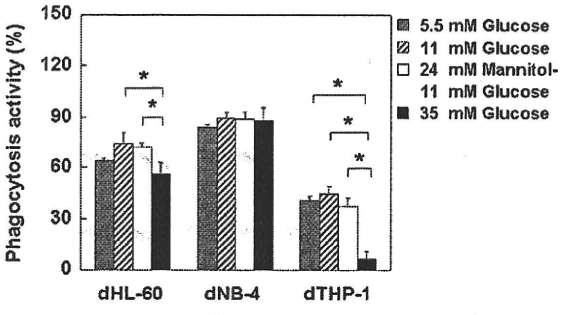

Figure 8. Effect of glucose on the phagocytosis activities of dHL-60, dNB-4, and dTHP-1. After differentiation, the cells were exposed to different concentrations of glucose (5.5 mM, 11 mM or 35 mM) for 24 h. Phagocytosis activity is expressed as the percentage of the cells ingesting. Data are mean ± SD from four independent experiments. *, $p < 0.001$.

The effect of glucose on the cellular phagocytosis was investigated. By culture with 35 mM glucose for 24 h, the phagocytosis of yeast particles by dHL-60 cells significantly decreased compared to the cultures with 11 mM glucose as well as 24 mM mannitol-11 mM glucose (Fig. 8, $p < 0.001$). In dTHP-1 cells incubated with 35 mM glucose for 24 h, phagocytosis activity extremely decreased compared to the cultures with the lower concentration of glucose and 24 mM mannitol-11 mM glucose ($p < 0.001$).

4 Discussion

To our knowledge, this study for the first time demonstrated that exposure of PMN-like differentiated cell lines to higher concentration of glucose (up to 35 mM) caused (i) the enhancement of cellular aggregation in dHL-60, (ii) the reduction of cellular fragility in dHL-60 and dTHP-1, (iii) the enlargements of intracellular vacuoles by PMA stimulation in dNB-4, (iv) the impairments of phagocytosis activities in dHL-60 and dTHP-1, and (v) the induction of superoxide generation by PMA stimulation. Because the culture of differentiated cell lines with 24 mM mannitol-11 mM glucose did not influence the aggregation, fragility or vacuolization, the osmotic effect can be excluded. The other studies showed that GM-CSF, which has a priming effect, stimulates neutrophil aggregation [28], but does not stimulate adherence to endothelial monolayers [29]. In contrast, tumor necrosis factor-α (TNF-α) and lipopolysaccharide, which are triggers, enhance neutrophil adherence to endothelium [30], but do not promote neutrophil aggregation [31]. Our study suggests that the expression of CD11b was not enhanced by high glucose treatment (data not shown). Vedder *et al.* [32] suggest that, as with adherence to endothelium, increased surface expression of CD11b is not the mechanism responsible for aggregation. Taken together, hyperglycemic conditions may play a role similar to GM-CSF in triggering immune responses of ATRA-induced differentiation.

The cytoplasmic vacuolization and/or toxic granulation of neutrophils have been demonstrated more often in bacteremic patients with high NBT reduction capacity [33]. Malcolm *et al.* [34] have reported that no significant differences in the extent of vacuolization were found among healthy donors, toxic patients without bacterial infections, and patients with bacterial infections in the absence of documented bacteremia. The extent of vacuolization was significantly greater in bacteremic patients when compared with the other patients, including patients with bacterial infection without bacteremia [34]. In our study, the effect of high glucose on the vacuolation of neutrophil-like cells by PMA stimulation to mimic bacterial infection was investigated. The dNB-4 cells under high glucose condition with 35 mM were extensively vacuolated by PMA stimulation, suggesting that the high glucose may enhance the ability of vacuolization due to some stimulation in neutrophil-like cells. The patients with type I diabetes mellitus were characterized by a significant impairment of the PMN-mediated phagocytosis [5, 35]. In our study, the phagocytosis activities of dHL-60 and dTHP-1 were significantly impaired in the cultures with high glucose for 24 h, suggesting the association with the pathogenicities in diabetes.

Oliveira *et al.* [36] have indicated that NBT reduction in incubation of rat pancreatic islets as well as neutrophils with 5.6, 8.3, and 16.7 mM of glucose for 1 h increased when compared with the absence of glucose. They further confirmed the involvement of NAD(P)H oxidase activation through protein kinase C (PKC) in the stimulatory effect of glucose by incubation with PMA (a PKC activator), bysindoylmaleimide (GF109203X) (a PKC-specific inhibitor), and diphenylene iodonium (an NAD(P)H oxidase inhibitor) to abolish the increase of NBT reduction induced by glucose. Although we do not know whether the NBT reduction in incubation of neutrophil-like cells with 5.5 mM glucose increases when compared with the absence of glucose, without PMA stimulation no superoxide production of differentiated cells was observed in incubation with any concentration of glucose in this study. Furthermore, the superoxide production by dHL-60, dNB-4, and dTHP-1 cells in incubation with higher glucose (11 or 35 mM) for long-term treatment (24 h) significantly increased when compared with 5.5 mM glucose (physiological concentration), in particular, the NBT reduction of dHL-60 in incubation with 35 mM glucose was extremely induced. However, no significant superoxide production in response to PMA was observed in the short-term treatment (5 h) of dHL-60 treated with 35 mM glucose, suggesting that longer-term treatment with high glucose seems to more enhance the induction of superoxide production. Since without PMA stimulation the NBT reduction of dHL-60 cells cultured with high glucose was not observed, the high glucose condi-

tion probably promotes the priming of neutrophil-like cells rather than the direct triggering.

Osmotic pressures of glucose-free RPMI, 5.5 mM glucose-containing RPMI, 11 mM glucose-containing RPMI, and 35 mM glucose-containing RPMI, which were estimated are approximately 250–290, 255–295, 265–300, and 300–350 mOsm/kg, respectively. In most of tissue culture media that are supplied by manufactures, the osmotic pressure is usually adjusted to the range of 260–320 mOsm/kg to keep isotonic condition. Osmotic pressures of 5.5 mM glucose- and 11 mM glucose-containing media are almost in the range. Only 35 mM glucose-containing medium may give the cell lines a little hypertonic shock. Therefore, we used 24 mM mannitol-11 mM glucose as such hypertonic control. In dHL-60 as shown in Fig. 7B, the ROS induction of 35 mM glucose (a little hypertonic)-treated cells was significantly increased by PMA stimulation when compared with those of the cells treated with 5.5 mM glucose (isotonic) and 11 mM glucose (isotonic), and 24 mM mannitol-11 mM glucose (the same osmotic condition as 35 mM glucose). This suggests that the increase of ROS production is dependent on very high glucose condition. In the cases of dNB-4 and dTHP-1, the ROS productions of 11 mM glucose (isotonic), 24 mM mannitol-11 mM glucose (a little hypertonic), and 35 mM glucose (a little hypertonic) were significantly increased by PMA stimulation when compared with 5.5 mM glucose (isotonic), suggesting that the increase of glucose concentration from 5.5 to 11 mM, but not hypertonic shock, seems to be related to the ROS induction. Thus, dHL-60 appears to become more sensitive by treatment with very high concentration of glucose such as 35 mM on ROS production by PMA stimulation, but dNB-4 and dTHP-1 are likely more sensitive by moderately high concentration (more than 5.5 mM and less than 35 mM) of glucose. The degree of effect may be dependent upon the sensitivity differences of those three cell lines against glucose concentration or treatment time, etc.

