linked cores and RGD ligands showed 10-fold higher efficiency than the ligand-less system without cross-linking (RGD (−) B-SH0% micelle). It is obvious that the effect of ligand installation was drastically enhanced by introducing disulfide cross-linking in the core. RGD (+) B-SH5% polyplex micelles achieved the highest transfection efficiency. Consequently, the combination of core cross-linking and ligand installation enhanced efficiency 20 times more than the polyplex micelles without ligands and cross-links.

Then, to further confirm that the increased transfection efficiency by RGD (+) micelles involves the receptor-mediated mechanism, a competitive assay using free cyclic RGD peptides was carried out for B-SH11% cross-linked micelles (Figure 3B). RGD (+) micelles showed a remarkably high transfection efficiency compared with RGD (−) micelles in the absence of free cyclic RGD peptides ($P < 0.01$). As the concentration of free cyclic RGD peptides increased, the transfection efficiency of RGD (+) micelles accordingly decreased, approaching the transfection level of RGD (−) micelles under the condition of 100 μM cyclic RGD peptides ($P = 0.104$). Thus, the results of the competitive assay indicate that $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$ integrin receptor-mediated endocytosis is involved in the transfection of the RGD (+) micelles against HeLa cells.

Analysis of Cellular Uptake of Polyplex Micelles. In general, the enhanced transfection by ligands has been attributed to an increased uptake of vectors.[14,20,21] Thus, the cellular uptake of the RGD (−) and RGD (+) micelles into the HeLa cells was evaluated using a system loaded with ^{32}P-labeled pDNA (Figure 3C). Regardless of ligand installation, cross-linked micelles tend to be taken up more efficiently than noncrosslinked micelles. The introduction of disulfide cross-links into the micelle core appreciably contributes to an increase in the stability of micelles under physiological conditions.[9] This implies that disulfide cross-links might prevent the micelles from dissociation in the extracellular medium, and consequently facilitate their internalization into the cellular compartment. Interestingly, there is no significant increase in micelle uptake even by installing RGD ligands, suggesting that other factors, including modulation of intracellular trafficking, may be involved in the enhancement of transfection efficiency by cyclic RGD peptide ligands.

Intracellular Distribution of Polyplex Micelles. Our previous report showed that RGD (+) polyplex micelles preferentially localize in the perinuclear region, unlike RGD (−) polyplex micelles,[11] suggesting that RGD ligands likely modulate intracellular trafficking of polyplex micelles. Therefore, detailed observation of the intracellular distribution was carried out using CLSM (Figure 4). The medium was replaced with fresh medium after 1 h incubation of polyplex micelles with cultured HeLa cells. Then CLSM observation was carried out after each reincubation without polyplex micelles in the medium. The CLSM images are shown in Figure 4A. The micelles localized in the inner-cytoplasm were quantitatively evaluated using the procedure described in the Experimental Section and shown in Figure 4B; the data are summarized in Figure 4C. After the 5 h reincubation (total 6 h incubation), the spots observed in the inner region of the cytoplasm were mainly the green spots of RGD (+) micelles (Figure 4A, left). On the other hand, the RGD (−) micelles, shown in red stayed mainly near the cell membrane (Figure 4A, left), but some fraction was observed in the inner-cytoplasm as red spots (RGD (−) micelle alone) or yellow spots (colocalizing with RGD (+) micelles). In the early stages, almost half of the cell population with fluorescence had only green spots, corresponding to the internalization of RGD (+) micelles (Figure 4C); this indicated that RGD (+) micelles were internalized into the inner-cytoplasm much faster than RGD (−) micelles. However, further reincubation resulted in the decrease in the cell fraction that showed only green spots and lead to an increase in the fraction that included yellow spots, corresponding to the colocalization of RGD (−) and RGD (+) micelles, as well as to an increase in red spots, corresponding to the presence of RGD (−) micelles alone. Note that there are two possibilities for the appearance of yellow spots. The first is that RGD (−) and RGD (+) micelles adsorbing to the cell membrane were simultaneously endocytosed by the cell. The second is that RGD (−) and RGD (+) micelles that were separately internalized into the cells subsequently colocalized through the possible fusion of the compartments in the inner-cytoplasm. On the other hand, the green spots still existed in a definite fraction of cells (G + GY + GRY) even after long-term reincubation, while the fraction of the cells including red spots (R + RY + GRY) continued to increase (Figure 4C). Thus, it is reasonable to assume that there may be distinct routes of internalization for RGD (−) and RGD (+) micelles, and eventually their final destinations may be different.

