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Feature Article

Gene Expression Profiles in Mouse Lung Tissue after Administration of Two Cationic Polymers Used for Nonviral Gene Delivery

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Purpose. This study compared gene expression profiles in mouse lungs after administration of the cationic polymers polyethyleneimine (PEI) or chitosan alone or formulated with a luciferase reporter plasmid (PEI–pLuc, chitosan–pLuc).
Methods. The polymers and formulations were administered intratracheally to Balb/c mice at doses judged to be nontoxic according to intracellular dehydrogenase activity and tissue morphology. RNA was isolated from the lungs 24 or 72 h after administration, and a dedicated stress and toxicology cDNA array was used to monitor the *in vivo* response to the gene delivery system in the lung tissue.
Results. The gene expression profiles differed between the PEI and chitosan groups with regard to both the total number and the type of expressed genes. Chitosan–pLuc upregulated genes that protect the cell from oxidative stress and inflammation, such as heme oxygenase-1 and catalase, whereas PEI–pLuc upregulated genes involved in inflammatory processes, such as the cyclooxygenases 1 and 2, indicating possible involvement in the development of adverse reactions. However, both polymers activated genes involved in reaction to stress, such as DNA damage repair. Furthermore, in the PEI group, chaperone genes and members of the p38 mitogen-activated protein kinase pathway were also upregulated, suggesting a possible explanation for the better performance of PEI in gene delivery systems.
Conclusions. The results indicate that gene expression profiling is a useful and sensitive tool for the evaluation of tissue responses after administration of polymers or gene delivery systems. The results also suggest a possible explanation for the differences in gene delivery performance between the two polymers in gene delivery systems.

KEY WORDS: cDNA array technology; chitosan; polyethyleneimine (PEI); pulmonary DNA delivery; toxicity.

INTRODUCTION

Although the two cationic polymers polyethyleneimine (PEI) and chitosan both offer potential for experimental nonviral gene therapy, their toxicity profiles are quite different. Unfortunately, PEI is associated with dose-dependent toxicity, which probably explains why it has not yet been used in human studies (1). Chitosan, on the other hand, is considered safe and nontoxic; it has been approved as a food additive and is used in wound-healing products (2). Information on the reasons for these different toxicity profiles is limited, and additional studies are needed to understand and eventually improve the toxicity profile of PEI and alternative polymers for use in gene technology.

PEI is considered by many to be the most effective cationic polymer for pulmonary gene delivery (3). PEI–pDNA complexes are bound to proteoglycans on cell surfaces and subsequently undergo endocytosis (4). After uptake, the high proton-buffering capacity of PEI results in rapid osmolysis of the endosomes, and the PEI–pDNA complexes escape into the cytosol (5) and are subsequently transported into the nucleus (6). Conventional high molecular weight chitosan is generally considered less effective in gene delivery systems than PEI *in vitro* and *in vivo* (7,8). However, it has a similar uptake mechanism to that of PEI. Thus, chitosan–pDNA is bound to cell surface proteoglycans and is internalized by endocytosis. However, in contrast to PEI, chitosan is degraded in the endosome and the material is then released into the cytoplasm, probably after hyper-osmotic rupture of the cell membrane caused by accumulation of the degradation products. The material is then transported to the nucleus (8). Earlier studies have shown that chitosan has immunostimulatory properties and it has therefore been used as a nasal adjuvant for protein antigen formulations in humans (9). In a recent study, we found that PEI can also stimulate the immune system (10) and, in an attempt to obtain more detailed information about how these polymers affect relevant tissues, we undertook this study on the local tissue effect after pulmonary delivery of the formulations to mice.