The reduction of NBT dye and the generation of ROS are thought to measure indirectly the bactericidal function of neutrophils. There is evidence demonstrating that bactericidal ROS production by activated PMN is reduced in diabetic patients with or without any infections [9] and in diabetic rats [37]. However, there is also evidence that it is normal [38] or increased [39] in patients with type 1 diabetes mellitus. Additionally, Nabi *et al.* and Bellinati-Peies *et al.* have suggested that reduction of NBT by neutrophils is not correlated with their bactericidal activity [35, 40]. They go on to suggest that only the complete absence of NBT reduction reflects low bactericidal activity in neutrophils. Based on those reports, a question is how we can explain exactly what the present findings of glucose-induced ROS production means in relation to bactericidal function of neutrophils in patients with type 1 diabetes. One of explanations is that the type 1 diabetes is much more complicated, because the patient blood with the diabetes included increased glycated proteins due to Maillard reaction, *e.g.* AGEs, as well as high glucose. Wong *et al.* [41] showed that AGEs stimulate an enhanced neutrophil respiratory burst mediated through the activation of cytosolic phospholipase A2 and generation of arachidonic acid. Moreover, De Toni *et al.* [42] have represented that in patients with diabetes the impact on PMN function is of multifactorial origin, and is probably correlated to the glucose level and to glycation of PMN protein, such as NADPH oxidase or myeloperoxidase. Alternatively, glucose in PMN may be reduced by aldose reductase to polyols, and this pathway requires NADPH, the coenzyme for the respiratory burst. They further found that superoxide production in response to opsonized zymosan was reduced in diabetic patients. The activation of protein tyrosine kinase is an important mechanism underlying transmembrane signaling and, the protein tyrosine phosphorylations, stimulated by zymosan receptor-mediated activation, might be caused by the activation of specific protein tyrosine kinase, whereas activation by PMA is probably mediated through another PKC type. In our study, therefore, we focused on the effect of high glucose alone (not including AGEs etc.) in ROS production at the mimicked initial step of maturated neutrophils released from bone marrow to blood using three differentiated cell lines.

Taken together, in response to glucose, HL-60 cells out of three cell lines seem to be better as a neutrophil model, but NB-4 and THP-1 (as well as HL-60) are still expected to have the potential responses similar to *in vivo* neutrophils under the consideration of sensitivity difference against glucose among those cell lines. By this possibility, we think that the three cell lines (HL-60, NB-4, and THP-1) are probably useful for better understanding of neutrophil function as a model *in vitro* experiment for long-term treatments (24 h or longer). Thus, this study provides significant and basic information regarding the effect of high glucose on the functional abnormalities of neutrophil-like differentiated cell lines.

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Genotoxicity and Estrogenic Activity of 3,3′-Dinitrobisphenol A in Goldfish

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3,3′-Dinitrobisphenol A (dinitro-BPA) is formed in a mixture of bisphenol A (BPA) and nitrite under acidic conditions. It shows genotoxicity in male ICR mice on a micronucleus test, but its estrogenic activity has not been examined *in vivo*. We examined its estrogenic activity using goldfish (*Carassius auratus*) by measuring plasma levels of vitellogenin (VTG) by the ELISA method. Expression of VTG didn't increase in the plasma of goldfish intraperitoneal injected with dinitro-BPA at a dose of 10 mg/kg of body weight.

We also examined the genotoxicity of dinitro-BPA by single-cell gel electrophoresis (comet assay) and a micronucleus test using goldfish. The DNA tail moment of blood cells increased after intraperitoneal injection of dinitro-BPA. Dinitro-BPA at the same dose significantly increased micronucleus frequency in gills of goldfish. On the other hand, BPA did not significantly increase the frequency of micronucleated cells.

In conclusion, we found that dinitro-BPA did not show estrogenic activity, but had genotoxic potency stronger than that of BPA.

Key words: goldfish; 3,3′-dinitrobisphenol A; vitellogenin; comet assay; micronucleus test

It is known that various chemicals in our environment show substantial influences on aquatic animals and mammals, including humans. Among these compounds, bisphenol A (2,2-bis(4-hydroxyphenyl)propane, BPA), which is used as an ingredient in the manufacture of epoxy carbonate, polycarbonate and polyester styrene, exhibits estrogenic activity. The estrogenic activity of BPA has been demonstrated using various assays. Krishnan *et al.* found that BPA showed estrogenic activity in a culture assay using MCF-7 human breast cancer cells.[1)] Hashimoto *et al.* revealed estrogenic activity of BPA using the yeast two-hybrid system.[2)] It has been reported that BPA bound with human estrogen receptor alpha, beta, and gamma.[3–5)] Hence it is said to be an endocrine-disrupting chemical (EDC). It is also used in food packaging and can-coating agents and in dental sealants, and is readily orally-ingested by humans. Consequently, it is important to examine the toxicity of BPA in human body after oral intake.

BPA has not been recognized as a mutagen by several *in vitro* and *in vivo* mutagenicity assays. Haworth *et al.* reported that it showed negative results in bacterial reverse mutation tests using *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537.[6)] Ivett *et al.* reported that it did not exhibit mutagenic activity in an *in vitro* genotoxicity test with Chinese hamster ovary cells.[7)] Gudi *et al.* also reported that it did not increase the frequency of micronucleated reticulocytes (MNRETs) in bone marrow of mice.[8)] Recently, however, there have been positive results to the effects that it shows mutagenic activity. Hilliard *et al.* reported that BPA exhibited positive effects in an *in vitro* chromosome aberration test using CHO cells without S9 mix.[9)] Tayama *et al.* demonstrated that it induced genotoxicity in CHO-K1 cells using sister-chromatid exchange and comet assay.[10)] Tsutsui *et al.* found that quinone compounds formed from BPA formed DNA adducts in an *in vitro* 32P-postlabeling assay.[11)] Thus BPA shows opposite effects on mutagenic activity in different test systems.

Humans regularly consume nitrite and nitrate through vegetables and tap water, and in their daily diet as food additives.[12)] Nitrate is readily reduced to nitrite by oral bacteria. Some scientists have reported that mutagenic/carcinogenic nitrosamines are formed by the reaction of nitrite and secondary amines in foodstuffs under acidic conditions.[13)] Several phenolic compounds also show mutagenic activity after nitrite treatment. Wakabayashi *et al.* found that some phenol and indole derivatives present in the environment are changed to mutagenic compounds by nitrosation.[14)] Kikugawa and Kato found

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that diazoquinone compounds, which showed strong mutagenicity, were formed by interaction between phenol and nitrite.[15,16] We have found that BPA showed mutagenic activity on treatment with nitrite under acidic conditions, and dinitro-BPA, which induced micronuclei in peripheral erythrocytes of ICR male mice, was formed in a reaction mixture of BPA and nitrite.[17] Additionally, we found that dinitro-BPA did not show estrogenic activity on *in vitro* assay. However, we have not yet determined whether dinitro-BPA shows estrogenic activity on an *in vivo* assay.

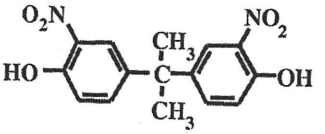
Some scientists have examined the estrogenic activity of endocrine disruptors by *in vivo* tests using aquatic organisms. Warner *et al.* demonstrated the estrogenic activity of BPA using fathead minnow.[18] Tabata *et al.* used Japanese medaka (*Oryzias latipes*) to confirm the endocrine disrupting action of BPA.[19] Ishibashi *et al.* also confirmed the estrogenic activity of nonylphenol by detecting the vitellogenin concentration in plasma of goldfish.[20] Goldfish can be bred easily and cheaply and are available all over the world. We have also examined the genotoxicity of various chemicals in river water using goldfish. Judging by these reports, goldfish is a useful organism in investigating the mutagenic and estrogenic activities of various chemicals.

In the present study, we examined the mutagenic and estrogenic activities of BPA and dinitro-BPA with goldfish.

Materials and Methods

Chemicals. BPA, 17β-estradiol (E2), acridine orange, methyl metahnesulfonate (MMS), and dimethylsulfoxide (DMSO) were purchased from Wako Pure Chemicals (Osaka, Japan). 3,3′-Dinitro-bisphenol A (dinitro-BPA) was synthesized as described by Masuda *et al.*[17] Figure 1 shows the chemical structure of dinitro-BPA. Low melting point (LMP) agarose and normal melting point (NMP) agarose were from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was from Gibco BRL (Grand Island, NY). Mitomycin C (MMC) and ethidium bromide were from Kyowa Hakko Kogyo (Tokyo) and Merck (Darmstadt, Germany) respectively.

Fish. We obtained goldfish weighing 11 ± 2 g from a local dealer in Hamamatsu, Japan. Before the experiment, goldfish were acclimatized for 2 weeks in a well-aerated aquarium at 18 ± 2 °C.



3,3'-dinitrobisphenol A (dinitro-BPA)

Fig. 1. Chemical Structure of Dinitro-BPA.