To examine whether or not RGD ligand installation in the micelles alters their intracellular trafficking, organelles were selectively stained using LysoTracker for the late endosomes and the lysosomes, and CT-B for the lipid rafts and the caveosomes (Figures 5 and 6).[22,23] In this experiment, the medium was replaced 1 h after the addition of polyplex micelles, followed by 11 h of reincubation. The rate of colocalization was quantified by the formula shown in the Experimental Section. The experiment with LysoTracker revealed that 39% of RGD (−) micelles and 28% of RGD (+) micelles in the inner-cytoplasm were localized in the late endosomes and lysosomes, indicating that the colocalization ratio of RGD (−) micelles with the late endosomes and lysosomes was significantly higher than that of RGD (+) micelles ($P = 0.0052$) (Figure 5B). On the other hand, the observation with CT-B revealed that 22% of RGD (−)

(20) de Bruin, K.; Ruthardt, N.; von Gersdorff, K.; Bausinger, R.; Wagner, E.; Ogris, M.; Bräuchle, C. Cellular Dynamics of EGF Receptor-Targeted Synthetic Viruses. *Mol. Ther.* **2007**, *15*, 1297–1305.

(21) Vinogradov, S.; Batrakova, E.; Li, S.; Kabanov, A. Polyion Complex Micelles with Protein-Modified Corona for Receptor-Mediated Delivery of Oligonucleotides into Cells. *Bioconjugate Chem.* **1999**, *10*, 851–860.

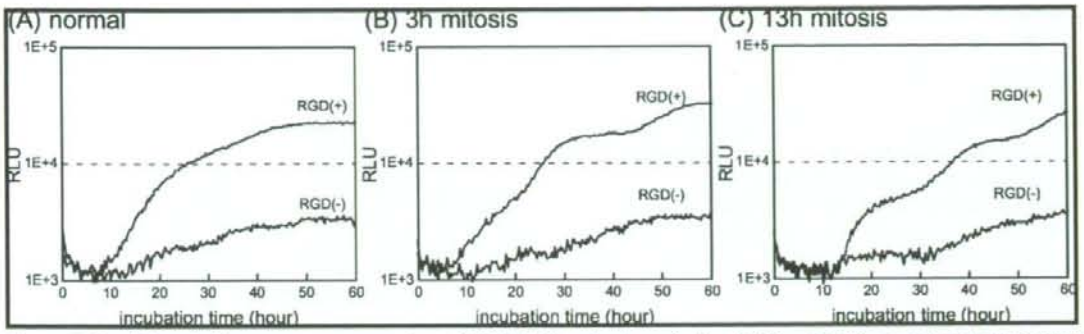

Figure 7. Real-time luciferase gene expression of B-SH11% polyplex micelles (N/P = 2) with or without cyclic RGD peptide ligands under the "normal" condition (A), the "3 h mitosis" condition (B), and the "13 h mitosis" condition (C).

micelles and 44% of RGD (+) micelles in the inner-cytoplasm were localized in the lipid rafts and caveosomes, respectively, indicating that the RGD (+) micelles had significantly higher localization ratios to the lipid rafts and caveosomes than the RGD (−) micelles ($P = 0.0004$) (Figure 6B). These results indicate that the polyplex micelles with cyclic RGD ligands were internalized preferentially through caveolae-mediated endocytosis by HeLa cells.

