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Reactive Oxygen Species and Intracellular Ca^{2+} Contribution to Micro-Discharge Plasma Gene Transfection

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ABSTRACT: We investigated on the mechanism of plasma gene transfection. It is suggested that reactive oxygen species (ROS) directly or indirectly acts on endocytosis for gene transfection. The intracellular Ca^{2+} concentration was increased by plasma irradiation, and it was confirmed there is some kind of relationship between ROS and release of the intracellular Ca^{2+}. It is clear that ROS are the most important chemical factors in plasma gene transfection and that Ca^{2+} is not a dominant factor but a subsidiary or consequential factor. Our studies also confirmed that the transfer mechanism for low molecular weight substances, such as YOYO-1, is different from the mechanism for molecules of higher weight, such as plasmid DNA. In the case of larger molecules, ROS play an important role by inducing endocytosis in plasma gene transfection. But in the case of smaller molecules, neither ROS nor ROS-dependent endocytosis is dominant. Some kind of "endocytosis-independent and ROS-free" transfer processes may exist for smaller molecules to be transferred into cells by plasm irradiation. To discuss the mechanism of micro-plasma gene transfection, it is important to note that the transfer mechanism is different for substances of different molecular sizes.

KEY WORDS: endocytosis, oxidative stress, endoplasmic reticulum, *N*-acetyl-L-cysteine, ruthenium red, plasma medicine

I. INTRODUCTION

Gene transfection is a technique for introducing nucleic acids into cells for phenotypic expression. Expected applications to medical and biological fields include gene therapies, regenerative medicine, drug development, and plant breeding.[1–5] Gene transfection is achieved by three primary methods: physical, chemical, and biological. Electroporation, which is one of the physical methods, transfers genes into cells by applying a high electric field pulse.[6] This method is known as a general method because of the short treatment time and effectiveness in target cells. However, this technique unfortunately is very damaging to the target cells. Lipofection is one of the chemical methods, in which a cationic lipid is employed to transfer genes into the target cells.[7] This method requires no special equipment; however, there are the problems of toxicity to the target cells and the cationic lipid reagent is expensive. The biological method uses a virus as a vector for gene delivery.[8] The viral vector method has *in vivo* applications and shows a high

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transfection efficiency. However, there is a risk of pathogenic expression or neoplastic transformation.[9] Therefore, new methods for gene transfer that do not have these side effects on the target cells are currently being sought in medical and biological fields.

In the past decades, tremendous progress has been made in the generation and control of atmospheric non-equilibrium plasmas (ANEP), and in the measurement and simulation of plasma parameters, such as electron density and electron temperature.[10–20] The applications of plasmas continue to expand in medical and biological fields. A novel gene transfection method by plasma irradiation was invented by Miyoshi et al. in 2002[21] and was published by Ogawa et al.[22] and Sakai et al.[23] After these publications, several methods using various plasma sources to transfect genes and molecules have been reported.[24–32] Although several studies on plasma gene transfection have been reported, the transfection mechanism is not clear yet.

From our previous study, the important factors of the contribution to plasma gene transfection are thought to be the electric factor during plasma irradiation and reactive oxygen species (ROS) after plasma irradiation.[33,34] In fact, H_2O_2, which is the long–term ROS, is one of the important factors in plasma gene transfection.[34,35] On the other hand, Sasaki et al. reported that the intracellular Ca^{2+} concentration is increased by plasma irradiation, and fluorescent dye YOYO–1 (Beckman Coulter) is transferred into the cells with the extracellular Ca^{2+}.[36] However, in our experimental procedure, gene transfection occurred despite the absence of the extracellular Ca^{2+}. Therefore, it was hypothesized that the extracellular Ca^{2+} is not related to gene transfection, but intracellular Ca^{2+} may be increased by use of plasma.

Intracellular Ca^{2+} is one of the factors regulating the function of cells and is known as a signaling molecule. The intracellular Ca^{2+} is stored in the endoplasmic reticulum (ER), which is an intracellular organelle, and the molecules responsible for the release of the intracellular Ca^{2+} are ryanodine receptors and the IP_3 receptor in the ER membrane.

