered into drug-loaded nanovesicles, and to enhance drug bioavailability at intracellular target sites, the nanocarriers may require membrane destabilizing characteristics (*e.g.*, proton buffering and membrane fusion) for endolysosomal escape [6,15–17].

High molecular weight biopharmaceuticals (protein-, peptide-, and gene-based drugs) also require carrying vehicles to attain cellular internalization processes via endolysosomes [18, 19]. For example, polymeric gene carriers are internalized into the cells via endocytic pathways and are trapped in acidic endolysosomal compartments. In order to reach their intracellular target sites, the nanosized gene carriers need to destabilize endolysosomal membranes to avoid endosomal sequestration and exocytosis [20–22]. Therefore, the role of local pH profiles and the rate of endosomal recycling are critical factors influencing polymeric gene delivery. To date, however, the role of MDR in biotherapeutics-based cancer therapies has not been well understood. In fact, although calcium phosphate- and lipoplex-mediated plasmid expression in wild-type murine embryonal carcinoma cells was approximately 2-fold and 4.3-fold higher than expression levels in their retinoid-resistant cell lines in Purpus and McCue's study [23], there was no discussion as to why drug-sensitive and drug-resistant cell lines showed different transgene expression profiles. Most polymeric gene deliveries have been applied without distinguishing between drug-sensitive and drug-resistant cells.

This study is designed to elucidate the roles of MDR in biotherapeutics-based cancer therapies. Of the common biological therapeutics, gene drugs were selected as the as first non-chemical candidates because various therapeutic genes have been used to kill tumors and investigate tumor mechanisms. Using representative polymeric vectors (poly(L-lysine) (PLL) with gene condensing property and branched polyethyleneimine (PEI) having both gene condensing and endosomolytic properties) and representative breast cancer cell lines (MCF7 cells and their multidrug-resistant subline), this study aimed to provide an understanding of the differences

in transfection against drug-sensitive cancer cell lines and MDR cancer cells with regard to transfection efficiency, cell viability, cellular uptake, intracellular trafficking, endosomal escape, and other relevant attributes of transfection.

2. Materials and methods

2.1. Materials

Branched polyethyleneimine (PEI; $M_w=25$ kDa), poly(L-lysine) hydrobromide (PLL; $M_w(\text{viscosity})=27.4$ kDa), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RPMI1640 medium, Ca^{2+} -free and Mg^{2+} -free Dulbecco's phosphate buffered saline ($\text{Ca}^{2+}(-)\text{Mg}^{2+}(-)\text{DPBS}$), fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RITC), triethylamine (TEA), dimethyl sulfoxide (DMSO), 4-(2-hydroxy-ethyl)-1-piperazine (HEPES), 2-(*N*-morpholino)ethanesulfonic acid (MES), nigericin, monensin, glucose, sodium bicarbonate, doxorubicin (DOX or adriamycin (ADR)), recombinant human insulin, Hoechst 33342 (HO), and paraformaldehyde (PFA) were purchased from Sigma-Aldrich (St. Louis, MO). Plasmid DNA (pDNA) encoding firefly luciferase (gWiz-Luc or pLuc) was purchased from Aldevron, Inc (Fargo, ND). Fetal bovine serum (FBS), penicillin-streptomycin antibiotics, trypsin-EDTA solution, LysoTracker® Red DND-99, and YOYO-1 were purchased from Invitrogen, Inc. (Carlsbad, CA). The Luciferase assay kit and BCA™ protein assay kit were bought from Promega Corporation (Madison, WI) and Pierce Biotechnology, Inc (Rockford, IL), respectively.

2.2. Cells and cell culture

In this study, MCF7 cells (a human breast adenocarcinoma cell line) and MCF7/ADR-RES cells (a DOX-induced multidrug resistant subline of MCF7 cells) were used. The cells were cultured in culture medium (*i.e.*, RPMI1640 medium) supplemented with insulin (4 mg/L), glucose (2 g/L), and 10% heat-inactivated FBS under humidified air containing 5% CO_2 at 37° C. To maintain the MDR characteristics of MCF7/ADR-RES cells, the cells were treated with DOX (400 ng/mL) weekly.