Treatment of fish. In a previous study, we injected chemicals from a river intraperitoneally to examine their mutagenic activities.[21] In a similar way, BPA and dinitro-BPA were dissolved in DMSO and injected intraperitoneally once at 1 and 10 mg/kg of body weight. Three to 5 fish were used in each group. In the negative control group, DMSO was injected intraperitoneally in place of the test chemical. In the positive control group, we injected intraperitoneally E2 (1.0 mg/kg of body weight), MMS (50 mg/kg of body weight) and MMC (4.0 mg/kg of body weight) for VTG determination and in comet assay and micronucleus test. For determination of VTG in plasma, at 96 h after injection blood was collected and centrifuged at 3,000 rpm for 20 min, and the plasma was divided into aliquots and stored at −20 °C until use. In the comet assay, 3 h after injection of chemicals, peripheral blood was collected. Peripheral blood and gills were obtained at 96 h after injection of chemicals in the micronucleus test.

Vitellogenin analysis. Several researchers have found that the VTG molecule structure of carp (*Cyprinus carpio*) is similar to that of other cyprinid fish. Zhong *et al.* determined VTG production of hepatocytes in a rare minnow (*Gobiocypris rarus*) by ELISA using an antibody against carp (*Cyprinus carpio*) VTG.[22] This antibody has been found to bind to VTG produced by other cyprinid species, such as goldfish and fathead minnow.[23] Hence the carp VTG antibody is used to measure VTG concentration in plasma of a wide variety of cyprinid fish, such as goldfish. In this study, a Carp VTG ELISA Kit (Trans Genic, Japan) was used to determine VTG contents in plasma of goldfish according to the manufacturer's instructions. The concentrations of VTG were calculated from the linear part of the log-transformed VTG standard curve. The detection limit of VTG was 0.04 μg/ml plasma.

Alkaline comet assay. The alkaline comet assay was performed according to the method of Tice *et al.*, with some modifications.[24] Five μl of blood was diluted with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS(-): 7.5 g of NaCl, 0.2 g of KCl, and 0.2 g of sodium bicarbonate dissolved in 1 liter of water), and samples were mixed with 75 μl of 1% low melting point (LMP) agarose. The mixture (75 μl) was layered on a 1% LMP agarose layer and covered with 75 μl of 1% LMP agarose. After preparation, the slide was immersed in lysing solution and refrigerated at 4 °C for 1 h. After lysis, the slide was placed in alkaline electrophoresis buffer for 10 min to allow salt equilibration and further DNA unwinding. Electrophoresis was performed at 30 V, 300 mA for 15 min at 4 °C. The slide was then neutralized with 0.4 M Tris buffer (pH 7.5) for 10 min. The cells were stained with 50 μl of ethidium bromide (20 μg/ml). Comet images were analyzed using a fluorescence microscope (magnification 200×) equipped with CCD camera. One hundred cells were examined

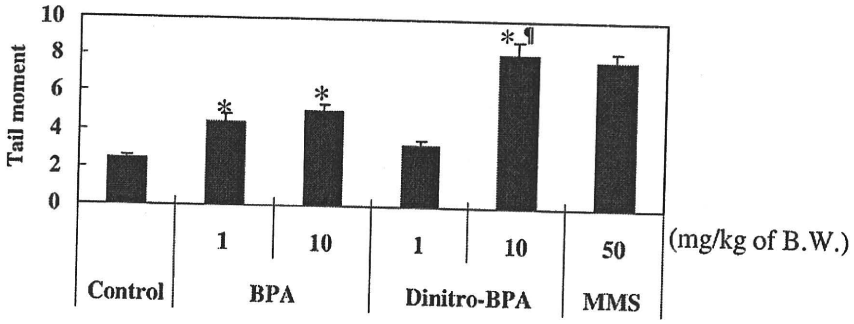


Fig. 3. Tail Moment of Peripheral Erythrocytes in Goldfish Due to Injection of BPA and of Dinitro-BPA. One hundred cells were counted per fish. The mean values were obtained from 500 cells. The bars represent S.E.M. values. $^{*}p < 0.01$ (*vs.* control), $^{¶}p < 0.01$ (*vs.* 10 mg/kg of B.W. BPA).

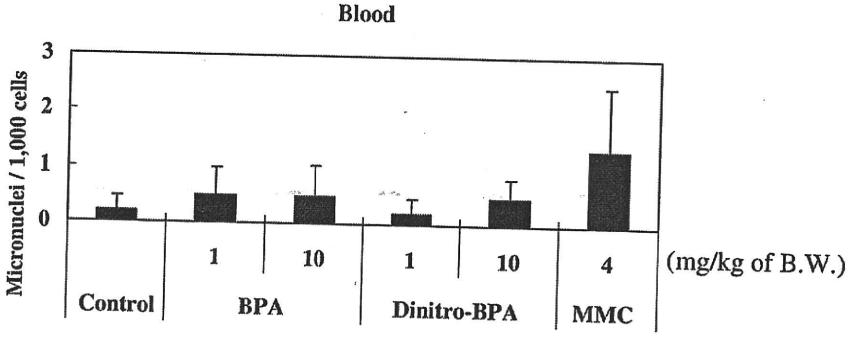


Fig. 4. Change in Frequency of Micronuclei in Peripheral Erythrocytes of Goldfish Injected with BPA and with Dinitro-BPA. Mean values were obtained from five fish. The bars represent S.D. values.

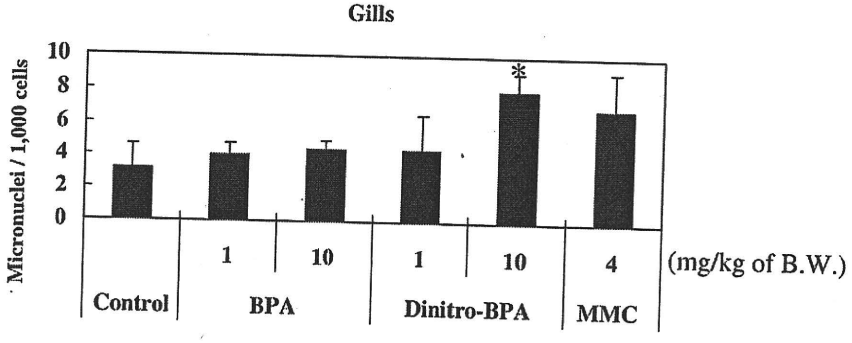


Fig. 5. Change in Frequency of Micronuclei in Gills of Goldfish Injected with BPA and with Dinitro-BPA. Mean values were obtained from five fish. The bars represent S.D. values. $^{*}p < 0.01$ (*vs.* control).

The frequency of micronuclei in gills increased significantly after intraperitoneal injection of dinitro-BPA at a dose of 10 mg/kg of body weight (8.00 ± 1.00) as compared with the control group ($p < 0.01$), but dinitro-BPA did not induce micronuclei in peripheral erythrocytes. Hayashi *et al.* have reported that mutagen-treated fish showed higher frequencies of micronucleated cells in gills than in peripheral erythrocytes, and recommended the use of gill cells in fish micronucleus assays.[26] BPA did not induce micronuclei in either gills or erythrocytes. Michael *et al.* have reported that BPA caused DNA damage due to apoptosis induced by BPA.[31] In this study, BPA showed DNA-damaging potency on the comet assay, but did not induce micronuclei on the micronucleus test at significant frequency. On the other hand, dinitro-BPA showed DNA damaging potency and induced micronuclei. These results suggest that the toxicity of BPA is different from that of dinito-BPA. Therefore, dinitro-BPA is assumed to induce abnormal chromosomes and to act as a genotoxic chemical.