In our experimental procedure, a micro-discharge plasma (MDP) irradiation system is used to directly treat cells and plasmid DNA. It is obvious that ROS generated by plasma act directly on cell membranes and the cells. The MDP irradiation system has the feature of high transfection efficiency and cell viability simultaneously by plasma gene transfection.[37] From our previous studies, it is known that gene transfection efficiency is decreased by 60% with the inhibition of H_2O_2 and by 80% with the inhibition of endocytosis.[35,38] Here, not only H_2O_2 but also other ROS such as O_2^- and OH are generated by plasma. Since about 80% of the gene transfection mechanism involves endocytosis, it is clear that ROS generated by plasma are directly or indirectly involved in endocytosis. Generally, endocytosis occurs by binding of the ligand to receptors on cell membranes. And, it is also known that the intracellular Ca^{2+} promotes endocytosis through Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα).[39] Therefore, it seems that ROS promotes endocytosis through some as yet unknown action on the cell membrane, or it acts inside the cells indirectly rather than directly. That is, it is considered that ROS may act on receptors in the cell membrane or inside the cells, and may help release the intracellular Ca^{2+} from the ER. In this paper, to elucidate further the transfection mechanism,

we focus on ROS generated by plasma and the intracellular Ca^{2+}.

II. MATERIALS AND METHODS

A. Effect of Reactive Oxygen Species for Plasma Gene Transfection

Figure 1 shows the MDP irradiation system for plasma gene transfection. A thin copper capillary was employed as a high voltage (HV) electrode applied by a HV power supply. The diameter of copper electrode is 70 µm. A grounded copper plate was used as the counter electrode and was placed under a 96-well microtiter plate. The distance between the tip of the capillary electrode and the surface of the cell mixed was set at 1 mm. The applied voltage waveform was 20 kHz sinusoidal, and the amplitude was 15 kV (peak to peak). A micro-plasma is generated at the tip of the capillary electrode. The plasma irradiation time was set at 5 msec.

To clarify the ROS for MDP irradiation system, *N*-Acetyl-L-cysteine (NAC) (Wako: 015-05132) was used as ROS inhibitor. This reagent is added in DNA solution, and its concentration ranged from 0.1 to 100 mM.

Our recent research suggested that the mechanism of the plasma gene transfection method is affected by ROS, endocytosis, and cell membrane puncture due to current charge. It is also reported that endocytosis accounts for more than 80% of the transfection mechanism,[38] and the remaining 20% is presumed to be other pathways attributed to ROS, current, charge or other factors. To further clarify the transfection mechanism, we examine how effective ROS generated by plasma are for gene transfer. In this paper, we will confirm the effect of ROS on gene transfection by inhibiting ROS with NAC.

The target adherent cells (L-929, mouse fibroblast cell, RCB1422:RBRC) were

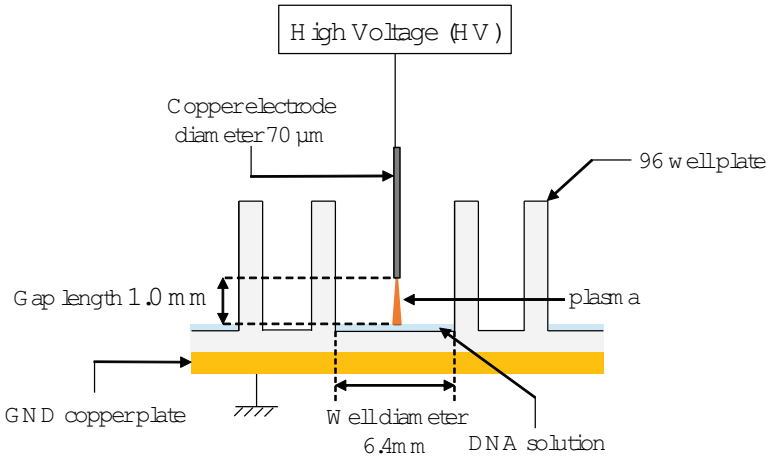

FIG. 1: Schematic of micro-discharge plasma (MDP) irradiation systems for plasma gene transfection