2.3. Preparation and physicochemical characteristics of polyplexes

Polyplexes were prepared using pDNA and polycations (branched PEI and PLL) in HEPES buffer (20 mM, pH 7.4) supplemented with 5% glucose (called as HBG). After mixing pDNA and polycations under predetermined complexation conditions, the polyplex solutions (20 μL per 1 μg pDNA) were incubated for 30 min at room temperature (RT). The complexation ratios of the polyplexes were based on amines (N) of polycations and phosphate groups (P) of pDNA.

The physicochemical characteristics (*e.g.*, particle size and surface charge) of polyplexes were measured using a Zetasizer 3000HS (Malvern Instrument, Inc, Worcestershire, UK) at a wavelength of 677 nm with a constant angle of 90° at RT. The concentrations of pDNA in the polyplex solutions were 2.5 $\mu\text{g}/\text{mL}$ and 5 $\mu\text{g}/\text{mL}$ for surface charge and particle size measurements, respectively.

2.4. In vitro transfection and cell viability

As previously reported [24–26], the transfection study was performed in six-well plates and the cells were seeded at a density of 5×10^5 cells/well. The seeded cells were cultured for 24 hr prior to adding polyplexes. One hour before transfection, the culture medium containing 10% FBS was replaced with serum-free and insulin-free medium. After dosing with the polyplexes (20 μL per 1 μg pDNA), the cells were transfected for 4 hr. Then, the cells were incubated for an additional 44 hr with a serum and insulin-containing medium. After completion of the transfection experiments, the cells were rinsed twice with $\text{Ca}^{2+}(-)\text{Mg}^{2+}(-)\text{DPBS}$ and then

lysed using a reporter lysis buffer. Relative luminescence unit (RLU) was evaluated by the manufacturer's protocol for the luciferase assay. Protein content in the cells was evaluated by the BCATM protein assay.

The MTT-based cell viability assay was the same as previously described for *in vitro* transfection except for the cell numbers (2.5×10^5 cells/well; 12-well plates) and the polyplex dose (10 μ L; 0.5 μ g pDNA). After finishing the 48 hr transfection procedure, MTT solution (0.1 mL; 5 mg/mL) was added to the cells (1 mL of culture medium). After an additional 4-hr incubation, the MTT-containing medium was removed. Formazan crystals produced by living cells were dissolved in DMSO and their absorbances were measured at 570 nm using a microplate reader.

2.5. Cellular uptake of polyplexes

As previously described, the cells were prepared in six-well plates. Polyplexes (20 μ L per 1 μ g pDNA) were prepared using YOYO-1-intercalated pDNA. After incubating for 4 hr in the transfection medium, the cells were detached and fixed using 4% PFA solution. The cells with fluorescent polyplexes were monitored using flow cytometry (FACScan Analyzer, Becton-Dickinson, Franklin Lakes, NJ) with a primary argon laser (488 nm) and fluorescence detector (530 ± 15 nm) for YOYO-1 dye. The polyplex uptake in the cells was analyzed from a gated viable population of at least 5,000 cells.

2.6. Intracellular pH measurement of polyplexes

The intracellular pH environments of polycation vectors were monitored using fluorescent dye-labeled polymers. PEI and PLL were double-labeled with pH-sensitive FITC and pH-insensitive RITC and designated FITC-PEI-RITC and FITC-PLL-RITC, respectively. FITC-PLL-RITC had approximately 2.3 mol% (based on the L-lysine unit) FITC and 1.2 mol% RITC, while FITC-PEI-RITC had approximately 1.6 mol% (based on the amines) FITC and 0.4 mol% RITC.

As previously described, cells were transfected using FITC-PLL-RITC/pDNA or FITC-PEI-RITC/pDNA complexes. To estimate the microenvironmental pHs of polymeric vectors at 0.5, 1, 1.5, 2, 3, and 4 hr post-transfection, the polyplexes that were not internalized by the cells were rinsed out using $\text{Ca}^{2+}(-)\text{Mg}^{2+}(-)\text{DPBS}$, and the transfected cells were detached from the culture plate. The cells were resuspended in $\text{Ca}^{2+}(-)\text{Mg}^{2+}(-)\text{DPBS}$ with 1% PFA to maintain their cellular and intracellular membrane structures.

