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Stilbene Analogs of Resveratrol Improve Insulin Resistance through Activation of AMPK

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Resveratrol (RSV), 3,5,4′-trihydroxy-*trans*-stilbene, is known to have many beneficial physiological activities. We have synthesized several stilbene anlogues and have reported that the hydroxyl group in the 4′ position of RSV exhibited strong radical scavenging action. Using stilbene analogs, we investigated the structure of RSV to explain its protective effect against obesity and type 2 diabetes. All six analogs used in this study inhibited the differentiation of 3T3-L1 adipocytes. 3-Hydroxy-*trans* stilbene (3(OH)ST), and 3,4′-dihydroxy-*trans* stilbene (3,4′(OH)$_2$ST) increased glucose uptake and induced adenosine monophosphate kinase (AMPK) phosphorylation in C2C12 myotubes independently of insulin. An *in vivo* study using mice fed high-fat diets indicated that 3(OH)ST was more effective than RSV in improving insulin resistance. In conclusion, RSV and its derivatives, particularly 3(OH)ST, inhibited adipocyte differentiation and enhanced glucose uptake in the myotubes, resulting in a reduction of obesity and an improvement in glucose tolerance *in vivo*.

Key words: resveratrol; stilbene analog; adenosine monophosphate kinase (AMPK); insulin resistance; myotube

Resveratrol (RSV) (3,5,4′-trihydroxy-*trans*-stilbene), a polyphenol abundant in grapes and red wine, possesses a range of biological activities including anti-aging,[1–5] anti-cancer,[5] repression of fat accumulation,[6,7] anti-oxidative activity,[8,9] and improvement of insulin sensitivity.[10] Especially, it is accepted as an activator of Sirtuin 1 (Sirt1), NAD-dependent deacetylase, which is related to longevity and calorie restriction, and has drawn the attention of many researchers. Some RSV derivatives with higher bioavailability are being tested in clinical trials for type 2 diabetes treatment,[11] but, information on the correlation between the physiological activities and the structural characteristics of RSV analogs is sparse.

We have investigated the physiological activities of RSV analogs using synthesized RSV derivative analogs showing various numbers and positions of the hydroxyl group on the stilbene backbone, and have reported that the 4′-hydroxy group on RSV is essential for strong anti-oxidative activity. On the other hand, RSV has been reported to induce chromosomal aberrations, micronuclei, and sister chromatid exchanges in mammalian cells in a bacterial reverse mutation assay[12] due to its strong radical scavenging action.[12–15] We found that the 4-hydroxy group on the RSV structure was responsible for *in vitro* clastogenic activity.[13] RSV and its analog 4-hydroxy-*trans*-stilbene (4(OH)ST), but not 3-hydroxy-*trans*-stilbene (3(OH)ST), scavenge the tyrosyl free radical of the R2 subunit of mammalian ribonucleotide reductase.[14] This observation should prove useful for safe drug design.

While most antioxidants are useful for human health, it is not known whether the many biological benefits of RSV are explained solely by its anti-oxidative function. In this study, we compared the effects of several stilbene analogs to clarify the structural importance of its hydroxyl groups in the prevention of obesity-induced disorders.

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Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; AMPK, adenosine monophosphate kinase; BAT, brown adipose tissue; cAMP, cyclic AMP; CamKKβ, calmodulin kinase kinase β; DMEM, Dulbocco's Modified Eagle Medium; FBS, fetal bovine serum; GLUT4, glucose transporter 4; GST, glutathione *S*-transferase; 3,4′(OH)$_2$ST, 3,4′-dihydroxy-*trans* stilbene; 3,3′(OH)$_2$ST, 3,3′-dihydroxy-*trans*-stilbene; 3,5(OH)$_2$ST, 3,5-dihydroxy-*trans*-stilbene; 4(OH)ST, 4-hydroxy-*trans*-stilbene; 3(OH)ST, 3-hydroxy-*trans* stilbene; HF, high fat; OGTT, oral glucose tolerance test; isoRSV, iso-resveratrol; ITT, insulin tolerance test; KRBB, Krebs-Ringer biocarbonate buffer; LF, low fat; NA, nicotine amide; NAD, nicotine amide dinucleotide; PPAR, peroxisome proliferator-activated receptor; RSV, resveratrol; Sirt1, sirtuin 1; WAT, white adipose tissue

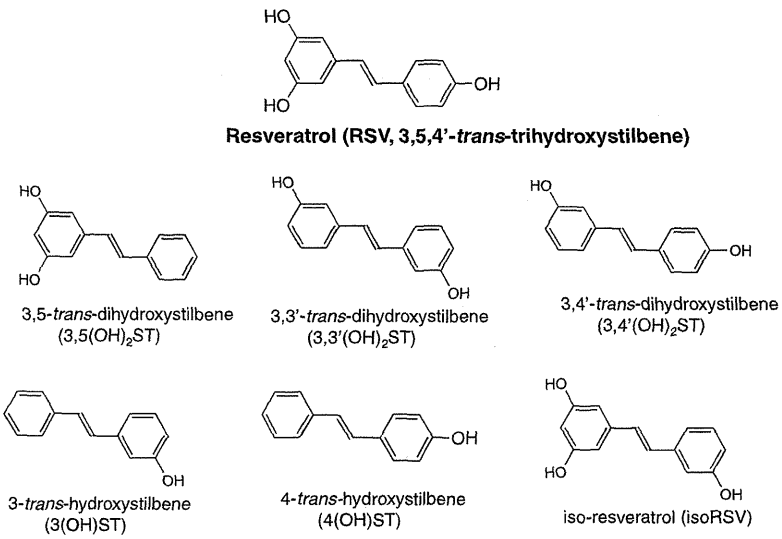

Fig. 1. Chemical Structures of RSV and the Stilbene Analogs.
Analogs showing different numbers and positions of hydroxyl groups on the stilbene backbone were synthesized as reported previously.[15,16]