The acute toxicity of PEI and chitosan has mainly been investigated previously by assessing the metabolic activity of

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cells [e.g., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay] after *in vitro* delivery or by histologic evaluation of the tissue after *in vivo* delivery (11,12). However, these techniques provide only limited information on acute toxicological responses in the target cells or tissues. One aim of this study was therefore to expand the toxicological response profiles of PEI, chitosan, and their corresponding complexes with reporter plasmid luciferase DNA (PEI–pLuc and chitosan–pLuc) to include effects at the site of administration. We hypothesized that such a profile would also provide insight into the reasons for the differences in efficacy between the molecules in gene delivery systems. We also planned to investigate the effects on the toxicity profiles of doses that are generally considered nontoxic *in vitro* according to standard toxicity assays. The intention was to investigate the sensitivity of cDNA arrays for assessing acute tissue responses to polymers and delivery systems. The doses chosen were thus lower than those evoking acute histologic changes or changes in the metabolic activity of lung tissue *in vitro* (8). Thus, the stress and toxicity responses of the whole-lung tissue (rather than those of any specific cell populations) were analyzed 24 h after intratracheal administration of the polymers/complexes to mice to capture the maximal induced luciferase gene expression (8). Previous studies have shown that whole organ tissues can be used to distinguish between patterns of differentially expressed genes, although the sensitivity of the gene expression analysis may be decreased (13). The tissues from all animals in each group were pooled as reported earlier (10,14), and total RNA was extracted for gene expression analysis using a cDNA array focused on stress and toxicological response.

The results of this study show that genes involved in stress reactions are induced by PEI and chitosan, indicating that it is possible to observe a wider range of acute stress and toxicological responses with array technology than with conventional metabolic and histologic examinations. In general, more genes involved in adverse reactions were upregulated by PEI than by chitosan, which supports previous indications that PEI is less biocompatible. However, PEI also upregulated genes that stabilize the reporter protein luciferase and other proteins, suggesting an explanation for the better performance of PEI in gene delivery systems. Our results show for the first time that gene expression profiling is a useful and sensitive tool for evaluation of local tissue responses after administration of polymeric delivery systems. The results also provide clues to the reasons behind the better gene delivery performance of systems using PEI compared with other polymer systems.

MATERIALS AND METHODS

Plasmids and Chemicals

A plasmid of GMP grade containing the pCMV-Luc reporter gene without immunostimulatory CpG sequences was a generous gift from Valentis, Inc. (The Woodlands, TX, USA). PEI, molecular weight 25 kDa, was purchased from Aldrich (Stockholm, Sweden) and ultrapure chitosan (Protasan UP G 210), molecular weight 162 kDa, was obtained from Pronova Biopolymer (Oslo, Norway).

Formulation of Polyplexes

PEI stock solution (10 mM) and PEI polyplexes (PEI–pLuc) at a charge ratio of 5:1 (positive amine/negative pDNA phosphate, +/–) (34 μg PEI: 50 μg pLuc) were prepared as previously described (15) with the exception that sterile water was used instead of 150 mM NaCl. A stock solution of chitosan (0.2 mg/mL) was prepared by dissolving the powder in sterile water at pH 6.2, followed by sterile filtration. Chitosan complexes (chitosan–pLuc) were formulated with a charge ratio of 3:1 by adding chitosan (160 μg) and then pLuc stock solutions to sterile water (1 mL final volume) under intense stirring on a vortex mixer (Heidolph REAX 2000, level 4, Kebo Lab, Spånga, Sweden). The polyplexes were then concentrated to around 70 μL by mild evaporation under vacuum and the tonicity of the polyplex solution was adjusted by adding 30 μL of a mannitol solution as described previously (8). The charge ratios of PEI and chitosan polyplexes (5:1 and 3:1, respectively) were optimized in an earlier study (8). In addition, solutions of free PEI polymer (34 μg) and free chitosan polymer (160 μg) were prepared by adding mannitol to a final volume of 100 μL. An isotonic mannitol solution (100 μL; vehicle only) was also prepared for use as a control. This control was selected to avoid overinterpretation of the gene expression related to the polymers and polymer formulations. The transfection efficiency of the PEI and chitosan complexes was maximal 24 and 72 h after intratracheal administration to mice, respectively (8). Therefore, we investigated the effects of both the polymer formulations at 24 and 72 h.