For the construction of a pH calibration curve, FITC-PLL-RITC/pDNA- or FITC-PEI-RITC/pDNA-transfected cells were resuspended in 0.5 mL of pH clamp buffers. To adjust the pHs (pH 7.4, 6.8, 6.0, 5.0, and 4.0) of the clamp buffers, $\text{Ca}^{2+}(-)\text{Mg}^{2+}(-)\text{DPBS}$ buffer (pH 7.4) and MES buffer (pH 4.0; 50 mM MES, 150 mM NaCl, 4 mM KCl, and 1 mM MgSO_4) were mixed. Additionally, monensin (20 μ M) and nigericin (10 μ M) were added to the pH clamp buffers to ensure that they were homogeneously applied to all intracellular compartments in the pH calibration cells.

The cells containing fluorescent polyplexes were monitored using flow cytometry with a primary argon laser (488 nm) and fluorescence detectors (530 ± 15 nm for FITC and $585 \pm$ nm for RITC). The average intracellular pH environments of polycations were determined using ratios of FITC to RITC intensities in a gated viable population of at least 5,000 cells. First, the correlation between pH and average RITC/FITC ratios of pH clamp cells was calibrated for polyplex-transfected MCF7 or MCF7/ADR-RES cells to adjust for differences in cellular autofluorescence backgrounds and laser intensity settings. A typical pH calibration plot is shown in Fig. S1(a). When transfected cells have a constant RITC intensity, their FITC intensity

decreases as the pH lowers. The relationship between clamp pH and average RITC/FITC was plotted in Fig. S1(b). Based on this pH calibration curve, the intracellular pHs of polymeric vectors in whole transfected cells were estimated. In order to estimate the major subcellular location of polyplexes from their intracellular pHs, whole fluorescent cell populations were further categorized into four different pH ranges using pH calibration cells (Fig. S1(c)): pH > 6.8 (most relevant pHs to the cytoplasm or the nucleus), 6.0 < pH < 6.8 (the early endosomes), 5.0 < pH < 6.0 (the late endosomes), and pH < 5.0 (the lysosomes).

2.7. Identification of pDNA location inside cells

The cells were seeded on coverslips, and the study was performed as previously described. Polyplexes (20 μ L; 1 μ g pDNA) were prepared by adding YOYO-1-intercalated pDNA to the cells. At 4 hr post-transfection (30 min prior to sampling), LysoTracker® Red dye and HO were added for staining acidic intracellular vesicles and the nucleus, respectively. The cells were rinsed with Ca^{2+} (-)/ Mg^{2+} (-)DPBS and were fixed with 4% PFA. The cells were evaluated using a laser scanning confocal microscope (FV1000, Olympus, Center Valley, PA) with excitation lasers (diode for 408 nm, Ar for 488 nm, and HeNe for 543 nm) and variable band-pass emission filters. Confocal images were collected in 500-nm sections and were used to construct images of whole cells.

3. Results and discussion

Prior to comparing polymeric transfection against MCF7 and MCF7/ADR-RES cells, optimum conditions of PEI- and PLL-based transfection for these cell lines were determined using less toxic complexation ratios of polymer/pDNA complexes. For PEI/pDNA complexes, N/P=5 was applied because generally higher N/P values of the polyplexes cause cytotoxicity and reduce transfection efficiency [27–29]. In the case of PLL/pDNA complexes, N/P=5 was used as an optimum condition for the highest transfection efficiency in the ranges of N/P=3 to N/P=10 (Fig. S2). The complexation ratio (*i.e.*, N/P=5) of PLL/pDNA complexes for MCF7/ADR-RES cells was also applied to MCF7 cells because MCF7/ADR-RES cells are a subline of MCF7 cells. Their particle size and surface charge were 83 ± 9 nm and 11 ± 6 mV, respectively, for the PEI/pDNA complexes and 92 ± 14 nm and 23 ± 10 mV, respectively, for the PLL/pDNA complexes.