Real Time Luciferase Gene Expression. In the conventional luciferase assay, transfected cells need to be lysed before measurement, and this restricts the evaluation of luciferase expression in real time. Alternatively, as explained in the Experimental Section, Kronos Dio allows us to measure real-time luciferase expression while maintaining the cell culture for a prolonged period.[24] Figure 7 shows the results of time-dependent gene expression with RGD (−) and RGD (+) B-SH11% micelles. Under the "normal" condition without any control over the cell cycle (Figure 7A), the luciferase expression with RGD (−) and RGD (+) micelles started almost simultaneously around 8 h after the micelles were added. The expression of genes reached a plateau after around 50 h of incubation regardless of the presence of RGD ligands. Considering that the half-life of luciferase is about 2−3 h in living cells,[25] the rate of luciferase production is expected to be equal to that of luciferase degradation in regions over 50 h. RGD (+) micelles showed higher transfection rates than RGD (−) micelles at all time points. Real-time gene expression was assessed for cells after aphidicolin, a DNA synthesis inhibitor, was used to synchronize the cell cycle (Figures 7B and 7C). HeLa cells incubated with aphidicolin for more than 16 h are arrested between the G1 and S phases,[17] and a change to a medium that dose not contain aphidicolin allows HeLa cells to progress into the S phase to divide 13 h later. We confirmed that almost all HeLa cells were in the S phase immediately after the medium replacement and were in the G2 phase 10 h after the replacement (data not shown). Under the "3 h mitosis" condition, where cell division started 3 h after polyplex micelles were added (Figure 7B), gene expression by RGD (+) micelles was detected 5 h after incubation, while that of RGD (−) micelles was below the Kronos Dio detection limit even after about 12 h of incubation. This implies that RGD (+) micelles can migrate into the nucleus during the first mitosis due to their early accumulation in the perinuclear region, whereas this is not the case for RGD (−) micelles because of their slow accumulation in that region. Under the "13 h mitosis" condition, where cell division started 13 h after polyplex micelles were added (Figure 7C), luciferase expression with RGD (−) and RGD (+) micelles was detected simultaneously at 13 h after incubation, which corresponds to the initiation of cell division. This result suggests that RGD (+) micelles move early to the perinuclear region but may not be actively transported into the nucleus in the nonmitotic condition. As shown in Figure 4, most of the RGD (−) micelles were internalized into the cell to accumulate in the perinuclear region at a level comparable to that of RGD (+) micelles after 24 h of incubation. Nevertheless, RGD (−) micelles under the "13 h mitosis" condition exhibited remarkably lower increases in luciferase expression at more than 30 h of incubation compared to RGD (+) micelles. Apparently, this cannot be explained by the difference in the migration rate between RGD (−) and RGD (+) micelles because of their similar levels of accumulation in the perinuclear region in this time period. Presumably, this might be explained by the difference in the final destinations of the polyplex micelles with and without cyclic RGD ligands due to the modulation of intracellular trafficking as shown in Figures 5 and 6.

(22) Orlandi, P. A.; Fishman, P. H. Filipin-Dependent Inhibition of Cholera Toxin: Evidence for Toxin Internalization and Activation through Caveolae-Like Domains. *J. Cell Biol.* **1998**, *141*, 905–915.

(23) von Gersdorff, K.; Sanders, N. N.; Vandenbroucke, R.; de Smedt, S. C.; Wagner, E.; Ogris, M. The Internalization Route Resulting in Successful Gene Expression Depends on both Cell Line and Polyethylenimine Polyplex Type. *Mol. Ther.* **2006**, *14*, 745–753.

(24) Takae, S.; Miyata, K.; Oba, M.; Ishii, T.; Nishiyama, N.; Itaka, K.; Yamasaki, Y.; Koyama, H.; Kataoka, K. PEG-Detachable Polyplex Micelles Based on Disulfide-Linked Block Catiomers as Bioresponsive Nonviral Gene Vectors. *J. Am. Chem. Soc.* **2008**, *130*, 6001–6009.