Material and Methods

Materials. RSV and iso-resveratrol (3,5,3′-trihydroxy-*trans*-stilbene) were purchased from Sigma (St. Louis, MO). 3(OH)ST, 4(OH)ST, 3,4′-dihydroxy-*trans*-stilbene (3,4′$(OH)_2$ST), 3,3′-dihydroxy-*trans*-stilbene (3,3′$(OH)_2$ST), and 3,5-dihydroxy-*trans*-stilbene (3,5$(OH)_2$ST) were synthesized as previously reported.[15,16] The chemical structures are shown in Fig. 1.

Cell culture. 3T3-L1 pre-adipocytes were purchased from ATCC (Rockville, MD) and were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Nissui, Tokyo) containing 10% fetal bovine serum (FBS, Bio West, Inc., Nuaillé, France) at 37 °C in a humidified atmosphere with 5% CO_2. Cells were plated at a density of 4×10^5 cells in a 75 cm^2 flask and passaged at 80% confluency. In each experiment, 15×10^4 cells were plated in 60 mm dishes, and the DMEM (low glucose) was changed every 2 d. At confluence, the medium was removed and replaced (day 0) with a differentiation medium comprising DMEM (high glucose) with 0.5 mM 3-isobutyl-1-methyl-xanthin, 10 μg/mL of insulin, and 1 μM dexthamethasone, maintained for 2 d, and then incubated with a maturation medium containing 5 μg/mL of the insulin until day 8. The cells were treated with RSV or stilbene analogs from day 0 to day 2.

The skeletal muscle cell line of C2C12 myoblasts was purchased from Riken (Tokyo), and was maintained in DMEM containing 10% FBS at 37 °C in a humidified atmosphere with 5% CO_2. Cells were plated at a density of 7.5×10^5 cells in a 75 cm^2 flask and passaged when it reached 80% confluence. In the glucose uptake experiments, 1.5×10^5 cells were plated in 6-well plates, and for Western blotting, 4.5×10^5 cells were plated in 60 mm dishes. At confluence (day 0), the medium was exchanged for differentiation medium, DMEM containing 2% horse serum (Invitrogen, San Diego, CA). Since myogenin mRNA expression levels increased (data not shown), it was thought that the C2C12 cells were differentiated into myotube cells at day 3 or 4. Hence, the cells were treated with RSV or stilbene analogs in differentiated myotube cells at day 4–6 in each experiment.

Oil Red O staining. Adipocytes were fixed using 10% formalin/PBS (−) for 10 min at day 8. Then they were stained with Oil Red O (Sigma, St. Louis, MO) and lysed with 60% isopropanol for 20 min.

Real-time RT-PCR. Total RNA was extracted from the mature adipocytes (at day 8) with Isogen (Nippon Gene, Tokyo) following the manufacturer's instructions. PCRs of the peroxisome proliferator-activated receptor γ (PPARγ), resistin, and adiponectin were performed for 40 cycles under the following conditions: denaturation 95 °C for 15 s, annealing and extension at 60 °C for 1 min by the 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The primers used had the following base sequences: for peroxisome proliferator-activated receptor γ (PPARγ), 5′-GCACTGCCTATGAGCACTTCAC-3′; for resistin, 5′-AGAGGTCCACAGAGCTGATTCC-3′, 5′-TGGAAACTACATGGTGGAAGGA-3′ and 5′-TGGTACTCAAGTGCCTCAGTGC-3′; and for adiponectin, 5′-GTGGATCTGACGACACCAAAAG-3′ and 5′-ACGTCATCTTCGGCATGACTG-3′.

Glucose uptake into C2C12 cells. Differentiated C2C12 myotube cells at days 4–6 were incubated in differentiation medium containing 50 μM RSV or stilbene analogs for 60 min. After they were washed twice with PBS (−), the cells were preincubated with KRBB (1 mL/well) for 40 min. Then 10 μL of 20 mM [^{3}H]2-deoxyglucose (0.68 mCi/mmol) was added (final concentration, 200 μM) and this was incubated for 5 min. After being washed 3 times with ice-cold PBS (−), the cells were dissolved in 0.1% SDS. The radioactivity of the cell lysate were measured by liquid scintillation counting.

Western-blot analysis. The differentiated myotube cells (at day 5) were incubated in serum-free DMEM for 3 h, and then 50 μM RSV and the stilbene analogs were added and this was incubated for a further 30 min. The cells, in 60 mm dishes, were washed with cold PBS(−) and harvested by cell scraping in 200 μL of lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% SDS, 10% glycerol, and 1 mM EDTA-2Na) supplemented with protease and phosphatase inhibitors (20 mM NaF, 30 mM sodium pyrophosphate, 1 mM $NaCO_3$, 1 mM aprotinin, 10 mM leupeptin, 200 mM PMSF, and 200 mM DTT). The cell lysates were centrifuged at 15,000 rpm for 10 min to obtain the supernatants, and were stored at −80 °C until use. For Western-blot analysis, cell lysates containing 10 μg proteins were resuspended in sample buffer (500 mM Tris–HCl pH 6.8, 20% glycerol, 8% SDS, and 0.004% bromophenol blue supplemented with 20% 2-mercaptoethanol), and heated for 2 min at 95 °C. Samples were separated by 9% SDS–PAGE and transferred to a PVDF membrane, which was blocked with 2% non-fat skim milk in PBS-Tween 20 (0.1% Tween20 in PBS) and phosphate blocking buffer (Phospho BLOCKER™ Blocking Reagent, Cell Biolabs, San Diego, CA) in PBS-Tween 20 and incubated with the primary antibody in PBS-Tween 20. The bound primary antibody was detected with a goat anti-rabbit IgG HRP-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit polyclonal antibody against AMPK alpha and phospho-AMPK alpha (Thr 172) was purchased from Cell Signaling Technology (Beverly, MA). Target protein bands were detected by the ECL plus Western Blotting Detection System (GE Healthcare UK, Buckinghamshire, UK).