seeded on a 96-well micro-titer plate. The cells were incubated with 100 μL of culture medium for more than 24 hr until the number of cells reached the semi-confluent stage under the ambient temperature of 37°C and the CO_2 concentration of 5%. The culture medium was aspirated and 6 μg/6 μL of the GFP gene encoded plasmid pAcGFP1 (Contech)-N1 was added to each well, then all wells were treated with the MDP irradiation system. After plasma irradiation, 100 μl of culture medium was added. After a 48-hr incubation, fluorescence expression was quantitatively measured with a Cytell imaging cytometer (GE Healthcare Bioscience).

B. Evaluation of the Intracellular Ca^{2+} Increased by Plasma Irradiation

Section II.A revealed that ROS generated by plasma are involved in gene transfection. However, it is still uncertain which part of the cell is affected by ROS. Here, gene transfection efficiency is decreased by 80% with the inhibition of endocytosis.[38] Therefore, it is obvious that ROS generated by plasma are considerably related to endocytosis directly or indirectly. In general, various mineral ions are present in cells and used, for example, for regulating various biological functions. Especially, intracellular Ca^{2+} is a second messenger and contributes to the intracellular signal transduction system. Based on the previous discussion, it was hypothesized that the intracellular Ca^{2+} might be related in some way as a process or as a result of gene transfection via ROS generated by plasma.

To clarify the relationship between plasma gene transfection and intracellular Ca^{2+}, we measured the intracellular Ca^{2+} level before and after plasma irradiation. Fluo-4 AM (F311: DOJINDO) was used for measuring intracellular Ca^{2+}. Fluo-4 AM binds to intracellular Ca^{2+}, and its fluorescence properties change. In this experiment, the fluorescence intensity (luminance value) measurements F_0 and F_1 were obtained from the fluorescent images before and after plasma irradiation, respectively. Then, the intracellular Ca^{2+} concentration is calculated as F_1/F_0. In this experiment, ruthenium red (RR) (189-03181: Wako), a reagent is used that inhibits the release of the intracellular Ca^{2+} from the endoplasmic reticulum by blocking ryanodine receptors. In this section, ruthenium red, NAC, and the extracellular Ca^{2+} are added; then the intracellular Ca^{2+} is measured with each of reagent. In addition, gene transfection is performed with each reagent, and the relationship between transfection efficiency and the intracellular Ca^{2+} is considered. RR is dissolved in the culture medium at 20 μM, added to the sample before plasma irradiation and incubated for 1 hr at 37°C. After aspiration of the culture medium, DNA solution was added and then plasma irradiation is performed with a standard protocol. NAC is dissolved in DNA solution at 10 mM and added to the cells before plasma irradiation. Also, the extracellular Ca^{2+} is dissolved in DNA solution at 2 mM. And furthermore, to confirm the influence of each reagent on gene transfection, gene transfection with different doses of each reagent was performed. In addition, combination experiments with each of reagent such as 20 μM of RR and 2mM of the extracellular Ca^{2+}; 20 μM of RR and 10mM of NAC; and 2 mM of extracellular Ca^{2+} and 10 mM of NAC were also used to confirm their influence.

C. Influence on Plasma Gene Transfection by the Difference of Molecular Weight

Previous studies have shown that the transfection mechanism varies depending on the difference in molecular weight and structure.[35] Therefore, in this experiment, the influence of the intracellular Ca^{2+} in plasma gene transfection is confirmed using YOYO-1 (1.27 kDa) as a low-molecular-weight fluorescent dye and pAcGFP1-N1 (2,900 kDa) as a high-molecular-weight fluorescent plasmid DNA. These two substances were used for transfection. YOYO-1 is used at 20 μM, and pAcGFP1-N1 is used at a concentration of 1 μg/μL. In this experiment, Pitstop 2-100 (Abcam) is used as a clathrin-dependent endocytosis inhibitor; MßCD (Sigma-Aldrich) is used as an endocytosis inhibitor; ruthenium red is used as an inhibitor of intracellular Ca^{2+} release from the ER; and NAC was used as an ROS inhibitor. Pitstop 2-100, MßCD, RR are dissolved in cell culture medium at 25 μM, 10 mM, 20 μM, respectively, then combined with 100 μL culture medium to cells before plasma irradiation and incubated at 37°C for 10 min. NAC is dissolved in DNA solution at 10 mM and added to the cells before plasma irradiation.