In Vivo Studies

The animal experiments were approved by The Swedish National Board for Laboratory Animals. Male Balb/c mice, 6–7 weeks old (Charles River, Sweden), were randomly separated into groups of two to three mice and anesthetized with ketamine–xylazine (5/20 vol %, 0.1 mL/10 g of body weight). The trachea was surgically exposed with a 0.5-cm incision. The study formulation or control solution was injected dropwise over 5 min into the trachea using a 27 G needle. After administration, the incision was sutured. The mice were killed (by CO_2) 24 or 72 h after administration. The lungs were perfused with 10 mL of cold phosphate-buffered saline (PBS, Life Technologies) through the left ventricle of the heart, removed, washed in PBS, and snap frozen in liquid nitrogen.

Gene Expression Profiling Using Array Technology

The frozen lungs from each group were pooled to compensate for individual variations (14,16) and the total RNA was extracted using Ambion's Totally RNA kit (Austin, TX, USA). DNA contamination was removed using DNaseI (Promega) and controlled by polymerase chain reaction (PCR). The samples were adjusted to a final concentration of 2.5 mg/mL and stored at −70°C. The Atlas Mouse Stress Array (Clontech, Palo Alto, CA, USA), incorporating 149 genes, was used according to the manufacturer's guidelines. The level of expression was quantified using a phosphor imager scanner (Molecular Dynamics, Sunnyvale, CA, USA).

The expression level of genes was not confirmed by reverse transcription–PCR (RT-PCR), as previous RT-PCR experiments indicated a reproducibility of 80–90% for the type of arrays used in this study (14,17).

Bioinformatic Analysis of the Gene Expression Data

Bioinformatic analysis was performed, as reported earlier (14). Briefly, the image data were processed using the software AtlasImage 1.01a (Clontech, Palo Alto, CA, USA). Signals lower than the average background level were filtered out. The adjusted intensity of each gene was obtained by calculating the average intensity (two measurements for each gene) after subtraction of the average background signal. To compare two or more arrays, the adjusted signal intensities of all genes were normalized to the data derived from the control sample using the global mode and the sum method. To filter for genes with significant expression changes, the following criteria were used: a more than 2-fold relative change compared with control in at least one sample and a difference in signal intensity of more than 100 across experiments (arbitrary units). The gene expression data were plotted using the program Excel and clustered using the software GeneCluster 1.0 (18). The functions of selected genes were studied using GeneCards (19).

Statistics

Gene expression background values were adjusted by 1 unit from 0 to 1. This negligibly small change in gene expression levels was implemented to allow logarithmic transformation of the data. The root-mean-square error (RMSE), i.e., the standard error of the regression, was used to measure the scatter of the gene expression data compared with control (C in the following equation).

$$RMSE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(\log y_{i,C} - \log y_{i,formulation})^2}$$

RESULTS AND DISCUSSION

General Stress and Toxicologic Responses

The stress and toxicological responses were investigated using pairwise comparisons of the gene expression data from