Table 1. Compositions of the Diets

Ingredient	Composition (%)			
	LF	HF	HF+RSV	HF+3(OH)ST
β-Starch	39.75	11.24	11.24	11.24
α-Starch	13.20	3.75	3.75	3.75
Casein	20.00	20.00	20.00	20.00
Sucrose	10.00	29.96	29.96	29.96
Cellulose	5.00	5.00	5.00	5.00
Mineral mix AIN-93G	3.50	3.50	3.50	3.50
Vitamin mix AIN-93G	1.00	1.00	1.00	1.00
L-Cystine	0.30	0.30	0.30	0.30
Choline hydrogen tartrate	0.25	0.25	0.25	0.25
t-Butylhydroquinone	0.0014	0.0050	0.0050	0.0050
Lard	7.00	25.00	25.00	25.00
Resveratrol			0.40	
3-Hydroxy-*trans*-stilbene				0.40

Animals and diets. Six-week-old female C57BL/6J mice were purchased from Tokyo Laboratory Animals Science (Tokyo). They were housed in rooms at a constant temperature of 22 ± 1 °C under a fixed 12-h light-dark cycle. After feeding of a CLEA Rodent Diet CE-2 (CLEA Japan, Tokyo) for 1 week, the mice were randomly divided into four groups (n = 6 per group) and fed the following diets: a lowfat (LF) diet (20% fat, 20% protein, and 60% carbohydrate); highfat (HF) diet (50% fat, 15% protein, and 35% carbohydrate); or HF with 0.4% 3(OH)ST. The compositions of the diets are shown in Table 1.

After 6 weeks of feeding, blood and tissues of the mice were collected. The weights of the liver, gastrocnemius, white adipose tissue (WAT) around the testis, and brown adipose tissue (BAT) were measured. All procedures were approved by the Animal Ethics Committee of Ochanomizu University.

Oral glucose tolerance test (OGTT). At the 5th week of the experiment, D-glucose (1.5 mg/g of body weight) was administrated to mice orally after overnight fasting. Blood samples were obtained from the tail at 0, 15, 30, 60, and 90 min after glucose administration. Blood glucose levels were measured with glucose analyzer Dexter Z II (Bayer, Tokyo).

Insulin tolerance test (ITT). At the 2 d after the OGTT, human insulin (0.40 mU/g the body weight) (Eli Lilly, Kobe, Japan) was injected into the mice intraperitoneally. Blood samples were obtained at 0, 15, 30, 60, and 90 min after insulin injection, and blood glucose levels were measured.

Measurement of glucose uptake into skeletal muscle. The soleus muscles of the mice were excised from both legs, and the tendons were tied by stainless-steel clips. These muscles were used for measurement of basal (without insulin) and insulin-stimulated glucose uptake respectively. To stabilize cell metabolism, the muscles were preincubated with continuous shaking at 37 °C in 4 mL of KRBB (Krebs-Ringer biocarbonate buffer at pH 7.4 containing 0.1%w/v BSA and 2 mM sodium pyruvate oxygenated with 95% O_2 and 5% CO_2) supplemented with 8 mM glucose and 32 mM mannitol for 30 min, and then incubated with or without 100 nM human insulin for 20 min. After washing with glucose free KRBB supplemented with 40 nM mannitol with or without insulin for 10 min, the muscles were incubated with KRBB supplemented with 1 mM [^{3}H]2-deoxyglucose and 39 mM [^{14}C]-mannitol with or without insulin for 15 min. Then they were washed in ice-cold PBS containing 0.1% BSA for 30 min, boiled in 1 mL of 1 N NaOH for 5 min, and neutralized with 60 μL of 5 N HCl. Radioactivity levels were measured by liquid scintillation counting. The amount of glucose uptake was determined by calculating specific glucose uptake by the ratio of [^{3}H]2-deoxyglucose to [^{14}C]-mannitol.

Statistical analysis. Results were expressed as mean ± SD. Data were analyzed by a one-way ANOVA, followed by Tukey's *post hoc* test for multiple comparisons. Significance was set at $p < 0.05$.