3.1. Transfection efficiency

The same transfection conditions for PEI- and PLL-based transfection were applied to MCF7 and MCF7/ADR-RES cell lines, and their transfection efficiencies are shown in Fig. 1. Regardless of polyplex type, the polymeric transfection efficiencies of MCF7 cells were higher than those of MCF7/ADR-RES cells. PEI/pDNA- and PLL/pDNA-transfected MCF7 cells demonstrated approximately 3.9-fold ($p=5 \times 10^{-7}$ by an unpaired Student's *t*-test) and 7.3-fold ($p=0.0002$ by unpaired Student's *t*-test) more gene expression than their transfected MCF7/ADR-RES cells, respectively. These findings are in accord with Purpus and McCue's non-viral (*i.e.*, calcium phosphates and liposomes) transfection results using murine embryonic carcinoma cells and their retinoid-resistant cells [23].

3.2. Cell viability

To better understand the differences in transfection properties between MCF7 and MCF7/ADR-RES cells, their transfection-induced effects on cell viability were investigated. It is well known that drug-resistant cells are more tolerant to chemical drugs than drug-sensitive cells [1]; however, there is no information available in the literature regarding whether this resistance extends to non-chemical drugs and large-sized biotherapeutics. Thus, polyplex toxicities against MCF7 and MCF7/ADR-RES cells were evaluated using MTT-based tests, which are influenced by cell viability, metabolic activity, and cell proliferation. When compared with the

cell viability of untransfected MCF7 cells (control, 100%), PEI/pDNA- and PLL/pDNA-transfected MCF7 cells had survival rates of $96\pm1\%$ ($p=0.0$ by unpaired Student's *t*-test) and $89\pm3\%$ ($p=0.0001$ by unpaired Student's *t*-test), respectively (Fig. 2). These numbers are contrary to the widely held opinion that PEI is more toxic than PLL [20]; PLL-polyplexes were more toxic than PEI-polyplexes to MCF7 cells in this study. This toxicity may be caused by PLL/pDNA complexes having a more positive surface charge (23 ± 10 mV vs. 11 ± 6 mV) than PEI/pDNA complexes; positive charges are strongly linked to cytotoxicity. Additionally, although the same N/P ratio was applied, more PLL was used compared to that of PEI because PEI has a higher charge density than PLL [20].

In the case of MCF7/ADR-RES cells, their viabilities were $98\pm2\%$ ($p=0.35$ by unpaired Student's *t*-test) for PEI/pDNA complexes and $93\pm2\%$ ($p=0.0006$ by unpaired Student's *t*-test) for PLL/pDNA complexes compared with the viability of corresponding control untransfected cells (Fig. 2). As was the case the for MCF7 cells, the MCF7/ADR-RES cells also showed less resistance to PLL-polyplexes than PEI-polyplexes under the experimental conditions in this study. Interestingly, MCF7/ADR-RES cells demonstrated more resistance against polyplex-induced toxicity than MCF7 cells although their differences were small (approximately 2~4 %) with low statistical significance ($p=0.53$ for PEI/pDNA complexes and $p=0.14$ for PLL/pDNA complexes by unpaired Student's *t*-test). However, this finding leaves open the possibility that there may be more significant differences between the cell viabilities of polyplex-transfected drug-sensitive cells and drug-resistant cells when more toxic conditions of polymeric transfection (high N/P ratios, high pDNA doses, more toxic polymers, etc) are applied, as is the case for chemical drugs.

3.3. Polyplex uptake

During polymeric transfection, transfection efficiency and cytotoxicity are affected by the number of polyplexes internalized into cells. In our transfection experiments, the transfection medium was applied for 4 hr and then the medium was replaced with the culture medium. Thus, the polyplexes that were not taken up by 4 hr post-transfection were removed. Only internalized polyplexes would be used for further gene expression processes. As shown in Fig. 3, the polyplex uptake of MCF7 and MCF7/ADR-RES cells was monitored at 4 hr post-transfection using polyplexes prepared with YOYO-1-intercalated pDNA. MCF7 cells took up more PLL/pDNA complexes than PEI/pDNA complexes. This finding is not unexpected; the cellular uptake of polyplexes generally increases with increasing positive surface charge due to the increased electrostatic attraction between the polyplexes and plasma membranes. Interestingly, MCF7/ADR-RES cells showed slightly more PEI/pDNA uptake than PLL/pDNA uptake although their difference was not significant.