The genotoxic effects of nitro compounds are generally linked to nitrate reductase. There are several kinds of NADPH-cytochrome *c* reductase,[32] xanthine oxidase,

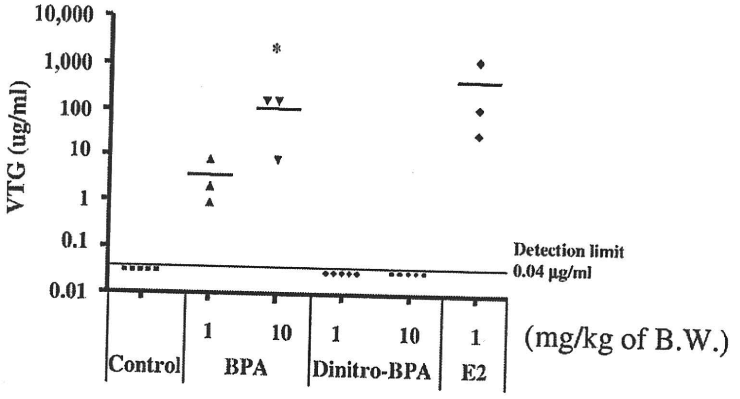


Fig. 2. VTG Concentration of Plasma in Goldfish by Injection of BPA and of Dinitro-BPA.
Mean values were obtained from three to five fish. The bars represent SD values. *$p < 0.01$ (*vs.* control).

per fish. The tail moment of DNA was measured using Comet Analyzer Youworks Bio Imaging Software.

Micronucleus test. The micronucleus test was performed according to the methods of Ueda *et al.*,[25] and Hayashi *et al.*[26] with some modifications. Peripheral blood of goldfish was collected from a caudal vein using a heparinized syringe. Ten µl of peripheral blood was diluted with 40 µl of fetal bovine serum. Nine µl of diluted blood was spread on an acridine orange-coated glass slide. Three gills on each side of the were excised and washed with PBS(-). Gills were transferred to 5 ml of PBS(-) and broken up with forceps. Tissue clumps and gill arches were removed and discarded. Free cells were collected by centrifugation (1,000 rpm, 5 min) and treated with 2 ml of 75 mM KCl hypotonic solution for 5 min. Then 0.1 ml of Carnoy fixative A (acetic acid and methanol, 1:3 by vol.) was added and centrifuged at 1,000 rpm for 5 min. A half volume of the supernatant with cells was suspended in 5 ml of Carnoy fixative B (acetic acid and methanol, 1:99 by vol.) and centrifuged. Then three fourth of the supernatant was removed and the cell suspension was dropped on a slide glass smeared with 0.0025% acridine orange solution. At least 1,000 erythrocytes and gill cells were observed with a fluorescence microscope (magnification, 400×) and the numbers of micronucleated cells were recorded.

Statistical analysis. Dunnett's test after one-way ANOVA was used to evaluate the significance of differences in VTG contents, tail moment in the comet assay, and micronucleus frequency in the micronucleus test between fish treated with BPA and with dinitro-BPA and the untreated group, A p-value lower than 0.05 was considered to be statistically significant.

Results and Discussion

VTG a phospholipoglycoprotein precursor of egg yolk protein, is synthesized in the liver of sexually mature oviparous females.[27] It is normally undetectable in the plasma of males and immature females. However, when male fish were treated with EDCs, VTG was detectable in their plasma. Hence VTG in plasma of male and juvenile fish treated with chemicals is used as a biomarker in evaluation of estrogenic activity of chemicals.[28]

In this study, we examined the estrogenic activities of BPA and dinitro-BPA by quantifying of VTG by the ELISA method with carp VTG antibody. The VTG concentration (113.5 µg/ml) in plasma of goldfish was significantly increased by treatment with BPA (10 mg/kg of body weight) ($p < 0.01$) (Fig. 2), but dinitro-BPA did not show any significant expression of VTG protein in plasma (n.d.). We have also found that the estrogen (α) binding potency of dinitro-BPA was weaker than that of non-treated BPA by *in vitro* assay.[17] In addition, we have reported that the estrogenic activity of 2-nitro-17β-estradiol, which is formed in a reaction mixture of 17β-estradiol and nitrite, was weaker than that of E2 using an ELISA kit.[29] Hence it is possible that binding potency to the estrogen receptor changed because the physico-chemical properties of BPA were transformed by nitration.

Comet assay is a rapid and sensitive method of detecting DNA single-strand breaks and alkali-labile sites in individual cells. Since these types of DNA damage can be the initial damage induced by genotoxic chemicals, comet assay is generally used as a method of detecting DNA damage due to exposure with mutagens and carcinogens.[30] Figure 3 shows the mean values of DNA tail moment in peripheral erythrocytes induced by dinitro-BPA and BPA. The mean tail moment values were increased by intraperitoneal injection of BPA (5.06 ± 0.37) and dinitro-BPA (8.20 ± 0.67) at 10 mg/kg of body weight. Variance analysis ($p < 0.01$) of these values showed a significant difference in DNA damage as between the dinito-BPA treated group and BPA-treated group.

Figures 4 and 5 show the frequencies of micronuclei in peripheral erythrocytes and gills of goldfish respectively.

DT-diaphorase,[33,34] and other enzymes. Nitro compounds show genotoxic activity through two pathways. The first reduction step is the formation of the nitrogen radical anion. Under aerobic conditions, this radical is reoxidized by O_2 and produces superoxide and hydroxyl radical anions. Under anaerobic conditions, reductive reaction leads to the formation of nitroso and hydroxylamine derivatives. Hydroxyl radicals cause DNA strand breaks.[35] Hydroxyl amino groups induce the transformation of DNA adducts.[36] These metabolic responses can cause DNA damage and abnormal chromosomes *in vivo*. Hence it is necessary to measure nitrogen reductase activity in goldfish.

Recently, many problems of nitrogen pollution have appeared in the water environment,[37] in acid rain,[38] and in the acidification of freshwater.[39] When these environmental conditions occur simultaneously, a nitro compound might be generated in the environment. Telscher *et al.* reported the formation of a nitrogen metabolite of nonylphenol isomer in soil/sewage sludge mixtures.[40] In the water phase, nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) can be formed by a photochemical reaction of PAH, with nitrite donating the nitro group.[41,42] There are various nitro compounds in the urban air and soil.[43–45]

The present study indicates that the estrogenic activity of dinitro-BPA was lower than that of BPA in goldfish. However, the genotoxic potency of dinitro-BPA was stronger than that of BPA. We also found that E2 and nitro-E2 showed similar behaviors.[29] Other nitro compounds might be formed and flow into the water environment. As a result, it is possible that aquatic organisms and human beings are exposed to nitro compounds. Hence we must investigate the formation of nitro compounds and evaluate their estrogenic and genotoxic activities *in vivo*. In addition, we must monitor dinitro-BPA and other nitro compounds in the environment.

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Review Article J. Clin. Biochem. Nutr., 43 Suppl. 1, 22–23, July 2008

Effect of Tea Extracts on Gastric Mucosal Erosion and Hemorrhage in *Helicobacter pylori* Infected Mongolian Gerbils

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Summary **In this study, we examined the inhibitory effect of green tea extract and catechins on the growth of *Helicobacter pylori* (*H. pylori*) and also on the improvement efficiency in *H. pylori* infected Mongolian gerbils. The possibility of eradication of *H. pylori* by oral administration of the catechins was also investigated. By administration of catechin adsorbed sucralfate for 10 days to *H. pylori* infected Mongolian gerbils, the colony forming units of *H. pylori* was significantly decreased. The combined effect of catechins and omeprazole, a proton pump inhibitor, was observed in an increase of pH as well as decrease of the mucosal hemorrhage. These results are supported by the epidemiological studies in green tea producing and non-producing area.**

***Key Words*: *H. pylori*, green tea, catechin, gastric ulcer**

The correlation between *Helicobacter pylori* (*H. pylori*) infection and gastric diseases, such as chronic gastritis, peptic ulcers, intestinal metaplasia and gastric cancer, has been widely investigated. In 1994, the World Health Organization/International Agency for Research on Cancer (WHO/IARC) concluded that *H. pylori* is one of the definite carcinogens based on the epidemiological findings. An epidemiological study shows that half numbers of Japanese are infected with *H. pylori*.