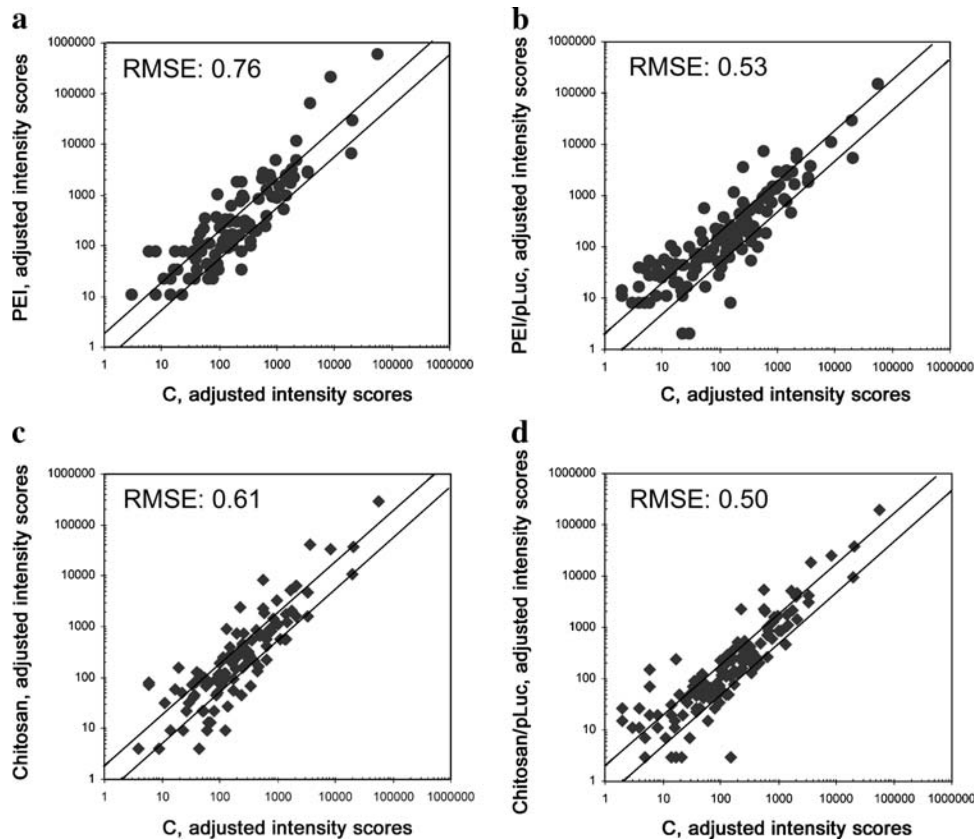

Fig. 1. Treatment-specific global gene expression patterns in mouse lungs 24 h after administration of polyethyleneimine (PEI), chitosan, their luciferase reporter plasmid complexes (PEI–pLuc, chitosan–pLuc) or vehicle (C). RNA was isolated and transformed to radioactively labeled DNA as described in Materials and Methods. The levels of cDNA for each gene were measured and processed by array technology. The plots represent pairwise comparisons of gene expression levels between each formulation and C. Each spot represents one gene and the lines represent 2-fold differences between the two samples. (a) PEI, (b) PEI–pLuc, (c) chitosan, (d) chitosan–pLuc. RMSE values, i.e., the standard error of the regression, were used as an indicator of the scatter of the data.

Table I. Number of Genes with Significant Expression Changes After Administration of Polyethylenimine (PEI), Chitosan or their Luciferase Reporter Plasmid Complexes (PEI–pLuc, Chitosan/pLuc) to Mouse Lung

Delivery system	No. of genes[a]	% of total no. of genes[b]
PEI	43	29
PEI–pLuc	25	17
Chitosan	30	20
Chitosan–pLuc	25	17

[a] The number of genes was compiled from Fig. 1a–d.
[b] The total number of genes on the array was 149.

the formulations and the control vehicle (Fig. 1). The scatter of the data and the RMSE values from tissue comparisons showed that PEI had a greater effect on genes in the lung than chitosan, whereas the formulated polymers (PEI–pLuc, chitosan–pLuc) had lower RMSE values.

The data were also filtered to select genes with significant changes in expression compared to control. PEI–pLuc, chitosan–pLuc, and chitosan affected 17, 17, and 20% of the genes on the array, respectively, whereas PEI changed the expression of 29% of the genes (Table I). The stronger effect of PEI on stress and toxicity gene expression levels is in agreement with earlier studies on PEI toxicity both *in vitro* and *in vivo* (20,21).

Treatment-Specific Gene Expression Changes

Cluster analyses of the gene expression data were performed to assess which genes were affected by each treatment; the data were filtered for significant gene expression changes and normalized across experiments to enable comparison of gene expression profiles (18). Six clusters, comprising 70 genes, were obtained (Fig. 2). This covered 47% of all genes represented on the array. There were 7 to 19 genes in each cluster. The clusters containing genes mainly activated by PEI were c0, c2, and c4, with a total of 44 genes (30%). The clusters containing genes mainly activated by the chitosan group were c1 and c3, with a total of 18 genes (12%). The functions of these genes were assessed using the classification provided by the array manufacturer as well as the public database GeneCard. Four main functional categories were found: (1) inflammation, (2) cell cycle regulation, (3) DNA damage repair, and (4) protein folding. The genes of particular interest in interpreting the effects are shown in Fig. 3 and summarized in Fig. 4.