Results

Effects of RSV and stilbene analogs on the differentiation of adipocytes

RSV and its simple analogs, 3(OH)ST, and 4(OH)ST, were examined for their effects on fat accumulation during the differentiation of 3T3 pre-adipocytes by Oil Red O staining. Fat accumulation decreased markedly in the presence of RSV, 3(OH)ST, and 4(OH)ST as compared with control (Fig. 2A–D). GPDH activities, the differentiation marker of adipocytes, were decreased by RSV, 4(OH)ST, and 3(OH)ST compared with control in a dose-dependent manner (data not shown). Furthermore, the mRNA expression levels of adiponectin in the small adipocytes were significantly increased by RSV, 3(OH)ST, and 4(OH)ST compared with the control. The effect of 3(OH)ST was the strongest (Fig. 2E). Hence, we compared the effects of other stilbene analogs with different numbers and positions of the hydroxyl group, as shown in Fig. 1. As Fig. 3 shows the mRNA expression levels of PPARγ, the master regulator of the differentiation of pre-adipocytes, were decreased by RSV (79%), 3(OH)ST (60%), 4(OH)ST (71%), 3,4′(OH)$_2$ST (41%), 3,3′(OH)$_2$ST (57%), 3,5(OH)$_2$ST (44%), and iso-RSV (63%) as compared to control. All the stilbene analogs, but not RSV, decreased the mRNA expression levels of resistin, the initiation factor for insulin resistance (Fig. 3B). This showed a significant decrease in 3,4′(OH)$_2$ST (25%), 3,3′(OH)$_2$ST (48%), and 3,5(OH)$_2$ST (51%) as compared with control.

Effects of RSV and stilbene analogs on glucose uptake in C2C12 myotube cells

RSV and the stilbene analogs did not influence the viability of C2C12 cells in the dose range between 1 and 100 μM, as assessed by MTT assay (data not shown). Since it has been reported that glucose uptake was increased by 50 μM RSV,[17] 50 μM RSV and stilbene analogs were added to the cells after differentiation for 1 h, and then glucose uptake into the C2C12 myotubes was measured. 3(OH)ST, 3,4′(OH)$_2$ST, and isoRSV significantly increased the amount of glucose uptake into the C2C12 myotubes, to 153%, 160%, and 147% respectively (Fig. 4A). Although neither RSV nor any of the stilbene analogs changed the ratio of the phosphorylated form of Akt (data not shown), they all

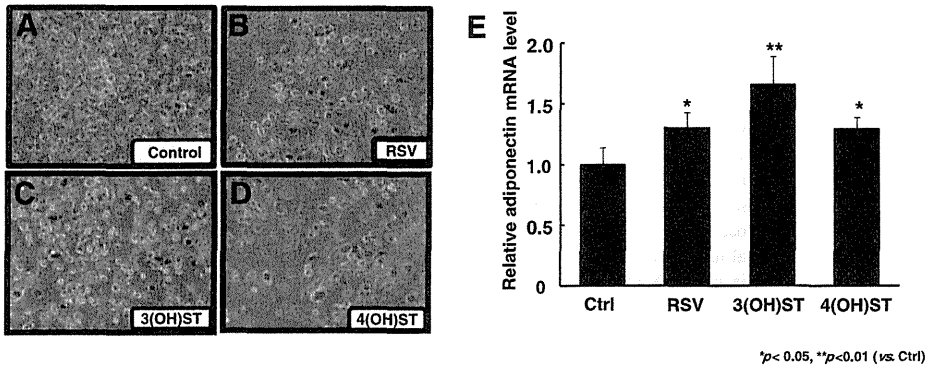

Fig. 2. Effects of RSV and the Stilbene Analogs on the Differentiation of 3T3-L1 Adipocytes.
3T3-L1 adipocytes were stained by Oil red O without treatment control (A), with 2.5 μM RSV (B), 2.5 μM 3(OH)ST (C), and by treatment with 2.5 μM 4(OH)ST (D). The levels of adiponectin mRNA were measured (E). Values were expressed mean ± SD (n = 3). Data were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test for multiple comparisons. Significant difference was expressed *vs.* control. $^{*}p < 0.05$; $p < 0.01$.

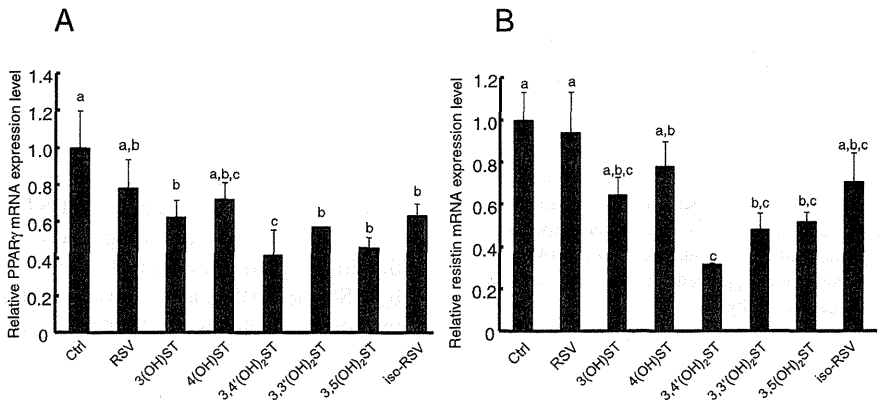

Fig. 3. Effects of RSV and the Silbene Analogs on 3T3-L1 Adipocytes.
Adipocytes treated with RSV or with stilbene analogs were collected at day 8, and total RNA was extracted. The mRNA levels of PPARγ (A) and resistin (B) were measured by quantitive RT-PCR. Data analyzed by one-way ANOVA, followed by Tukey's *post hoc* test for multiple comparisons. Different superscripts by column indicate significant differences ($p < 0.05$).

induced AMPK phospholylation significantly (Fig. 4C). Especially, the extent of AMPK activation by 3(OH)ST (196%), 3,4′(OH)$_2$ST (233%), and 3,3′(OH)$_2$ST (226%) was greater than AICAR (192%), the activator of AMPK. We also evaluated AMPK activation in the presence and the absence of nicotine amide (NA), an inhibitor of Sirt1 (Fig. 4D). All the stilbene analogs activated the phospholylation of AMPK without NA, but, activation by 3(OH)ST and 3,3′(OH)$_2$ST was suppressed in the presence of NA, while that of RSV and 3,4′(OH)$_2$ST did not change. These results suggest that both 3(OH)ST and 3,3′(OH)$_2$ST activated AMPK *via* Sirt1 activation, and that the process of activation by RSV and 3,4′(OH)$_2$ST, which holds a 4′ position of OH, was independent of Sirt1.