In a clinical field, the eradication is now performed by combined administration of amoxicillin, clarithromycin of antibiotics and lansoprazole of proton pump inhibitor. In recent year, side effects and resistance to bacteria are often observed after the administration of these medicines. Therefore, usage of natural products has been desired for the eradication of *H. pylori* infection diseases. Several investigators demonstrated the suppression effects of daily food stuffs containing Wasabi on *H. pylori*-induced gastric in Mongolian gerbils (M. Iimuro *et al.*, 2002, S. Matsubara

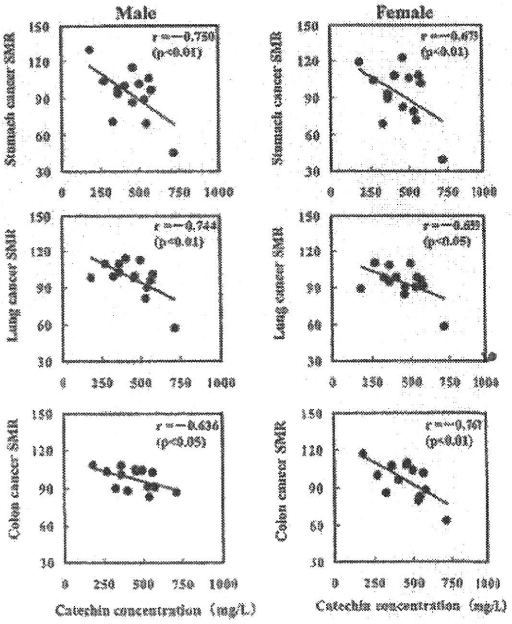

Fig. 1. Relationship between catechin concentration in the brewed green tea and cancer SMR in 14 areas (1995–1999)

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Table 1. Combined effect of green tea catechins and anti-*H. pylori* agents

a) Combined effect of green tea catechins and anti-*H. pylori* agents

Test systems	MBC (μg/ml)	
	NCTC	YS50
AMPC + GTC (1.25 mg/ml)	0.186	0.047
AMPC	0.186	0.186
CAM + GTC (1.25 mg/ml)	0.8	0.8
CAM	6.4	6.4

MBC: Minimal Bactericidal Concentration
GTC: Polyphenon 70S (Tokyo Food Techno, Tokyo Japan) contains 73.4% catechins (EGC: 33.5%, ECG: 10.1%, EGC 17.4%, EC: 8.6%)

b) Combined effect of green tea catechins and sucralfate in *H. pylori* infected mongolian gerbils

Experimental group (No. of animals)	GTC administration (mg daily)	Intragastric pH[a]	Bacterial count (log CFU/stomach)[b]
I Control (4)	0	2.7 ± 0.9	5.690 ± 0.268
II Sucralfate (4)	0	2.8 ± 0.5	5.655 ± 0.071
III GTC solution (4)[c]	20	3.3 ± 0.3	5.353 ± 0.197
IV GTC-Sucralfate (6)[d]	5	2.6 ± 0.4	5.538 ± 0.197
V GTC-Sucralfate (6)[d]	10	2.8 ± 0.7	5.088 ± 0.339
VI GTC-Sucralfate (6)[d]	20	2.8 ± 0.4	3.839 ± 0.476*

*Significantly reduced compared with the Control group, at $p<0.05$ Sucralfate: Anti-gastritis for gastric membrene erosion GTC, green tea catechin; Polyphenon 70S

[a] The average pH of the stomach contents. The values for results are expressed as means ± SDs. Bonferroni's test was used for statistical analysis.

[b] I The average bacterial count each group was calculated. The values for results are expressed as means ± SDs. Bonferroni's test was used for stastistical analysis

[c] The contcentration of GTC solution was 20 mg Polyphenon 70S/ml distilled water. The animals were orally administered 1 ml of the solution daily for 10 days

[d] The contcentration of GTC-Sucralfate was 20 mg Polyphenon 70S/ml Ulecermin suspension. The animals were administered 0.25 ml (group IV), 0.5 ml (group V), and 1 ml (group VI) of GTC-Sucralfate daily for 10 days

et al., 2003; H. Masuda *et al.*, 2004, T.Y. Oh *et al.*, 2005). Recently, it was found that tea catechins inhibited the growth of *H. pylori* under the neutral condition (K. Mabe *et al.*, 1999). *H. pylori* was eradicated in 10 to 36% of the catechin treated Mongolian gerbils with significant decreases in mucosal hemorrhage and erosion.

In this study, we present the inhibitory effect of green tea extract and catechins on the growth of *H. pylori* and also on the improvement efficiency in *H. pylori* infected with Mongolian gerbils. The possibility of eradication of *H. pylori* by the oral administration of the catechin was also investigated. By Oral administration of catechin adsorbed sucralfate for 10 days to Mongolian gerbils infected with *H. pylori*, the colony forming units of *H. pylori* was significantly decreased. The combined effect of catechins and omeprazole, was demonstrated by the increase of pH as well as decrease of the mucosal hemorrhage. These results are supported by the epidemiological data in tea producing and non-producing areas.

再生医学

マイクロアレイとNIA array analysisを用いた心筋分化誘導因子の探索

Exploration of the cardiomyogenic differentiation factor using DNA micro array and NIA array analysis

マイクロアレイを用いた心筋分化誘導因子の探索

近年の研究から心筋細胞の発生過程において，骨形成因子(bone morphogenetic protein：Bmp)や線維芽細胞増殖因子(fibroblast growth factor：Fgf)などのサイトカインによって誘導されるCsx/Nkx2.5やGata4，Mef2cのような転写因子は，その心筋細胞の運命決定因子として重要な役割を担っている[1]．このような数多くの心筋細胞の発生に関与する主要な転写因子群の機能は明らかになりつつあるものの，心筋分化誘導のマスタージーンは確認されていない．これは心筋細胞の発生には複数の遺伝子が経時的に関与するため，あるいはこれらの転写因子群の機能を制御している別の因子が存在しているためであると考えられている．このような背景から心筋分化に関連する遺伝子の選択のために，マイクロアレイを用いた網羅的な遺伝子発現解析が行われてきた．しかし，複数の細胞のマイクロアレイの結果をもとに特定の遺伝子を選択することは非常に困難である．そのため，統計解析を用いた特定の細胞群特異的な発現を示す遺伝子を選択する手法が必要となる．

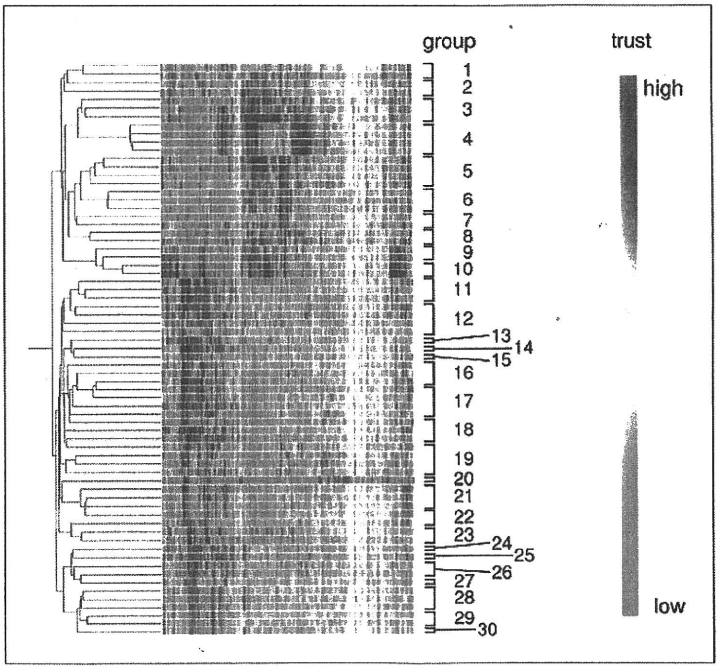

図1　69種類の培養ヒト細胞の遺伝子発現解析

GeneSpring softwareを用いた69種類のヒト細胞の遺伝子発現解析結果．発現データの近似している細胞をグループ化したところ，30グループにまとめることができた．

NIA array analysisによる心筋分化誘導因子の選択と今後の展望

これまで著者らのチームは，月経血や胎盤などの初代培養細胞とマウス胎児心筋細胞の共培養による心筋分化誘導に成功してきた[2-4]．この知見をもとに，これら心筋分化しやすい細胞と分化しにくい細胞のマイクロアレイデータ，ならびにヒト胚性幹細胞などのマイクロアレイデータ(NCBI Gene Expression Omnibus データベースから取得)の合計69種類のデータを用い，GeneSpring GX softwareにて遺伝子発現を比較した．さらに，近似した遺伝子発現データを示す細胞を任意にグループ化したところ，30グループに区分できた(図1)．この30グループのうち21グループは，共培養法