Differences in cellular autofluorescence and experimental conditions prevent absolute comparisons in the polyplex uptakes of these two cell types. However, histograms of untransfected cells suggested that relative comparisons could be made. As shown in Fig. 3, both polyplexes were more internalized into MCF7/ADR-RES cells than MCF7 cells. The difference between PLL/pDNA uptake and PEI/pDNA uptake in MCF7/ADR-RES cells was reduced compared with that in MCF7 cells. As is the case for chemical drugs, polyplex-containing endosomes can also be sequestered and recycled (or exocytosed) [30]. Unlike PEI/pDNA complexes, PLL/pDNA complexes do not have protonable secondary and tertiary amines, and therefore lack endosomal disrupting characteristics [20]. MDR cells also have more exocytosis activity than drug-sensitive cells. Based on these facts, it can be inferred that PLL/pDNA complexes are more easily exocytosed than PEI/pDNA complexes in MCF7/ADR-RES cells.

Exocytosis of PLL/pDNA complexes in MCF7/ADR-RES cells was further confirmed by the observation of time-dependent relative pH-insensitive RITC polyplex intensities as shown in

Fig. 4. Polyplex uptake in each polyplex-transfected cell at 4 hr post-transfection was set at 100% because of different RITC graft ratios in PLL and PEI and different autofluorescence backgrounds in the two cell lines. For the most part, the polyplex-transfected MCF7 and MCF7/ADR-RES cells showed continuously increasing relative RITC intensities (*i.e.*, increasing polyplex uptake) with increasing post-transfection time, the exception being PLL/pDNA-transfected MCF7/ADR-RES cells. PLL/pDNA uptake in MCF7/ADR-RES cells reached a saturated level within one hour post-transfection and the level was maintained throughout the experiment. This finding suggests that PLL/pDNA complexes in MCF7/ADR-RES cells have no endolysosomal escape ability and can be easily exocytosed.

3.4. Intracellular environments of polyplexes

As previously mentioned, polyplexes can be sequestered in the endolysosomes of MDR cells in the same way that chemical drugs can [30]. Unlike the exocytosis of polyplexes, their sequestration cannot be monitored by polyplex uptake studies. Intracellular environments, including sequestration of polyplexes or pDNA, are very important for understanding effective polymeric transfection processes. Increased nuclear localization and decreased endolysosomal sequestration of polyplexes (or pDNA) caused higher polymeric transfection efficiency. Confocal microscopy-based techniques can track intracellular localizations and pHs of polyplexes in a single cell or multiples cells [31]. This method can microscopically quantitate polyplexes located at certain intracellular compartments or exposed at certain pHs. Also, using the fluorescent distributions of at least several thousand cells, flow cytometry has macroscopically predicted the average pHs of transfected cells resulting from intracellular polyplexes (or pDNA) [32,33].

This study was conducted using polymeric gene carriers having a pH-sensitive fluorescent dye (FITC) and a pH-insensitive fluorescent dye (RITC). The ratios of RITC and FITC intensities obtained from flow cytometry were used to estimate the average intracellular pHs of PEI/pDNA- and PLL/pDNA-transfected MCF7 and MCF7/ADR-RES cells (Fig. 5). When MCF7 cells were transfected with PLL/pDNA complexes, the cells were quickly exposed to pH ~6.8 for 0.5 hr post-transfection. Their average intracellular pHs slowly dropped to approximately pH 6.6 until 2 hr post-transfection and then slightly recovered. However, in PEI/pDNA-transfected MCF7 cells, their pHs were approximately 7.3–7.4 for 1 hr post-transfection and then were decreased to approximately pH 7.0 and little lower. Similarly, when these polyplexes were applied to drug-resistant MCF7/ADR-RES cells, the acidification rates of PLL/pDNA-containing cells were greater than those of PEI/pDNA-containing cells. That is, the average intracellular pHs of PLL/pDNA-transfected MCF7/ADR-RES cells dropped sharply to approximately 5.1–5.2 within the initial 0.5 hr post-transfection and then slowly increased to around 6.1–6.2 at 4 hr post-transfection.