The target cell (L-929) and the standard experimental protocol, which is MDP irradiation, cell incubation, and fluorescence observation, was identical to that described in Section II.A.

III. RESULTS

A. Effect of Reactive Oxygen Species for Plasma Gene Transfection

In order to clarify the relationship between plasma gene transfection and ROS, gene transfection was carried out with NAC, which is an ROS inhibitor. The results are shown in Figs. 2 and 3. In Fig. 2, normalized transfection efficiency of plasmid DNA by the MDP irradiation decreased by 80% in the presence of 10 mM NAC. Normalized cell mortality at this concentration was only about 5%. Therefore, it became clear that ROS are involved in gene transfer. We reported that gene transfection is decreased by 80% with the inhibition of endocytosis.[38] Figure 4 shows that relevant effects of endocytosis and ROS are less than 60%. However, whether ROS are directly or indirectly effective for gene transfection cannot be determined with only this result.

B. Evaluation of the Intracellular Ca^{2+} Increased by Plasma Irradiation

The ratio of intracellular Ca^{2+} concentration before and after plasma irradiation was measured. The results are shown in Table 1. Normalized transfection efficiencies are also shown in Table 1. Here, to evaluate the significance level of experimental results, the *t*-test (versus Standard) was used. The concentration of the intracellular Ca^{2+} is calculated as the ratio of the luminance values before and after plasma irradiation.

Table 1 shows that the ratio of the intracellular Ca^{2+} concentration was 4.2 in the

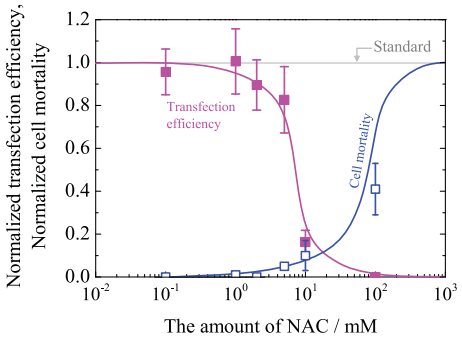

FIG. 2: The relationship between the amount of NAC and normalized gene transfection, the amount of NAC and normalized cell mortality. Solid squares represent normalized transfection efficiency, and open squares represent normalized cell mortality.

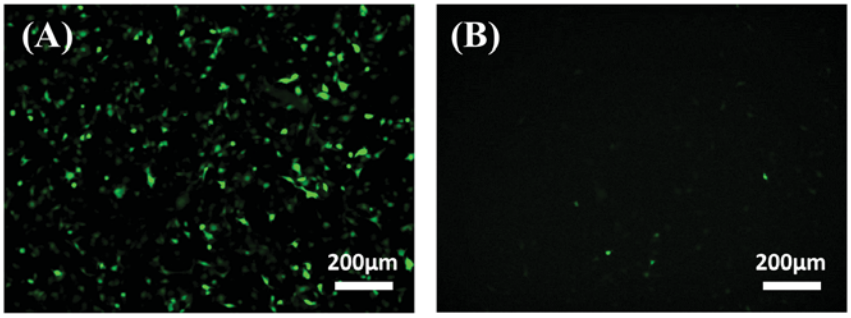

FIG. 3: Fluorescence microscope images by plasma irradiation without 10 mM of NAC (A), and with 10 mM of NAC (B)