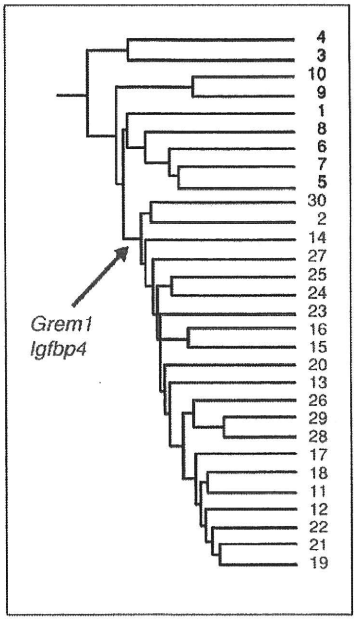

図2　培養ヒト細胞の階層的クラスタ分析

図1でまとめられた30グループに対してNIA array analysisを用いた階層的クラスタ分析の結果．このとき，心筋分化が可能な細胞群(21グループ；赤字)特異的な遺伝子として*Grem1*と*Igfbp4*を選択できた．

や胚体形成によって心筋細胞に分化誘導が可能な細胞グループであった．そこで，この21グループ特異的な遺伝子を同定するために，マイクロアレイの結果を簡便に統計解析処理できるwebアプリケーションNIA array analysis (http:/ /lgsun.grc.nia.nih.gov/ANOVA/)[5]を用いて階層的クラスタ分析を行った．この結果，この21グループ特異的遺伝子として，2つの心筋分化誘導候補遺伝子(*Grem1*, *Igfbp4*)を選択できた(図2)．この2遺伝子の心筋分化誘導能を検証したところ，Bmpアンタゴニストである Grem1は分化誘導初期のBmpシグナルを阻害することで[6]，Igfbp4は分化誘導後期のcanonical Wnt/β-cateninシグナルを阻害することで[7]，胚性細胞の心筋分化を促進していることが明らかになった．

これらの結果から本手法は心筋細胞のみならず，何らかの特徴的な性質をもつ細胞群のマイクロアレイデータを用いることで，臓器特異的な分化誘導因子や，近年注目されているクロマチンリモデリング因子の探索に役立つ可能性がある．さらに，NCBI Gene Expression Omnibusにアップロードされているデータも利用できるため，*in silico* での解析や考察の確認にも有用である．今後はこれらの手法を組み合わせ，あらたな心筋分化誘導因子の探索とその評価解析も同時に行っていく．

1) Shiojima, I. and Komuro, I.：Cardiac developmental biology：from flies to humans. *Jpn J. Physiol.*, **55**：245-254, 2005.
2) Okamoto, K. et al.：Working' cardiomyocytes exhibiting plateau action potentials from human placenta-derived extraembryonic mesodermal cells. *Exp. Cell Res.*, **313**：2550-2562, 2007.
3) Hida, N. et al.：Novel cardiac precursor-like cells from human menstrual blood-derived mesenchymal cells. *Stem Cells*, **26**：1695-1704, 2008.
4) Nishiyama, N. et al.：The significant cardiomyogenic potential of human umbilical cord blood-derived mesenchymal stem cells *in vitro*. *Stem Cells*, **25**：2017-2024, 2007.
5) Sharov, A. A. et al.：A web-based tool for principal component and significance analysis of microarray data. *Bioinformatics*, **21**：2548-2549, 2005.
6) Kami, D. et al.：Gremlin enhances the determined path to cardiomyogenesis. *PLoS ONE*, **3**：e2407, 2008.
7) Zhu, W. et al.：IGFBP-4 is an inhibitor of canonical Wnt signalling required for cardiogenesis. *Nature*, **454**：345-349, 2008.

上　大介，石井隆雅，梅澤明弘，渡邉昌俊／Daisuke KAMI[1], Ryuga ISHII[3], Akihiro UMEZAWA[3] and Masatoshi WATANABE[2]

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糖尿病・内分泌代謝学

劇症1型糖尿病――最新動向

Recent progress in fulminant type 1 diabetes

劇症1型糖尿病とは

劇症1型糖尿病は2000年に報告された“非常に急速でほぼ完全な膵β細胞破壊の結果生じる糖尿病”と定義される糖尿病のサブタイプである[1]．日本人急性発症1型糖尿病の約20%を占め，GAD抗体などの膵島関連自己抗体がおおむね陰性であることが特徴であり，他の80%に相当する自己免疫性1型糖尿病と対照的である．そのほかに，ケトアシドーシスを伴って非常に急激に発症することや発症時に著明な高血糖を認めるにもかかわらず，HbA_{1C}は正常または軽度上昇にとどまる，といった臨床的な特徴も明らかになっている．

劇症1型糖尿病発症に関与する遺伝因子として，HLA(human leukocyte antigen)遺伝子が知られていた．すなわち，class II HLAのDR-DQハプロタイプのなかで，劇症1型糖尿病ではDR4-DQ4の頻度が高く，とくにこのハプロタイプをホモで有する場合のオッズ比は13.3と，非常に高い値を示すことが報告されていた．ちなみに，DR4-DQ4は通常 *DRB1*0405-DQB1*0401* という遺伝子型によりコードされる．

劇症1型糖尿病とCTLA-4

今回HLAに加えて，やはり免疫反応に関与する分子であるCTLA-4(cytotoxic T lymphocyte antigen-4)遺伝子と劇症1型糖尿病との関連が明らかにされた．

CTLA-4は抗原提示細胞(マクロファージや樹状細胞)上に発現する分子で，T細胞のB7-1(CD80)

表1　CTLA-4遺伝子多型(文献[2]より作成)

	+49GG	CT60AA
劇症1型(n=55)		
オッズ比(95%CI)		2.68(1.13～6.37)
p value	NS	0.021
急性発症1A型(n=90)		
オッズ比(95%CI)	2.26(1.41～3.60)	
p value	0.0005	NS

はじめに
Introduction

渡邉昌俊
Masatoshi WATANABE
横浜国立大学大学院工学研究院機能の創生部門過程の機能と安全分野（医工学）

ナノテクノロジーの発展はすさまじく，いろいろな分野への応用が期待されている．当然のことながら医学と工学の連携で，drug delivery system(DDS)や分子イメージングなど医療への応用が図られている．

ナノ粒子メディスンという言葉は，ナノテクノロジーのなかで重要な技術・素材であるナノ粒子と医療を合わせた造語である．ナノ粒子はバルクと異なり，活性度と反応性が飛躍的に高まり，その特性である電磁気的・光学的・機械的性質などが大きく変わることが知られている．本特集では，このナノ粒子の再生から癌治療にわたる医療への応用を中心に組ませていただいた．

一方，ナノテクノロジーのリスク，とくにナノマテリアルの細胞毒性が問題となっているが，その生体への影響について十分な知見は得られていない．本年(2009)3月には厚生労働省より“ナノマテリアルに対するばく露防止等のための予防的対応について”(通知)がだされた．このような状況で，多層カーボンナノチューブによる実験動物での中皮腫の発生に関して異なる報告がだされている[1)]．著者自身，病理学者として，重要な科学技術であるナノマテリアルの細胞毒性に十分注意を払いつつ，評価し使用していく必要があると考える．また，ナノ粒子が原因不明の肉芽種の原因であるという説も出ており，病因論的には非常に興味深い．

本特集号では，2007年の化学工学会第39会秋期大会シンポジウム(マイクロプロセスからみた細胞・組織工学の展開)，2008年の異分野融合ナノテクノロジー横浜コロキウム，2008年の第55回日本臨床検査医学会学術集会シンポジウム(医学領域におけるナノ粒子展開をめぐる話題)などで講演を行っていただいた研究者に執筆をお願いした．この特集を読まれてナノ粒子をめぐる問題を理解していただき，ナノ粒子を含むナノテクノロジーを用いた医療のさらなる発展に力となれば幸いである．

1) Sakamoto, Y. et al.：Induction of mesothelioma by a single intrascrotal administration of multi-wall carbon nanotube in intact male Fischer 344 rats. *J. Toxicol. Sci.*, **34**：65-76, 2009.