Regardless of cell type, the intracellular pHs of PLL/pDNA complexes were lower and fell more quickly than those of PEI/pDNA complexes. This phenomenon might have been caused by the proton buffering activity of the PEI/pDNA complexes, which can delay endosomal acidification and cause a quick disruption of endosomal compartments. The different acidification rate profiles of the two polyplexes were consistent with previous studies using confocal microscopy and flow cytometry [31,33]. In addition, after a pH drop, their recovered intracellular pHs might be related to increasing numbers of polyplexes that are exposed to the cytoplasm or the nucleus [31].

Compared to the intracellular pHs of PLL/pDNA complexes in MCF7 cells, those in MCF7/ADR-RES cells were much lower. These results indicate that the endosomal acidification rates of MCF7/ADR-RES cells (drug-resistant cells) are faster than those of MCF7 cells (drug-sensitive cells). For PEI/pDNA complexes in MCF7/ADR-RES cells, their proton buffering capacities resulted in a delayed drop of their intracellular pHs like PEI/pDNA complexes in

MCF7 cells. Polyplex-transfected MCF7/ADR-RES cells showed quicker intracellular pH recovery for PLL/pDNA complexes and higher intracellular pHs for PEI/pDNA complexes than polyplex-transfected MCF7 cells. These findings might be the result of higher cytosolic and nuclear pHs in MCF7/ADR-RES cells than in MCF7 cells [1,5,34].

The average ratios of RITC and FITC intensities (*i.e.*, average intracellular pHs) from polyplex-transfected whole cells can impact the acidification rates of polyplexes but not intracellular compartments containing polyplexes. Thus, a whole fluorescent cell population was further divided into four different pH groups using pH calibration cells. Altan's report on the intracellular pHs of endosomes (pH 6.6 ± 0.1 vs. pH 6.1 ± 0.1), lysosomes (pH > 5.8 vs. pH 5.1 ± 0.1), cytosol (pH 6.75 ± 0.3 vs. pH 7.15 ± 0.1), and nucleus (pH 7.1 ± 0.1 vs. pH 7.2 ± 0.1) in drug-sensitive MCF7 cells and their DOX-resistant counterparts [35] was used to predict the major intracellular compartments of polyplexes from their intracellular pHs in the transfected MCF7 and MCF7/ADR-RES cells.

As shown in Fig. 6, the major population of PLL/pDNA-transfected MCF7 cells increased from 46% at 0.5 hr post-transfection to 61% at 4 hr post-transfection and had an average intracellular pH in the range of 6.57–6.65. These pH ranges were close to the endosomal and cytosolic pHs of MCF7 cells reported in the literature, suggesting that the intracellular polyplexes may be exposed to endosomes (probably early endosomes) or cytosol [35]. The second major cell population (~20%) was exposed to pH 7.23–7.34 suggesting that their PLL/pDNA complexes may be primarily in the nucleus. Less than 20% of the transfected cells had pH 5.41–5.45 or pH ~4; these PLL/pDNA complexes may be exposed to the lysosomes or the late endosomes.

In the case of PEI/pDNA-transfected MCF7 cells, most cells (> 80% at each time point) had an average intracellular pH of 7.0–7.38. This pH range suggests that PEI/pDNA complexes may quickly disrupt the endolysosomal compartments and localize to the nucleus or the cytoplasm. By 3 hr post-transfection, cells having pH 5.68–6.16 were identified as making up approximately 7–13% of total cells. In these cells, polyplexes may have been exposed in the maturation process from the endosomes to the lysosomes. However, the sequestered PEI/pDNA complexes in vesicles that were too acidic (the lysosomes) may be very rare, as indicated by the low cell population (< ~6%) having a pH close to 4.