Inflammation and Tissue Injury

The cyclooxygenases 1 and 2 (COX-1, COX-2) are key enzymes in the biosynthesis of prostaglandins and are expressed in cells involved in inflammatory processes. The COX-2 gene, which is upregulated rapidly after stimulation, causes the release of prostaglandins, which not only mediate inflammation but can also contribute to tumor growth (22). Increases in the expression of COX-1 were greater with PEI and in particular with PEI–pLuc than with the chitosan group. COX-2 was also highly induced by PEI–pLuc, but was downregulated by chitosan (Fig. 3a).

Heme oxygenase-1 (HO-1) is induced by oxidative stress, heat shock, inflammatory cytokines, and prostaglandins (23). It is thought to be important for protection of the lungs during inflammatory reactions (24) such as hyperoxia-induced lung injury caused by altered expression of p53 or proteins induced by damaged DNA (25). Induction of HO-1 to moderate levels (2- to 5-fold) is beneficial for cell survival but, if levels are increased by more than 15-fold, accumulation of toxic metabolites can have the opposite effect (26). At the dose levels used in this study, the high levels of HO-1 induced by PEI–pLuc indicate a potential for subsequent adverse events. In comparison, the levels of HO-1 induced by the other formulations seemed to be more benign (Fig. 3a).

Three mitogen-activated protein (MAP) kinases (MAPK12, MAPKAPK2, AND MAP2K3, members of the p38 MAPK signaling pathway) were selectively activated by PEI–pLuc but not by the other formulations (Fig. 3a). MAPKs are activated by factors such as stress and the inflammatory response (27). The p38 MAPK pathway is also known to induce COX-2 (28). The induction of three different MAPKs by PEI–pLuc provides additional support for the hypothesis that an inflammatory response was evoked, despite earlier studies indicating that no inflammatory response was provoked in lung tissue after administration of PEI polyplexes at the investigated charge ratio (29).

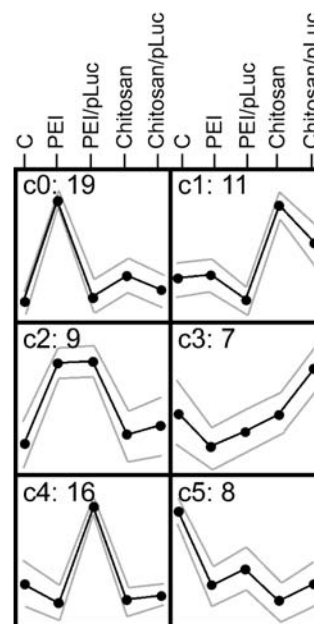

Fig. 2. Visualization of gene expression profiles. Genes with similar expression pattern 24 h after administration of polyethyleneimine (PEI), chitosan, their luciferase reporter plasmid complexes (PEI–pLuc, chitosan–pLuc) or vehicle (C) were sorted into the same cluster (clusters c0–c5). Each square (c0–c5) represents one cluster and the numbers indicate the number of genes in the clusters. Genes affected by PEI and PEI–pLuc were found in c0, c2 and c4, whereas chitosan- and chitosan–pLuc-dependent genes were found in c1 and c3. Note that the clusters with similar expression pattern do not necessarily contain genes with similar function. They are therefore named by numbers such as c1 for cluster 1, etc. The *y* axis represents a relative scale with normalized gene expression levels and the samples are indicated at the top of the clusters. Lines connecting the dots indicate mean values; the outer lines indicate the SD (n = 8–19).