Prevention of HF diet-induced insulin resistance by RSV and 3(OH)ST

To investigate the effect of RSV and the involvement of its chemical structure, we compare the effect of 3(OH)ST, the simplest analog RSV, with RSV on HF diet-induced insulin resistance *in vivo*. We fed C57BL/6J mice, six mice in each group, the diet shown in Table 1. As shown in Fig. 5A, the HF diet mice (HF group) had a higher mean body weight at the end of the experimental period, as indicated by a 20% increase as compared to the LF diet mice (LF group). The body weight of the RSV-fed mice (RSV group) decreased to the same level as the LF group, and the addition of 3OH to the HF diet decreased in 73% from HF. There was no difference in energy intake among experimental groups (data not shown). No abnormalities were observed in the appearances of the organs. The values for aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glutathione *S*-transferase (GST) were not changed by 3(OH)ST treatment (data not shown). The weight of WAT around the testis in the HF group was 1.7 fold higher than in the LF group, but the addition of RSV to the HF diet decreased the WAT weight to the level for the LF group. Furthermore, 3(OH)ST treatment reduced the WAT weight to half of the LF groups (Fig. 5B).

We tried OGTT and ITT after 5 weeks of dietary intervention. The addition of 3(OH)ST to the HF diet improved glucose intolerance in the mice fed the HF diet alone to an extent similar to LF diet mice, but RSV failed to reproduce this (Fig. 5C and D). The HF group also showed aggravation of insulin resistance as compared the LF group, but both RSV and 3-OH improved insulin tolerance to the LF group. We further examined glucose uptake into isolated skeletal muscle with and without insulin induction (Fig. 5E). The basal levels of

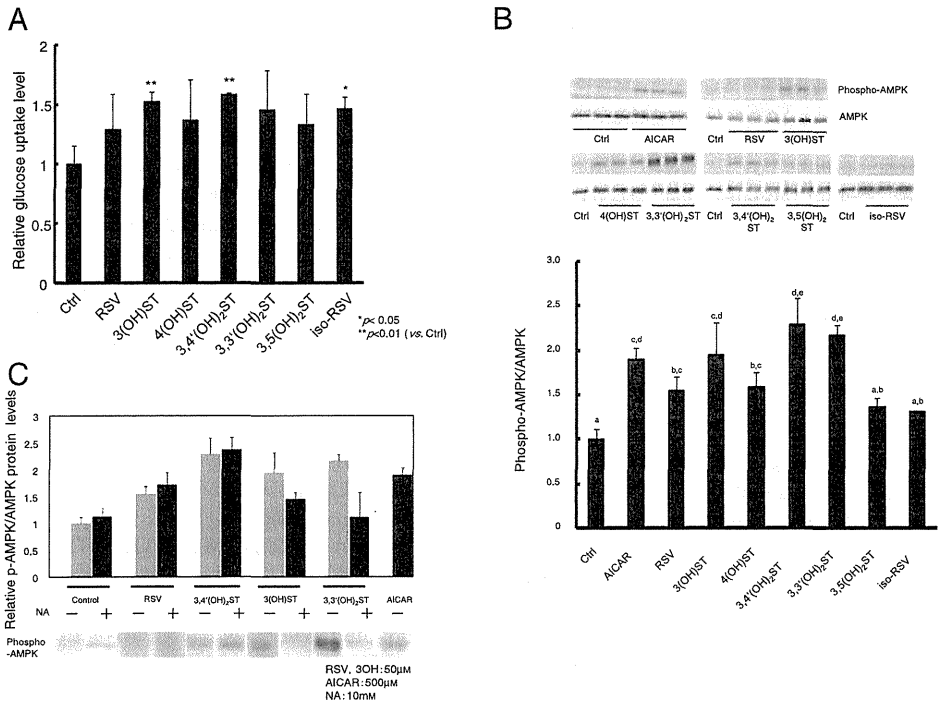

Fig. 4. Effects of RSV and the Stilbene Analgs on C2C12 Myotube Cells.

The incorporation of [^{3}H]2-deoxyglucose into differentiated C2C12 myotube cell were measured as described in "Materials and Methods." Values are expressed as uptake ratio relative to control (A). Western-blot analysis was used and the measure the phospholylation of AMPK (B). AMPK activation in the presence and the absence of 10 mM NA. One of the three individual images of Western-blotting is shown (C). All values were expressed mean ± SD (n = 3). Data were analyzed by a one-way ANOVA. Significant difference was expressed *vs.* control. $p < 0.05$; $p < 0.01$. Different superscripts by column indicate significant differences ($p < 0.05$).