When MCF7/ADR-RES cells were transfected with PLL/pDNA complexes, the cell populations for pH 4.0 (lysosomal pHs), pH 5.40 (probably late endosomal or lysosomal pHs), and pH 6.31 (probably early endosomal pHs) were approximately 22%, 17%, and 11%, respectively, at 0.5 hr post-transfection (the cell number at 4 hr post-transfection was set at 100%). The percentage of cells exposed to pH ~6.19–6.44 (probably early endosomes) increased up to 55% at 4 hr post-transfection. However, the cell populations with pHs in the 5.40–5.57 range and around pH 4 (*i.e.* the late endosome or lysosome-related pHs) increased to 48% at 2 hr post-transfection and 26% at 1 hr post-transfection, respectively, and then decreased to 35% and 5% at 4 hr post-transfection, respectively. However, their reduced cell populations did not cause an increase in the percentage of cells having cytosolic or nuclear pHs (approximately 5%). This finding might further support the hypothesis that most PLL/pDNA complexes in the late endosomes and lysosomes of MCF7/ADR-RES cells are exocytosed while few polyplexes can be released into the cytosol and further localized to the nucleus. The possible exocytosis of polyplexes in the late endosomes and lysosomes of MCF7/ADR-RES cells may be related to the fact that the lysosomal compartments of human breast malignant cells are mostly located at the cell periphery [36].

For PEI/pDNA-transfected MCF7/ADR-RES cells, their largest cell population (from 75–80% to 50%) with time had a pH of 7.4, suggesting cytosolic or nuclear localization. The percentage

of cells having pH 6.46–6.60 increased from 3.5% at 0.5 hr post-transfection to 34% at 4 hr post-transfection. In these cells, the endosomal release process of PEI/pDNA complexes may be progressing. Also, the cells having pH 5.51–5.62 (probably late endosomal pHs or lysosomal pHs) were detectable 3 hr post-transfection, and their population was approximately 12% at 4 hr post-transfection.

Compared to polyplex-transfected MCF7 cells, more polyplex-transfected MCF7/ADR-RES cells were exposed to acidic intracellular environments. In MCF7/ADR-RES cells, PLL/pDNA complexes were more often exposed to late endosomal pHs and lysosomal pHs than those in MCF7 cells. This could indicate that PLL/pDNA complexes are more frequently trapped in acidic endolysosomal compartments in drug-resistant cells than in drug-sensitive cells.

For further supporting evidence of the intracellular localization of polyplexes, the polyplexes prepared with YOYO-1-intercalated pDNA were tracked by a combination of LysoTracker®-stained acidic vesicles (probably late endosomal and lysosomal compartments) and HO-stained nuclei. Although this study does not distinguish between polyplexes in the early endosomes and the cytoplasm, the localization of polyplexes in the nucleus, the early endosomes/cytoplasm, and the late endosomes/lysosomes were predicted.

As shown in Fig. 7, most PLL/pDNA complexes in MCF7 cells were distributed in both the cytoplasm/early endosomes and the nucleus, whereas few complexes were localized to acidic vesicles. However, in PEI/pDNA-transfected MCF7 cells, PEI/pDNA complexes had a more nuclear than cytoplasmic distribution. Unlike the PLL/pDNA complexes in MCF7 cells, PEI/pDNA complexes were very rarely trapped in acidic vesicles. In MCF7/ADR-RES cells, PLL/pDNA complexes were almost evenly distributed to the nucleus and the early endosomes/cytosol, whereas slightly more PEI/pDNA complexes accumulated in the nucleus than the cytoplasm/early endosomes. However, PLL/pDNA complexes were much more likely to be trapped in acidic compartments compared to PEI/pDNA complexes in these cells. Compared with polyplex-transfected MCF7/ADR-RES cells, the polyplexes of MCF7 cells were more localized in the nucleus and were less sequestered in acidic compartments. These confocal results are in line with the localization predictions made using flow cytometry techniques.

In this study, our findings suggest that polymeric gene transfection may be similar to the delivery process of chemical anticancer drugs. That is, regardless of polyplex type, the polymeric transfection efficiency was lower in drug-resistant cells than in drug-sensitive cells. MDR cells have an increased tolerance to polymer-based gene drugs compared to drug-sensitive cells. In addition, when polyplexes with no proton-buffering capacity were applied, they were more frequently exocytosed from or trapped in drug-resistant cells compared with the polyplexes of polymeric transfection of drug-sensitive cells. These findings might explain why the transfection efficiencies of PLL/pDNA- (with no proton buffering capacity) in MCF7 and MCF7/ADR-RES cells were larger than those of PEI/pDNA- (with proton buffering capacity) transfected cells. These striking similarities between gene drugs and chemical drugs in MDR cells might be applicable to other biological therapeutics such as peptides and proteins.