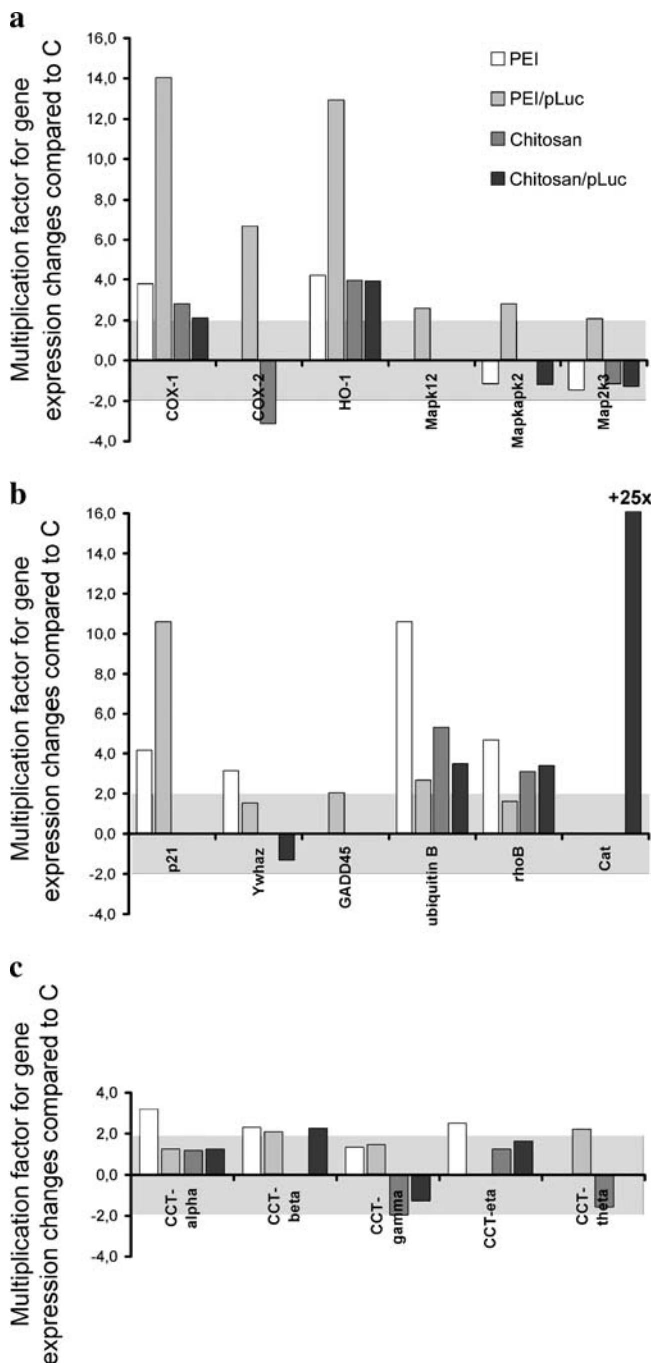

Fig. 3. Gene expression changes associated with toxic reactions after administration of polyethyleneimine (PEI), chitosan, their luciferase reporter plasmid complexes (PEI–pLuc, chitosan–pLuc) or vehicle (C) to mouse lung. (a) Inflammation, (b) cell cycle regulation and DNA damage, and (c) protein folding. Gene expression levels that changed more than 2-fold compared with C (indicated by columns outside the shaded area in the figure) were considered significant (see Materials and Methods). The shaded area represents a change in expression level of greater than 100 units but less than 2-fold compared with C.

Taken together, the results suggest that an inflammatory response is induced in the lung tissue 24 h after PEI–pLuc delivery. This response could have been mediated by a transient upregulation of the NFκB pathway at an earlier time point than 24 h and was therefore not detected in this study. However, the inflammatory response could be downstream events of an activation of the NFκB pathway (30). The inflammatory responses after delivery of PEI and the chitosan group were not as pronounced as that after PEI–pLuc, as shown by a moderate increase in COX-1 levels and the zero or low level of activation of HO-1 and the MAPKs with these formulations (Figs. 3 and 4). The more intense inflammatory response caused by PEI–pLuc could be used to stimulate a strong immune response in tissues other than the lung, provided that the toxic responses can be suppressed (10).