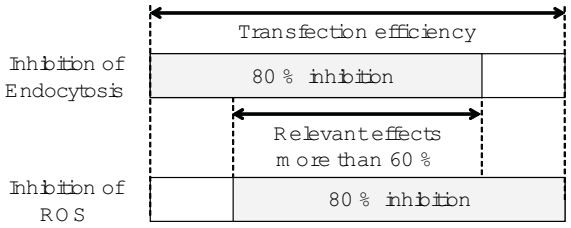

FIG. 4: Relevant effects of the action of endocytosis and ROS

Standard, 1.7 in 20 μM RR, 0.8 in 10 mM NAC, and 3.9 in 2 mM extracellular Ca^{2+}. On the other hand, normalized transfection efficiency was 0.73 in 20 μM of RR, 0.16 in 10 mM of NAC, and 0.98 in 2 mM extracellular Ca^{2+}. Here, looking at the results of the combined experiment, such as 20 μM RR and 2 mM extracellular Ca^{2+}; 20 μM RR and 10 mM NAC; 2 mM extracellular Ca^{2+} and 10 mM NAC, it is clear that extracellular

Ca^{2+} increased the intracellular Ca^{2+} concentration.

C. Influence on Plasma Gene Transfection by the Difference of Molecular Weight

To elucidate the transfection mechanism to the difference in molecular weight, several reagents were used. The results are shown in Fig. 5. In this figure, normalized transfec-

TABLE 1: Ratio of the intracellular Ca^{2+} concentration before and after plasma irradiation. The significance levels of experimental results by *t*-test are also shown. Also, normalized transfection efficiency with each reagent is shown. The concentration of the intracellular Ca^{2+} is calculated as F_1/F_0 (F_0 obtained by fluorescence image before plasma irradiation; F_1 obtained by fluorescence image before plasma irradiation).

	Ratio of the intracellular Ca^{2+} concentration		Normalized transfection efficiency
n = 4	**F_1/F_0**	***t*-Test (vs Standard)**	
Standard	4.2 ± 0.18	–	1 ± 0.05
RR 20 μM	1.7 ± 0.09	$p < 0.05$	0.73 ± 0.09
Ca^{2+} 2 mM	3.9 ± 0.38	–	0.98 ± 0.05
NAC 10 mM	0.8 ± 0.14	$p < 0.05$	0.16 ± 0.06
RR 20 μM & Ca^{2+} 2 mM	2.7 ± 0.15	$p < 0.05$	0.82 ± 0.08
RR 20 μM & NAC 10 mM	1.3 ± 0.11	$p < 0.05$	0.15 ± 0.02
Ca^{2+} 2 mM & NAC 10 mM	2.2 ± 0.17	$p < 0.05$	0.20 ± 0.02

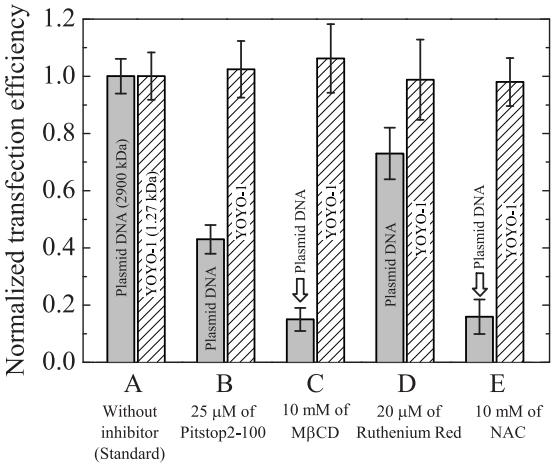

FIG. 5: Normalized transfection efficiency of plasmid DNA 2,900 kDa (gray bar) and YOYO-1 1.27 kDa (hatched bar) by plasma irradiation without inhibitor (A), with 25 μM of Pitstop 2-100 (B), with 10 mM of MßCD (C), with 20 μM of ruthenium red (D), and with 10 mM of NAC (E)

tion efficiency of plasmid DNA by plasma irradiation decreased by 60% with Pitstop 2-100, by 80% with MßCD, by 30% with RR, and by 80% with NAC. In contrast, normalized transfection efficiency of YOYO-1 isn't changed by the presence of any reagent used in these experiments. From this result, it can be said that the transfection mechanism is different depending on molecular weight.