磁性ナノ粒子を用いたiPS細胞の誘導

Generation of the iPS cells using magnetic nano particles

上　大介(写真)　梅澤明弘　渡邉昌俊

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◎人工多能性幹細胞(iPS細胞)は，体細胞に4遺伝子(*Oct3/4*，*Sox2*，*cMyc*，*Klf4*)を導入することにより，胚性幹細胞と同等の多分化能(pluripotency)をもつ細胞である．このiPS細胞の研究は特定疾患のメカニズムの解明や新薬開発，再生医療に用いられる細胞ソースなど，多岐にわたる活躍が期待されている．一般的に，iPS細胞作製における遺伝子導入にはレトロウイルスやレンチウイルスが用いられている．しかし，ホスト細胞のゲノムDNA内にウイルス由来DNA配列が残存する，細胞が癌化するといった問題点があるため，現状のままでは医療への応用には適していない．そこで，ウイルスを用いないあらたなiPS細胞作製方法が求められている．本稿では，非ウイルス性であり高効率な遺伝子導入を可能とする磁性ナノ粒子を用いた手法の利点と進行状況について報告する．

Key word：磁性ナノ粒子，非ウイルス性遺伝子導入，人工多能性幹細胞(iPS細胞)

ナノ粒子の医学・生物分野への開発と応用

近年，ナノデバイスの研究は，その素材の開発から利用・応用にかけて盛んに行われており，産業界におけるナノデバイスの活躍がおおいに期待されている．ナノデバイスのひとつであるナノ粒子はナノメートル(10^{-9}m)オーダーの微粒子であり，同一素材のマイクロ(10^{-6})オーダーサイズの粒子と比べて，その流動性の向上，表面積の増加といった性質を示す．このようなナノ粒子の物性変化を応用することで，粒子の圧縮成形時の密度向上，吸着容量の増大，化学反応触媒としての機能向上，他の物質との複合化を簡便に可能とする技術の開発が期待されている．

ナノ粒子の生化学や分子生物学，医学分野への応用はすでに行われており，ナノ粒子表面に何らかの標識化合物を結合させて特定の分子構造を同定，検出，定量，可視化することなどに用いられている．とくに磁性ナノ粒子は，外部からの磁場により体内での移動や固定が可能である．また，磁性ナノ粒子の粒子径は数nmから数十nm範囲で制御が可能であり，蛋白質(5～50 nm)や遺伝子(幅2 nm)と比較しても同等以下の大きさにコントロールできる．このため磁力による外部モニタリングも容易なため，標識としても使用されている．さらに，磁性ナノ粒子は外部からの磁力を熱エネルギーに変換することが可能なため，腫瘍細胞を加温により死滅させるハイパーサーミアの発熱体として利用されようとしている．

このほかにも磁性ナノ粒子は，細胞の磁気による選別と単離(magnetic cell separation system：MACS)，薬剤のキャリア(drug delivery system：DDS)，MRI(magnetic resonance imaging)の造影剤としても利用されている．通常，このような磁性ナノ粒子には鉄やコバルト，マンガンなどの金属を酸化させたものが用いられる．しかし，金属酸化物の粒子表面を標識物質で被膜するとより凝集性が強くなるため，凝集した粒子の二次粒子径が大きくなり，その機能が低下するという欠点をも

つ．なぜなら，この磁性ナノ粒子の溶液中の安定性は，粒子径，熱エネルギーおよび引力(van der Waals および相極子間相互作用)，斥力(立体および静電)の釣り合いで決定するからである．そこで，界面活性剤による立体障害斥力，あるいは電気二重層相互作用による斥力によって磁性ナノ粒子の分散を維持させることが重要となってくる(「サイドメモ1」参照)[1]．

磁性ナノ粒子を用いた遺伝子導入法の開発と，iPS細胞作製への応用

このような背景のなか，著者らは磁性ナノ粒子(γ-Fe_2O_3, d=3 nm, 横浜国立大学・一柳優子准教授提供)[2]を用いた培養細胞への遺伝子導入方法を開発している．現在，培養細胞への遺伝子導入は，①polyethylenimine(PEI)に代表されるトランスフェクション試薬を用いたもの，②ウイルス(アデノウイルス，レトロウイルス，レンチウイルス)を用いたもの，③エレクトロポレーション法を用いたもの，に大別することができる．

近年話題になっている人工多能性幹細胞(induced pluripotent stem cell：iPS細胞)も，当初はレトロウイルスを用いた遺伝子導入法で作製されてきた[3,4]．このiPS細胞は体細胞をホスト細胞としながらも，胚性幹細胞(embryonic stem cell：ES細胞)と非常によく似た性質をもっており，多分化能と自己複製能，ジャームライン・トランスミッション(「サイドメモ2」参照)ですら可能な驚くべき細胞である．現在，すでにヒトiPS細胞の作製は成功しており，種々の遺伝病患者由来iPS細胞も作製されており，あらたな治療法や新薬の開発が期待されている[3,5]．実際に疾患モデルマウス由来iPS細胞を用いて同マウスに細胞移植したところ，疾患モデルマウスの病態が改善したという報告もあり[6,7]，iPS細胞を用いた新しい医療の可能性が示された．

しかし，iPS細胞の作製には高効率な遺伝子導入が必要である．そのため，レトロウイルスやレンチウイルスを用いた作製法は，核ゲノム内にウイルス由来DNA配列が挿入されるうえに，導入遺伝子による細胞の癌化の危険性も指摘されている．そこで，2008年にウイルスを用いない非ウイルス性のiPS細胞作製法が開発された[8]．このiPS細胞作製法は，CAGGSプロモーター下流にiPS細胞作製に必要な4遺伝子(*Oct3/4*, *Sox2*, *cMyc*, *Klf4*)を配置したプラスミドDNAとトランスフェクション試薬を用いていた．しかし，この手法はウイルスを用いた方法に比べて遺伝子導入効率が低く，iPS細胞への誘導効率が悪い[8]．これは，トランスフェクション試薬で導入した場合，プラスミドDNAは培養液中をランダムな方向に漂うためとされている(図1-A)．このため，トランスフェクション試薬を用いた遺伝子導入効率は低いという欠点がある．

そこで著者らは，磁性ナノ粒子をプラスミドDNAのキャリアとした遺伝子導入法の確立を目的とした．本手法の特徴はランダムに漂うプラスミドDNAを磁性ナノ粒子に吸着させる点にある．このプラスミドDNAに吸着させた磁性ナノ粒子は，培養プレート底に設置したマグネットシートの磁力によって培養細胞方向に引き寄せることが可能となり，遺伝子導入効率が上昇すると予想される(図1-B)．

磁性ナノ粒子を用いた遺伝子導入の問題点

磁性ナノ粒子をプラスミドDNAのキャリアと

サイドメモ1　界面活性剤と分散剤

界面活性剤は，磁性ナノ粒子の表面を被膜し，その立体障害によって粒子どうしの凝集を抑制し，溶媒中に安定に分散する．分散剤は，高分子窒素化合物などでナノ粒子表面を被覆することで，ナノ粒子表面をプラスに帯電させ，電気二重層の作用のみによってナノ粒子の分散を安定的に可能にする．

サイドメモ2　ジャームライン・トランスミッション

ES細胞やiPS細胞などの多能性幹細胞が生殖細胞に分化でき，多能性幹細胞由来の遺伝子がキメラを介して次世代に伝わることを意味する．これにより，その多能性幹細胞に由来する個体が産まれる．