(25) Ignowski, J. M.; Schaffer, D. V. Kinetic Analysis and Modeling of Firefly Luciferase as a Quantitative Reporter Gene in Live Mammalian Cells. *Biotechnol. Bioeng.* **2004**, *86*, 827–834.

Discussion

In the present study, two distinctive implementations, that of environment-sensitive cross-links in the core and that of cyclic RGD peptide ligands on the surface, were integrated into the polyplex micelles formed by PEG-PLys block copolymers and pDNA. The physicochemical characteristics of these micelles were quite similar regardless of the thiolation degree or the introduction of RGD ligands (Table 1). The PEG palisade surrounding the polyplex core shielded the charges of the micelles to maintain very small absolute values in the ζ-potentials. The cumulant diameter of polyplex micelles was around 100 nm. This indicates that all of the polyplex micelles possess favorable characteristics relevant to future *in vivo* application. In transfection experiments using cultured cells, polyplex micelles with cyclic RGD ligands achieved higher transfection efficiency than polyplex micelles without cyclic RGD ligands against HeLa cells appreciably expressing $\alpha_v\beta_3$ integrin receptors (Figure 3A). Interestingly, RGD ligands' effect on transfection was further enhanced by the introduction of disulfide cross-linking in the micelle core. Disulfide cross-links have been reported to stabilize polyplex micelles against the counter polyanion exchange reaction under nonreductive conditions.[9] Therefore, cross-linked micelles might acquire greater stability in the medium compared to noncrosslinked micelles, indicating the enhanced effect of cyclic RGD ligands. Note that RGD (+) B-SH5% micelles achieved the highest transfection efficiency among all micelles. Excessive cross-linking into the cores of polyplex micelles has been reported to overstabilize the micelles and impede the release of pDNA,[9] which is considered to be a cause of the lower transfection efficiency of B-SH11% micelles compared to that of B-SH5% micelles. The inhibitory experiment using free cyclic RGD peptides (Figure 3B) certainly confirmed that receptor-mediated uptake by RGD ligands contributed to the enhancement of gene expression. It is worth noting that the enhancement was not due to an increase in the cellular uptake of polyplex micelles (Figure 3C), suggesting that cyclic RGD peptide ligands may modulate the intracellular trafficking of the polyplex micelles, leading to increased transfection efficiency. In this regard it should be noted that, in our previous study, cyclic RGD ligands facilitated the transport of the polyplex micelles to the perinuclear region.[11] Other studies found that some ligands, such as b-FGF[26] and lactose,[27] contribute to the change in the intracellular trafficking of gene vectors. Time-dependent CLSM observation revealed that RGD (+) micelles were internalized into the cytoplasm and moved to the perinuclear region much earlier than RGD (−) micelles (Figure 4). This is consistent with the results of real-time luciferase expression under the "3 h mitosis" condition, where RGD (+) micelles exhibited earlier onset of gene expression with high efficiency (Figure 7B). The CLSM observation also clarified the variation in final localization in the cytoplasm between the two micelles (Figure 4). CLSM observation with the staining of acidic endosomes and lysosomes (Figure 5) or lipid rafts and caveosomes (Figure 6) revealed that RGD (+) micelles were distributed in the acidic organelles at lower levels than RGD (−) micelles, and were preferentially internalized via caveolae-mediated endocytosis. Considering that pDNA degradation occurs in late endosomes and lysosomes by enzymatic hydrolysis, RGD (+) micelles are more likely than RGD (−) micelles to protect entrapped pDNA from enzymatic degradation. It is known that cells uptake particles of different sizes through different routes: macropinocytosis (>200 nm), clathrin-mediated endocytosis (100−200 nm), and caveolae-mediated endocytosis (<100 nm).[28] The average particle size of RGD (−) and RGD (+) micelles was around 110 nm, with a moderate size distribution (polydispersity index = 0.14−0.18). Thus, these micelles are likely to be internalized by both clathrin- and caveolae-mediated endocytosis. Also, cyclic RGD peptides selectively recognize both $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors. $\alpha_v\beta_5$ integrins, which adenoviruses use for their internalization into target cells, are known to facilitate clathrin-mediated endocytosis,[29] while $\alpha_v\beta_3$ integrin-mediated internalization occurs via caveolae-mediated endosytosis.[30,31] Since $\alpha_v\beta_3$ integrins have 10-times higher binding affinity to cyclic RGD peptides than $\alpha_v\beta_5$ integrins,[32] it is reasonable to assume that RGD (+) micelles might preferably recognize $\alpha_v\beta_3$ integrins and thus to induce the caveolae-mediated endocytosis as the internalization route into HeLa cells. Alternatively, RGD (−) micelles, based on their size, might be primarily internalized by clathrin-mediated endocytosis and subsequently delivered to the acidic compartment of a lysosome. Caveolae-mediated endocytosis is not associated with a pH decrease, and is