Cell Cycle Regulation

When cells are exposed to stress or toxic compounds, mechanisms are activated that arrest the cell cycle to limit the transfer of damaged DNA to new cells (31). Induction of DNA repair proteins is also initiated. In this study, PEI–pLuc activated MAPKs (Fig. 3a), indicating not only the presence of an inflammatory reaction but also the arrest of the cell cycle, which is mediated by phosphorylation of the tumor suppressor protein p53 (32). The lack of upregulation of p53 in our study may have been the result of predominantly posttranslational mechanisms of activation of the protein (33). The PEI group also activated the cyclin-dependent kinase inhibitor p21 (Fig. 3b). p21 acts as a downstream mediator of p53 and is important in the coordination of the cell cycle, DNA replication, and repair of damaged DNA (34). At 24 h after administration, PEI further upregulated Ywhaz (also called 14-3-3 delta or phospholipase A2) (Fig. 3b). Ywhaz, which is activated by p53, is also involved in the arrest of the cell cycle (35). The antitumor GADD45 gene was also moderately upregulated by PEI–pLuc (Fig. 3b). The antitumor properties of GADD45 are manifested by its maintenance of the p38 MAPK pathway and its c-*jun*-NH_2-kinase (JNK)-MAPK activity after genotoxic stress to arrest the cell cycle via p53 (36). Taken together, these results indicate that the PEI group had a significant effect on cell cycle arrest via p53. Furthermore, p53 is also involved in cell death (37) and, therefore, activation of this pathway could be a major mediator of the observed toxicity of PEI–pLuc (Fig. 4). In contrast, the chitosan group showed lower levels of activation or even no activation of these genes.

DNA Repair

One characteristic of the majority of the clusters in Fig. 2 was the presence of genes associated with the repair of DNA. The PEI group and chitosan each upregulated five genes (in clusters c0, c1, c2, and c4; Fig. 2a), whereas chitosan–pLuc activated only one gene (in cluster c3; Fig. 2). In addition to its involvement in cell cycle regulation and apoptosis, GADD45a activates DNA excision repair (38) (Fig. 3b). GADD45a, along with RhoB and ubiquitin, can be induced by various types of DNA damage (39,40). These three genes were moderately activated by the PEI group and chitosan (Fig. 3b). It is therefore plausible that the activation of these genes was part of a general reaction to stress in the lung tissue as a consequence of the treatment. It is interesting

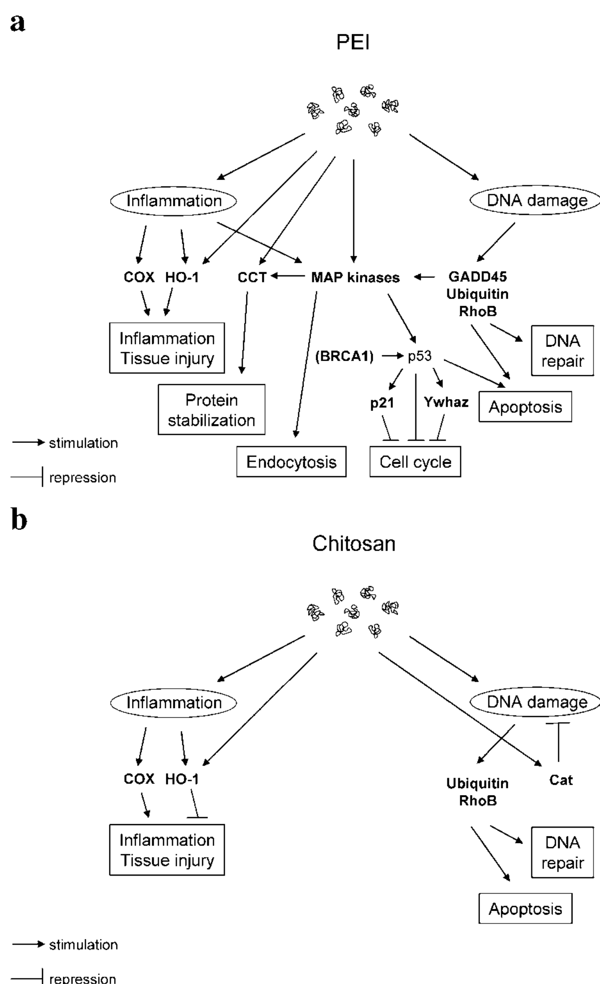

Fig. 4. Activation of stress and toxicity pathways in mouse lung tissue after administration of polyethyleneimine (PEI), chitosan, or their luciferase reporter plasmid complexes (PEI–pLuc, chitosan–pLuc). (a) PEI-induced pathways. The PEI group upregulated genes taking part in signal transduction pathways ending in inflammation, tissue injury, protein folding, endocytosis, cell cycle arrest, apoptosis, and DNA repair. (b) Chitosan-induced pathways. The chitosan group upregulated genes taking part in signal transduction pathways ending in inflammation, tissue injury, apoptosis, and DNA repair. Upregulated genes are shown in bold (compiled from references 22,23, 27,32,34–41,43,48,49).