IV. DISCUSSION

In this study, in order to clarify the contribution of ROS to the micro-discharge plasma gene transfection, ROS inhibitor NAC was used. As a result, gene transfection efficiency of plasmid DNA by plasma irradiation decreases by 80% when ROS were inhibited by 10 mM of NAC. In Fig. 2, it is suggested that ROS are the most important chemical factors in plasma gene transfection. However, it is unclear how ROS contribute to the transfection.

The ratios of the intracellular Ca^{2+} concentration before and after plasma irradiation with inhibitors are shown in the Table 1. Comparing the cases of Standard and the case with 10mM of NAC, it was confirmed that the intracellular Ca^{2+} concentration doesn't increase and that the normalized transfection efficiency has decreased by 80% with ROS inhibition. In the case when the intracellular Ca^{2+} inhibited by 20 μM of RR, the reduction of gene transfection efficiency to 73% of the Standard was also confirmed. Therefore, it is suggested that ROS generated by plasma contribute to the increase of the intracellular Ca^{2+}concentration. From these results, the relation between the normalized transfection efficiency and the ratio of the concentration of the intracellular Ca^{2+} before and after plasma irradiation is shown in Fig. 6. It becomes clear that the transfection efficiency strongly depends on whether ROS are present. When ROS exist, the gene transfection efficiency also depends on the intracellular Ca^{2+} concentration. However, when ROS are inhibited, the gene transfection efficiency doesn't depend on the intra-

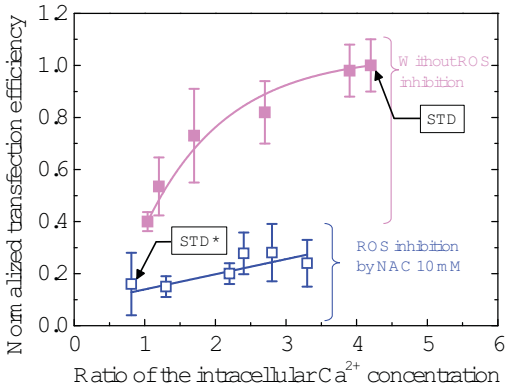

FIG. 6: Normalized transfection efficiency to the concentration of the intracellular Ca^{2+} before and after plasma irradiation. Solid squares show the effect of ROS, open squares show ROS inhibition with 10 mM of NAC. STD, Standard; STD*, 10 mM of NAC.

cellular Ca^{2+} concentration so much as the case when ROS are not inhibited. Since the intracellular Ca^{2+} concentration is adjusted by the extracellular Ca^{2+} in this experiment, it is assumed that the intracellular Ca^{2+} has some relationship with transfection, and that the extracellular Ca^{2+} does not. Therefore, ROS play an important role for gene transfection rather than intracellular Ca^{2+}. In other words, Ca^{2+} is not a dominant factor, but it is a subsidiary or consequential factor; although ROS are the one of the dominant causes of the transfection.

Though it is reported that the intracellular Ca^{2+} promotes endocytosis,[39] in this study gene transfection does not occur with increasing extracellular Ca^{2+} without plasma irradiation. This means that sufficient gene transfection cannot occur by merely increasing the intracellular Ca^{2+} concentration without ROS generated by plasma irradiation. The essential factors for plasma gene transfection are ROS and electrical components, and they have synergistic effects in plasma gene transfection.[38] It is suggested that ROS promote the release of intracellular Ca^{2+} in the processes of plasma gene transfection. However, the cause of ROS and intracellular Ca^{2+} increase and the process initiated by an increase in intracellular Ca^{2+} are not clear. They must be investigated in more detail to elucidate the whole process of plasma gene transfection.