(26) Fisher, K. D.; Ulbrich, K.; Subr, V.; Ward, C. M.; Mautner, V.; Blankey, D.; Seymour, L. W. A Versatile System for Receptor-Mediated Gene Delivery Permits Increased Entry of DNA into Target Cells, Enhanced Delivery to the Nucleus and Elevated Rates of Transgene Expression. *Gene Ther.* **2000**, *7*, 1337–1343.

(27) Hashimoto, M.; Morimoto, M.; Saimoto, H.; Shigemasa, Y.; Sato, T. Lactosylated Chitosan for DNA Delivery into Hepatocytes: The Effect of Lactosylation on the Physicochemical Properties and Intracellular Trafficking of pDNA/Chitosan Complexes. *Bioconjugate Chem.* **2006**, *17*, 309–316.

(28) Grosse, S.; Aron, Y.; Thevenot, G.; Francois, D.; Monsigny, M.; Fajac, I. Potocytosis and Cellular Exit of Complexes as Cellular Pathways for Gene Delivery by Polycations. *J. Gene Med.* **2005**, *7*, 1275–1286.

(29) Wickman, T. J.; Filardo, E. J.; Cheresh, D. A.; Nemerow, G. R. Integrin $\alpha_v\beta_5$ Selectively Promotes Adenovirus Mediated Cell Membrane Permeabilization. *J. Cell Biol.* **1994**, *127*, 257–264.

(30) Wary, K. K.; Mainiero, F.; Isakoff, S. J.; Marcantonio, E. E.; Giancotti, F. G. The Adaptor Protein Shc Couples a Class of Integrins to the Control of Cell Cycle Progression. *Cell* **1996**, *87*, 733–743.

(31) Wary, K. K.; Mariotti, A.; Zurzolo, C.; Giancotti, F. G. A Requirement for Caveolin-1 and Associated Kinase Fyn in Integrin Signaling and Anchorage-Dependent Cell Growth. *Cell* **1998**, *94*, 625–634.

(32) Marinelli, L.; Gottschalk, K.-E.; Meyer, A.; Novellino, E.; Kessler, H. Human Integrin $\alpha_v\beta_5$: Homology Modeling and Ligand Binding. *J. Med. Chem.* **2004**, *47*, 4166–4177.

known to be a nondigestive route of external substances into the cellular compartment.[33] Some nonenveloped viruses, such as simian virus 40, utilize this route for transfection to host cells and accumulate in a smooth endoplasmic reticulum compartment.[34,35] Thus, RGD (+) micelles internalized by caveolae-mediated endocytosis may be able to avoid pDNA degradation in acidic organelles, leading to high transfection efficiency. Moreover, as seen in the real-time luciferase assay under the "13 h mitosis" condition (Figure 7C), the luciferase expression of RGD (+) micelles detected at 30 h was remarkably higher than that of RGD (−) micelles, despite the comparable levels of cellular uptake between the two (Figures 3C and 4). Obviously, this result cannot be explained by the difference in the migration rate, because there was sufficient time for both RGD (+) and RGD (−) micelles to accumulate in the perinuclear region before mitosis. The higher gene expression of RGD (+) micelles is consistent with their preferential localization in caveosomes due to distinctive intracellular trafficking through the nonacidic and nondegradable route of caveolae-mediated endocytosis.