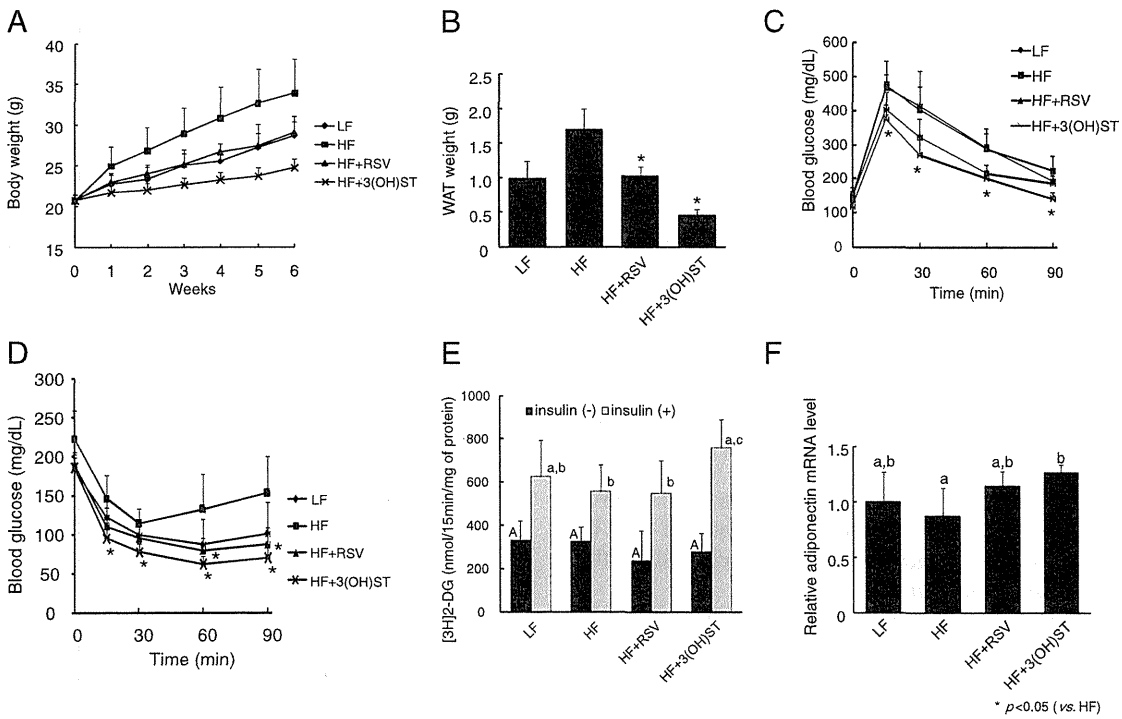

Fig. 5. Effects of RSV and 3(OH)ST on Glucose Tolerance and Insulin Resistance in HF Induced Obese Mice.

C57BL/6J mice were fed a high-fat diet with 0.4% RSV or 3(OH)ST for 6 weeks. Changes in body weight during the experimental period (A). Weights of WAT around the testis (B). Changes in blood glucose levels during OGTT (C) and ITT (D). Incorporation of [^{3}H]2-deoxyglucose into the soleus muscle (E). Levels of adiponectin mRNA in adipose tissue (F). Values were expressed mean ± SD (n = 6). Data were analyzed by one-way ANOVA. Significant difference was expressed *vs.* control. $p < 0.05$; $p < 0.01$. Different superscripts by column indicate significant differences ($p < 0.05$).

glucose uptake without induction of insulin in all experimental groups were similar. As a result of insulin induction, the HF diet increased glucose uptake by 1.5 fold, while LF increased it twice. Addition of RSV to the HF diet recovered to an extent similar to LF. 3(OH)ST increased glucose uptake by 2.6-fold after insulin induction. These results indicate that 3(OH)ST was effective in improving insulin resistance in the skeletal muscle. As shown in Fig. 5F, adiponectin mRNA levels were suppressed by HF (89%) as compared with LF, and increased by RSV (105%) and 3(OH)ST (127%).

Discussion

To determine how the position of hydroxyl groups on stilbene molecules influences their biochemical role in fat metabolism, we investigated the *in vitro* and *in vivo* effects of several stilbene molecules. We found that even the simplest analogs were more effective than RSV itself in preventing obesity and insulin resistance.

RSV is known to suppress body weight gain induced by a high-calorie diet in rats[10,18] and to reduce the differentiation of 3T3-L1 pre-adipocytes into adipocytes in 3T3-L1 pre-adipocytes. RSV and 3(OH)ST reduced the body weight gain as compared with HF diet. 3(OH)ST 0.4% (w/w) was much more effective, by 89%, at reducing fat accumulation than RSV at the same concentration. We found a similar effect using lower concentrations, (0.1 and 0.2% w/w) of 3(OH)ST (data not shown), while RSV as well as 3(OH)ST and 4(OH)ST suppressed the differentiation of 3T3-L1 preadipocytes and the mRNA expression levels of PPARγ (Fig. 3A). The other analogs tested, resistin and adiponetin, also protected the adipocytes against obesity-induced disorders (Fig. 3B). Based on these data, we speculate that 3,4′(OH)$_2$ST, 3,3′(OH)$_2$ST, and 3,5′(OH)$_2$ST are more effective than RSV *in vivo*.

Skeletal muscle is the main target tissue of insulin. It maintains blood glucose levels. In C2C12 myotubes, 3(OH)ST, 3,4′(OH)$_2$ST, and isoRSV increased the amounts of the glucose uptake (Fig. 4A). This is consistent with the results obtained *in vivo*. RSV and 3(OH)ST did not change glucose uptake by the soleus muscle without insulin, but increased it in the presence of insulin (Fig. 5E). Hence, we conclude that the increase in glucose uptake induced by RSV and 3(OH)ST in the presence of insulin improves insulin sensitivity and reduces obesity in the whole body. Moreover, in that RSV and 3(OH)ST activated AMPK and consequently promoted glucose uptake in C2C12 cells, they might also act on skeletal muscle cells in the insulin-resistant state *in vivo*.