In addition, our results may provide insight into the disparities between polymeric transfection in animal solid tumors and mono-layered cell culture models. Efforts to develop polymeric vectors have led to the finding that polyplexes often exhibit remarkable transfection efficiencies, which are several orders of magnitude greater than naked pDNA in cell culture experiments [20]. However, in animal tests, polymeric transfection efficiency rarely exceeds one order of magnitude greater than naked pDNA. Although *in vitro-in vivo* disparities with regard to transfection efficiency are not yet fully understood, the poor efficacy *in vivo* may be related to extracellular and intracellular barriers prior to transfection [20]. Additionally, different drug resistances of tumor cells could be attributed to the fact that three dimensional

in vivo solid tumors and *in vitro* cell culture models having more drug resistance than mono-layered cells [37,38]. These facts, combined with our findings, suggest that effective polymeric gene vectors for solid tumor cells should be designed with consideration of the differential drug sensitivity of target cells.

CONCLUSION

Compared to drug sensitive cells, MDR cells exocytosed polyplexes more actively, which is the typical MDR cell response to chemical anticancer drugs. MDR cells also trapped polymeric gene carriers in acidic compartments to a higher degree. These observations explain why transfection efficiencies for polymeric vectors were lower in MDR cells than in non-MDR cells regardless of polymeric proton buffering capacities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Transfection efficiency of PEI/pDNA- and PLL/pDNA-transfected MCF7 and MCF7/ADR-RES cells. (***) $p < 0.001$ determined by an unpaired Student's *t*-test; means $\pm$ SEM; $n \geq 39$)

Fig. 2. Cell viability of PEI/pDNA- and PLL/pDNA-transfected MCF7 and MCF7/ADR-RES cells. The cell viability of each untransfected control was set at 100%. (* $p < 0.05$ and *** $p < 0.001$ compared with cell viability of untransfected cells (control) as determined by an unpaired Student's *t*-test; means $\pm$ SEM; $n \geq 35$)

Fig. 3. Histograms of PEI/pDNA- or PLL/pDNA-uptake in MCF7 and MCF7/ADR-RES cells at 4 hr post-transfection.

Fig. 4. RITC intensity-based PEI/pDNA- or PLL/pDNA-uptake in MCF7 and MCF7/ADR-RES cells within 4 hr after polymeric transfection. Each RITC intensity at 4 hr post-transfection was set at 100% (means $\pm$ SEM; n=3).

Fig. 5. Average intracellular pH of PEI/pDNA- or PLL/pDNA-uptake in MCF7 and MCF7/ADR-RES cells within 4 hr after polymeric transfection (means $\pm$ SEM; n=3).

Fig. 6.

Intracellular pH distributions of PEI/pDNA- or PLL/pDNA-uptake MCF7 and MCF7/ADR-RES cells within 4 hr after polymeric transfection. The pH of each subcellular compartment in polyplex-transfected cells and the number of cells in each subcellular compartment at a given time point following transfection are represented in the following dot plots and column plots, respectively. Intracellular pHs for polyplex-transfected cells relevant to pHs of the lysosomes (black circle, ●), the late endosomes (gray inverse triangle, ▼), the early endosomes (dark gray square, ■), and the cytosol/the nucleus (bright gray diamond, ◆) are represented in dot plots. Their corresponding % cell numbers are represented as ■, ■, ■, and ■ in the column plots. The cell number at 4 hr post-transfection was set to 100% (means±SEM; n=3).

MCF7 **MCF7/ADR-RES** **Fig. 7.**

Intracellular localization of pDNA delivered with PEI or PLL in polyplex-transfected MCF7 and MCF7/ADR-RES cells at 4 hr post-transfection. Nuclei, pDNA, and acidic vesicles were distinguished using HO (blue), YOYO-1 (green), and LysoTracker[®] dye (red).