In Fig. 7, the schematic of the transfection processes by plasma irradiation is shown. Since the electrical charges, one of the electrical factors, attaches to the cell membrane and changes its polarity from negative to positive,[33] due to the Coulomb force the colli-

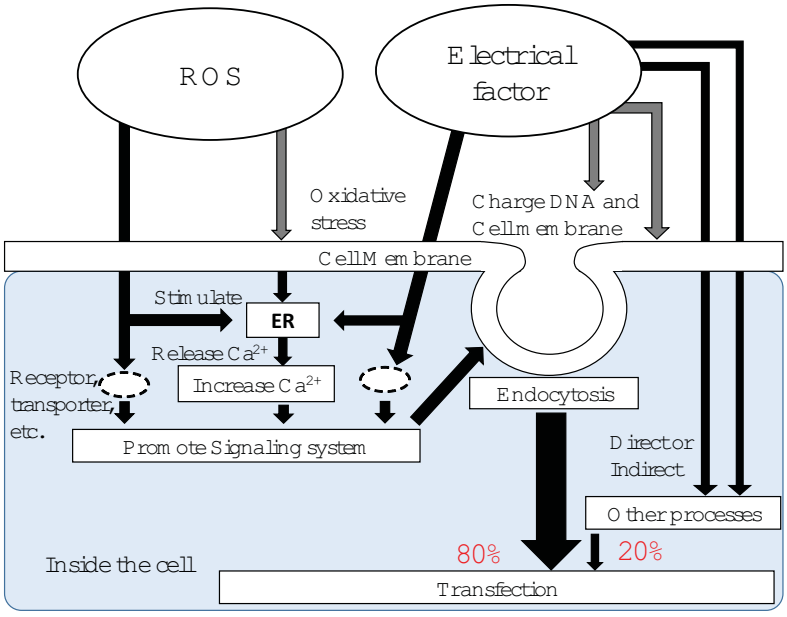

FIG. 7: Schematic of the contribution of ROS and electrical factor, the intracellular Ca^{2+} for gene transfection. ER, endoplasmic reticulum.

sion frequency between cell and plasmid DNA, whose polarity is negative, is increased by plasma irradiation. This leads to plasmid DNA attachment on the cell membrane, followed by endocytosis. The oxidative stress is brought by ROS to the cell membrane, which stimulates or promotes the release of the intracellular Ca^{2+} from ER. Furthermore, it is speculated that both ROS and Electrical factors promote the intracellular signaling system and act as a switch for inducing endocytosis.

Figure 5 shows that the low-molecular-weight materials such as YOYO-1 are transferred into the cells, even if both endocytosis and ROS are inhibited. On the other hand, the high-molecular-weight materials such as plasmid DNA are not transferred into the cells, even if either or both endocytosis and ROS are inhibited. These results show that the transfection mechanism is different between the introduced materials with low molecular weight and those with high molecular weight. In the case of large molecules, endocytosis and ROS play important roles in plasma gene transfection. On the other hand, both endocytosis and ROS are not dominant, and some kind of "endocytosis-independent and ROS-free" transfer processes exists for smaller molecules to be transferred into cell by plasma irradiation. These results also show that we must be careful of the molecular weight. We should not discuss the mechanism in case of the high-molecular-weight compounds by the results obtained with small molecules, and vice versa.

V. CONCLUSION

We evaluated the contribution of ROS and intracellular Ca^{2+} level to plasma gene transfection. It becomes clear that ROS are the most important chemical factors in plasma gene transfection and that Ca^{2+} is not a dominant factor but a subsidiary or consequential factor.

We also discovered a difference of the transfer mechanisms by size of molecules. In the case of large molecules such as plasmid DNA, ROS play an important role in facilitating endocytosis in plasma gene transfection. But in the case of small molecules such as YOYO-1, neither ROS nor ROS-dependent endocytosis is dominant. Some kind of endocytosis-independent and ROS-free transfer processes exist for low-molecular-weight substances to be transferred into the cell by plasm irradiation. To discuss the mechanism of micro-plasma gene transfection exactly, it is important to note that the transfer mechanism varies for molecules of different sizes. We should discuss the mechanisms for large and small molecules separately.

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