Glucose uptake is generally promoted downstream of the insulin signal by translocation of GLUT4 subsequent to Akt phosphorylation.[19] GLUT4 translocation is also mediated by AMPK phosphorylation.[20,21] It has been reported that RSV promotes glucose uptake in L6 and C2C12 myotube cells.[17,22] Activation of AMPK promotes glucose uptake into skeletal muscle.[23,24] RSV and the stilbene analogs activated the phosphorylation of AMPK but not of Akt, consistently with the results of Breen *et al.*, who found that activation of Akt did not mediate the stimulation of glucose uptake.[17] On the other hand, Deng *et al.* reported that phosphorylation of Akt *via* activation of estrogen receptor α promoted glucose uptake, suggesting that the estrogen receptor is an important determinant of glucose uptake by RSV.[25] Since RSV has a structure similar to estradiol, RSV and stilbene analogs might function as phytoestrogens. Phenol red, which binds to the estrogen receptor, is present in the DMEM we used in our study, so it might compete with RSV and the stilbene analogs to inhibit the Akt signal. Thereafter, we confirmed that Akt phosphorylation in phenol red free DMEM was not activated (data not shown). These results suggest that RSV and the stilbene analogs improved insulin sensitivity enhancing glucose uptake in skeletal muscle cells through direct activation of AMPK, but not Akt.

The process of AMPK activation appears different depending on whether the stilbene backbone has the hydroxyl group at the 4th position. As shown in Fig. 4F, NA, an inhibitor of Sirt1, failed to suppress AMPK activation by RSV and 3,4′(OH)ST, suggesting that RSV and 3,4′(OH)$_2$ST did not depend on Sirt1 activation to activate AMPK, whereas 3(OH)ST and 3,3′(OH)$_2$ST activated AMPK *via* Sirt1 activation.

RSV activates AMPK in several ways. Like other polyphenols, RSV reduces ATP levels by inhibiting ATP synthase,[26] and Dasgupta *et al.* found that RSV activated AMPK through a Sirt1-independent pathway without altering the AMP-to-ATP ratio.[27] A recent study found that competitive inhibition of cAMP-degrading phosphodiesterases induced by RSV led to elevated cAMP levels, increased intracellular Ca^{2+} levels, and activates the CamKKβ-AMPK pathway. Thus, it appears that RSV increases NAD^+ and the activity of Sirt1.[28] Since we did not measure the activity of Sirt1 directly, we cannot speculate as to how these analogs act on AMPK.

Adiponectin is secreted specifically from adipose tissue, and it is known that younger, smaller adipocytes secrete more adiponectin than mature ones.[29] Obesity and/or a high-fat diet suppresses the secretion of adiponectin.[30] Adiponectin increases the expression of PPARγ, a master regulator of the differentiation of adipocytes,[31] and improves insulin resistance through the activation of AMPK.[17,31,32] Based on these reports, we suggest that one function of 3(OH)ST *in vivo* is increased secretion of adiponectin which activates AMPK in the skeletal muscle by suppression of pre-adipocytes differentiation.

Lagouge *et al.* have reported that RSV reduced fat accumulation in the whole body as a result of an increase in the basal metabolic rate and thermogenesis.[4] RSV activity was associated with the induction of genes related to oxidative phosphorylation and mitochondrial biogenesis, and was largely explained by an RSV-mediated decrease in PGC-1α acetylation and an increase in PGC-1α activity. In addition, AMPK directly induced the phosphorylation of PGC-1α.[24] In a study using AMPK knockout mice, RSV failed to reduce fat mass in mice fed a HF diet, or to increase insulin sensitivity, mitochondrial biogenesis or physical endurance, suggesting that AMPK was the central target of the metabolic effects of RSV.[25] In our study, the weights of BAT were decreased by RSV and 3(OH)ST treatment (data not shown), but we did not examine UCP1 expression in BAT. Mitochondrial function and thermo-

genesis are the subject of further investigation in our laboratory.

In conclusion, the 3′-hydroxy group of RSV was important to the protective effect of RSV against obesity-induced disorders, such as inhibition of adipocyte differentiation and activation of AMPK, but the 4′ hydroxyl group of RSV, which associated with strong antioxidant and genotoxic activity, was not essential. These results provide a basis for a safe drug design without loss of the therapeutic potential of RSV.

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細胞・組織加工製品の開発環境整備に向けたレギュラトリーサイエンス研究

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Regulatory science research to facilitate the development of cell/tissue-processed products

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Regulatory science research to facilitate the development of cell/tissue-processed products

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Regenerative medicine is regarded as innovative therapy for severe diseases and damages caused by tissue loss and functional impairment. In Japan, regenerative medicine is one of the most important subjects issued by Council for Science and Technology Policy and also referred to in Medical Innovation of New Growth Strategy. Cell/tissue-processed products are living cells, which have been manipulated or processed for the purpose of regenerative medicine, and are extensively developing. Human somatic cells, somatic stem cells, embryonic stem cells, and induced pluripotent stem cells are cell sources used for regenerative medicine. Since we lack in experiences with cell/tissue-processed products, technical development of safety and quality assessment is urgently needed. National Institute of Health Sciences has carried out a mission of Regulatory Science and worked on safety assessment of pharmaceuticals and medical devices and their guideline development. The objective of our study is to develop safety and quality assessment methods for cell/tissue-processed products derived from stem cells, based on recent progresses in life science. We are currently developing methods to evaluate products as follows; a) useful and quantitative tumorigenicity tests to detect contamination of undifferentiated and/or abnormal cells in products, b) quality assessment by gene expression analysis and detection of genetic stability in a manufacturing process, and c) analysis of quality attributes associated with propensity of undifferentiated cells to set acceptable criteria of cell banks. We will be able to provide indicators to control the quality, efficacy and safety of stem cell-processed products and support efficient and economical promotion of the products. Especially, this study would help translate stem cell science into therapeutic products to patients with severe and life-threatening diseases, consequently contributing to administrative policy of Ministry of Health, Labor and Welfare.