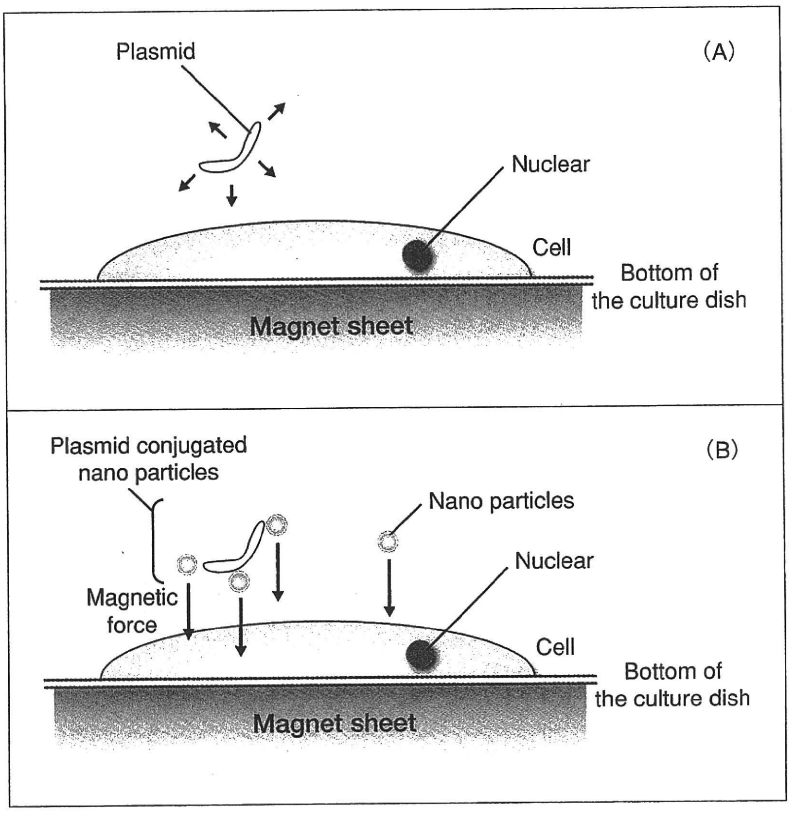

図 1　ナノ粒子を用いた細胞へのプラスミドDNA導入モデル

A：通常のトランスフェクション方法の略図．トランスフェクション試薬で導入する際，プラスミド DNA は培養液中をブラウン運動に従ってランダム方向に移動していると予想される．

B：開発中のトランスフェクション方法の略図．プラスミド DNA と磁性ナノ粒子を接着させた複合体を磁力で細胞表面に輸送することで，接触できるプラスミド DNA 数を上昇させ，遺伝子導入効率を向上させる．

して用いるには，磁性ナノ粒子の分散と磁性ナノ粒子とプラスミド DNA 間の結合方法について考慮すべきである．なぜなら粒子径が 100 nm 以下のナノ粒子の分散制御は非常に困難で，何らかの分散剤を用いてナノ粒子表面を修飾する必要があり，プラスミド DNA とナノ粒子の吸着はプラスミド DNA の配列を損ねず安定的に遺伝子発現できるようにすべきだからである．これらのことから，著者らはトランスフェクション試薬として用いられている PEI に着目した．というのも，PEI はトランスフェクション試薬だけでなく，粒子表面を修飾して電気二重層相互作用を示す分散剤としても用いられているからである．また，PEI はカチオン性のため，アニオン性のプラスミド DNA と電気的に結合すると予想される．そこで PEI を用いてナノ粒子表面を被膜化し，ナノ粒子の分散とプラスミド DNA の結合を同時に試みた(図 2)．

現在，適当な条件下で磁性ナノ粒子 γ-Fe_2O_3 と PEI の被膜化は成功しており，良好な分散状態が観察された．PEI によって被膜化していない粒子(図 3-B)は被膜化したナノ粒子(図 3-A)に比べて早く凝集し沈降した．さらに，磁性ナノ粒子は PEI で被膜した後も磁力によって凝集(図 4-A)と再分散(図 4-B)が可能であった．今後はこの磁性ナノ粒子を用いたプラスミド DNA の吸着，ならびに細胞への遺伝子導入を試みていく．

磁性ナノ粒子を用いた遺伝子導入法の利点と今後の展望

本手法の最大の利点は，ウイルス由来 DNA 配列が挿入されない遺伝子導入が可能である点と，理論上，通常のトランスフェクション法に比べて高効率的な遺伝子導入が可能な点である(図 1)．

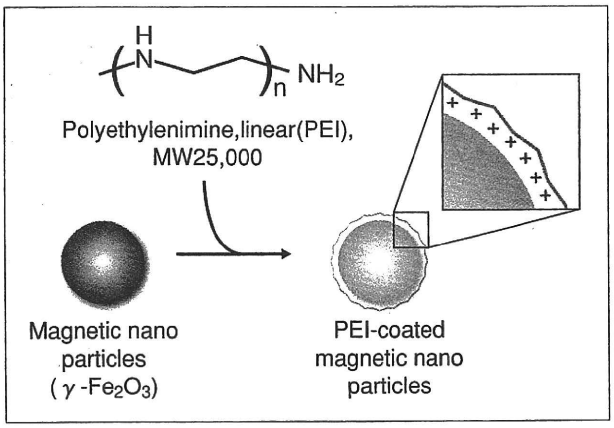

図 2　磁性ナノ粒子へのPEI被膜化と粒子表面の電荷
磁性ナノ粒子 γ-Fe_2O_3は直鎖状の PEI(分子量 25,000)で被膜化する．粒子表面は被膜化した PEI によって＋に帯電すると予想している．

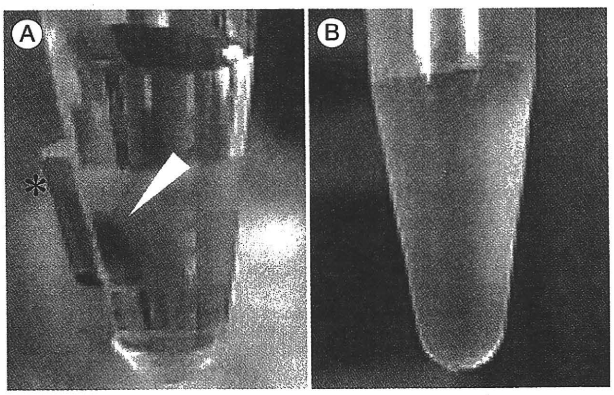

図 4　磁性ナノ粒子の吸着と再分散
A：磁性ナノ粒子をネオジム磁石(＊)で吸着した(矢尻)．
B：再度かき混ぜたところ，再分散した．

この 2 点は iPS 細胞の作製にたいへん有利な特徴である．現在，iPS 細胞の作製にはホスト細胞への 4 遺伝子導入にレトロウイルスやレンチウイルスを用いている．このため，ウイルス由来 DNA 配列は iPS 細胞のゲノム DNA 内に挿入され，残存している．このウイルス由来 DNA 配列の残存は，iPS 細胞の性質に影響を与えている可能性が高い．というのも，レンチウイルスで作製した iPS 細胞に挿入された 4 遺伝子の DNA 配列を Cre/loxP システムを用いて排除した(正確には一部の DNA 配列は残存している)ところ，排除していない iPS 細胞に比べてマイクロアレイにて ES 細胞に近い遺伝子発現パターンを示したからである[9]．本手法で作製された iPS 細胞はこの外来性 DNA 配列がホスト細胞のゲノム内に残りにくいため，

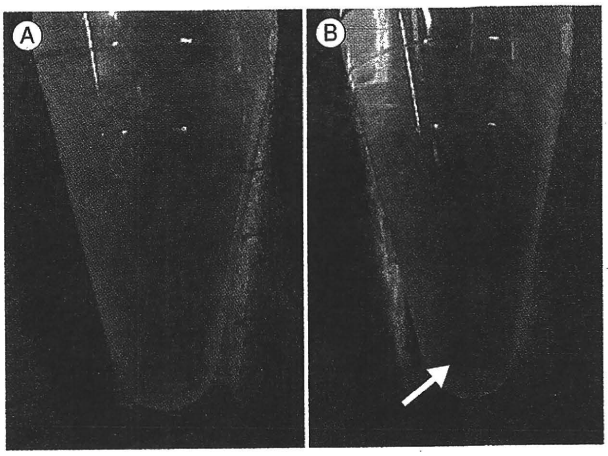

図 3　PEI被膜化した磁性ナノ粒子と，PEI被膜化していない磁性ナノ粒子の分散性
分散剤である PEI で被膜化した磁性ナノ粒子は，室温に 30 分間おいても沈殿しなかった(A)．一方，PEI で被膜化していない磁性ナノ粒子は，同様の条件下で沈殿が確認された(B，矢印)．

Cre/loxP システムを用いた手法より ES 細胞に近い性質を示す可能性が高い．

このように本手法によって作製された iPS 細胞は，特定疾患のメカニズムの解明や新薬開発，再生医療に用いられる細胞ソースとして重要な役割を果たす可能性がある．その一方で，遺伝子導入に使用した磁性ナノ粒子の細胞毒性についても検討する必要があるため，解析を進めている．今後はより遺伝子導入に適した磁性ナノ粒子表面の修飾法の検討と本手法を用いた iPS 細胞を作製していく．

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