that in contrast to PEI–pLuc, chitosan–pLuc upregulated catalase (Cat in Fig. 3b). This enzyme, which is induced by oxidative stress, removes hydrogen peroxide and prevents generation of hydroxyl radicals, thereby protecting the lung tissue from DNA damage (41). In summary, both the PEI and chitosan groups induced genes involved in DNA damage repair, indicating a general stress reaction in the lung tissue after administration of the polymers and the formulations (Fig. 4).

Protein Stabilization and Endocytosis

One significant difference between the gene expression profiles of the PEI and chitosan groups was the overrepresentation of genes coding for the chaperone CCT that assist in the folding of proteins. CCT is activated in mammalian cell cultures during recovery from chemical stress (42). The PEI group was associated with upregulation of four CCT subunit genes (Fig. 3c). Furthermore, members of the p38 MAPK pathway can activate heat shock protein 27 (43) and because CCT is a member of the heat shock protein family, it is possible that the MAPKs were also responsible for the activation of CCT (Fig. 4). The upregulation of subunits of CCT by the PEI group is interesting because it has been reported that CCT is involved in the folding of luciferase (44,45), the reporter gene product used in this and many other studies of PEI. This might partly explain earlier observations that PEI–pLuc is associated with higher and more prolonged luciferase expression than chitosan–pLuc, which affected fewer CCT subunits to a lower extent (7,8). Which CCT subunits are involved in the binding of luciferase is, however, unknown. Nonetheless, it has been speculated that different CCT subunits are responsible for different substrates (45,46). More importantly, although genes other than luciferase are more effective when delivered with PEI, their interactions with CCT have not been studied in any detail. However, in a recent publication, it was demonstrated that CCT can interact with another reporter gene product, green fluorescent protein, supporting the hypothesis that CCT is a factor in the increase in efficacy of PEI in gene delivery systems (47).

MAP-kinases (e.g., MAPK12) are also involved in the activation of endocytosis *in vivo* (48). It is therefore possible that the observed upregulation of three p38 MAPK pathway members by PEI–pLuc also resulted in activation of endocytosis of the formulation, although this needs to be confirmed. This activation may have contributed to the effective gene delivery properties of PEI–pLuc (Figs. 3a and 4).

Late Stress and Toxicity Responses

To further study the stress and toxicity profiles, lungs were analyzed 72 h after polyplex delivery. PEI–pLuc significantly changed the expression of 55 (37%) genes, whereas chitosan–pLuc affected only 20 (13%) genes. The gene expression data displayed a similar pattern received after 24 h, i.e., that PEI–pLuc induced genes connected to inflammation and tissue injury (COX-1, HO-1, MAPKs), cell cycle regulation (p21, Ywhaz), DNA repair (RhoB, ubiquitin), and protein folding (CCT-alpha, CCT-beta). Additionally, the p53 coactivator BRCA1, which is known to increase the transcription of p21 (49), was highly upregulated (34 times) at 72 h, which further strengthen the results received after 24 h indicating an arrest in the cell cycle (data not shown).

CONCLUSIONS

The results of this study show for the first time that gene expression profiling is a useful and sensitive tool for the initial characterization of local tissue responses to polymers and delivery systems. This methodology allowed us to distinguish between PEI and chitosan with respect to their acute tissue effects at dose levels evoking no apparent tissue

responses according to standard metabolic and histologic techniques. Clear differences in gene expression patterns related to stress and toxicological reactions were observed between the polymers and between their corresponding delivery systems. In general, upregulation of genes involved in adverse reactions occurred more often with PEI than with chitosan, which supports previous indications that PEI is less biocompatible. However, genes that stabilize the reporter protein luciferase and other proteins were upregulated in mice receiving PEI, suggesting that this molecule improves protein stability and may increase endocytosis, two factors that could contribute to the better performance of PEI in gene delivery systems. Thus, focused cDNA arrays have potential for use in screening early tissue reactions to pharmaceutical excipients and formulations.

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