It should be noted that the results in Figures 5 and 6 indicate that not all of the RGD (+) micelles were internalized by caveolae-mediated endocytosis. Clathrin-mediated endocytosis, a relatively slow uptake pathway, could also contribute to the internalization of a portion of RGD (+) micelles, possibly due to the nonspecific interaction between the micelle and the cell membrane, even though polyplex micelles are covered with PEG to minimize nonspecific interaction. Nevertheless, the ζ-potentials of the polyplex micelles still take small positive values, suggesting that PEG charge shielding is incomplete. This slight positive charge might induce the nonspecific interaction of polyplex micelles with the negatively charged cell membrane. If this is the case, an increase in the PEG density of polyplex micelles may reduce the interaction, increasing the ligand's effects on the uptake and gene expression of polyplex micelles. An alternative explanation on the nonspecific uptake of polyplex micelles is available by considering the amphiphilic character of the PEG molecule. A PEG chain under concentrated conditions, as found in the shell layer in the micelle system, might have the ability to interact with the plasma membrane components through hydrophobic interaction or indirectly through a bridge of hydrated water molecules.[36] Research to clarify the underlying mechanism is now under way in our laboratory, and the results will be reported elsewhere in the near future.

Conclusions

In conclusion, polyplex micelles with integrated implementations of cyclic RGD peptide ligands on the micelle surface and disulfide cross-linking in the core achieved remarkably enhanced transfection efficiency against HeLa cells expressing $\alpha_v\beta_3$ integrins on the surface. The RGD ligands were effective not for increasing uptake but for modulating intracellular trafficking of polyplex micelles. RGD (+) micelles were distributed in the perinuclear region at an early period and were preferentially internalized by caveolae-mediated endocytosis via nonacidic and nondegradable intracellular compartments. These results indicate that polyplex micelles with cyclic RGD ligands and disulfide cross-links are promising approaches to facilitate cell-specific transfection by controlling intracellular trafficking as well as by the environment-sensitive release of encapsulated pDNA in the target cells.

Cyclic RGD peptide is well-known to selectively recognize $\alpha_v\beta_3$ integrin receptors identified as a marker of angiogenic vascular tissue,[37] and thus is a good candidate as a ligand for gene vectors used for diseases including tumor characterized by neovascularization. Indeed, nonviral gene vectors, in which cyclic RGD peptide ligands are installed have been applied to delivery pDNA and siRNA to tumor vasculature, effectively suppressing tumor growth.[13,14] Thus, polyplex micelle with cyclic RGD ligands and disulfide cross-links may be a useful system for cancer gene therapy through a systemic administration.

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MP800070S

(33) Pelkmans, L.; Kartenbeck, J.; Helenius, A. Caveolar Endocytosis of Simian Virus 40 Reveals a New Two-Step Vesicular-Transport Pathway to the ER. *Nat. Cell Biol.* **2001**, *3*, 473–483.

(34) Norkin, L. C.; erson, H. A.; Wolfrom, S. A.; Oppenheim, A. Caveolar Endocytosis of Simian Virus 40 Is Followed by Brefeldin A-Sensitive Transport to the Endoplasmic Reticulum, Where the Virus Disassembles. *J. Virol.* **2002**, *76*, 5156–5166.

(35) Conner, S. D.; Schmid, S. L. Regulated Portals of Entry into the Cell. *Nature* **2003**, *422*, 37–44.

(36) Kataoka, K.; Kwon, G. S.; Yokoyama, M.; Okano, T.; Sakurai, Y. Block Copolymer Micelles as Vehicles for Drug Delivery. *J. Controlled Release* **1993**, *24*, 119–132.

(37) Brooks, P. C.; Clark, R. A.; Cherish, D. A. Requirement of Vascular Integrin $\alpha_v\beta_3$ for Angiogenesis. *Science* **1994**, *264*, 569–571.