Keywords: cell/tissue-processed products, induced pluripotent stem (iPS) cells, regenerative medicine

研究目的

再生医療は，身体の一部の機能不全や欠損による重篤な疾患や障害を治療できる革新的な方法として注目されており，総合科学技術会議の提言や「新成長戦略」のメディカルイノベーションなどにおいても最重要課題とされている．平成25年１月11日閣議決定の『日本経済再生に向けた緊急経済対策』でも，iPS細胞等を用いた再生医療等に係る研究開発・実用化を支援する環境整備に取り組むことが明記されている．平成25年２月には再生医療等の新規医療産業の国際競争力を高める司令塔機能として，内閣官房に『健康・医療戦略室』が設置された．また，平成25年４月26日成立の『再生医療推進法』には，再生医療の迅速かつ安全な研究開発及び提供並びに普及の促進に関する施策を総合的に策定及び実施する責務を国が有することが示されている．

再生医療（や細胞治療）に使用することを目的に生きた細胞を加工して製造される製品は細胞・組織加工製品と呼ばれ，国内外で活発に研究・開発が行われている．

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細胞ソースとしてはヒト体細胞に加え，近年ではヒト体性幹細胞，胚性幹細胞（ES細胞）などの幹細胞が対象とされてきている．また最近，生命倫理的な問題や免疫学的な拒絶をクリアできると考えられる人工多能性幹細胞（iPS細胞）が登場し，再生医療が社会的に大きな期待を集めている．しかしながら細胞・組織加工製品は，臨床使用経験が少ないために知見の蓄積も乏しく，国内指針やICH，WHOなどの生物製剤製造国際ガイドライン等にある従来の品質・安全性評価法が役立たないケースが頻出しており，新たに適切な評価技術を樹立することが火急の課題となっている．本研究では，新たなガイドライン作成に資する細胞・組織加工製品，特に幹細胞加工製品の品質・安全性評価法の開発を行うことを最終目的とする．具体的には，幹細胞の加工過程における未分化細胞／異常細胞の混入は，幹細胞加工製品においてがん化を引き起こすとして最も懸念される．最終製品に含まれるこれらの細胞の高感度かつ定量的な測定方法は開発が遅れている．そこで，本研究では汎用性・定量性のある幹細胞加工製品の造腫瘍性試験法の開発を目指した実験研究を展開する．また細胞の培養・加工過程での細胞の形質安定性も考慮に入れる必要があることから，培養工程での遺伝子発現の動態解析による品質評価法および遺伝子安定性評価法の開発に関する研究を行う．さらに安全性上の懸念として，ヒト多能性幹細胞株間での各種目的細胞への分化のし易さ（分化プロペンシティ）のバラツキがある．原材料の細胞の分化プロペンシティの情報は細胞株の選択に必要不可欠であり，その評価系は最終製品を見据えた細胞バンクの構築に必要である．未分化細胞において分化プロペンシティの評価系を含んだ細胞特性解析法を確立することにより，幹細胞由来加工製品の品質の一定性・有効性・安全性のさらなる確保に繋がることが期待される．

行政への貢献

本研究の成果により幹細胞加工製品の有効性・安全性に関する品質評価に必要な指標・試験法が示され，製品の迅速かつ経済的な開発を推進することが可能になることにより，特に治療困難な重篤な疾患に対して期待の大きい再生医療・細胞治療が実用化され普及することに貢献できる．国民に安全かつ有用性の高い再生医療・細胞治療をいち早く提供するという厚生労働行政の施策に大きく寄与するものと考えられる．具体的には，以下のことが挙げられる．1）幹細胞加工製品の合理的な製法，工程管理に必要な要件，品質評価方法の確立に貢献できる．2）均一性・再現性を確保するための原材料（幹細胞株／バンク）のあり方において，より合理的な規格の設定が可能となり，適切な開発が推進される．3）生命科学の進歩に見合ったガイドラインや基準の策定及び改訂に役に立つ．4）幹細胞加工製品の科学的規制に関する国際調和に貢献できる．5）幹細胞加工製品の先進性，有用性に関する理解が深められる．

研究の進捗状況

平成24年度の研究としては，1．「汎用性・定量性のある幹細胞加工製品の造腫瘍性試験法の開発を目指した実験研究」として，新規免疫不全動物NOGへアレスマウスを用いた造腫瘍性試験法の開発にむけた動物コロニーの拡大，および試験方法の条件検討を行った．2．「培養工程での遺伝子発現の動態解析に基づく品質・安全性評価指標の開発に関する研究」として，幹細胞の*in vitro*培養工程における遺伝子発現の動態解析による品質評価技術の開発を行い，ヒト骨髄由来間葉系幹細胞のがん化と相関すると予想される遺伝子に関する機能解析を行った．3．「製造工程における遺伝子安定性評価法の開発に関する研究」として次世代シークエンサーを用いた遺伝子変異解析の性能評価を行った．また，4．「幹細胞（未分化細胞）における分化プロペンシティの評価系の開発に関する研究」として，ヒト多能性幹細胞の分化プロペンシティを予測するための指標の同定の基盤となるデータの収集を行った．

1．新規免疫不全動物を用いた造腫瘍性試験法の開発

我々は，重度免疫不全動物であるNOGマウスとヌードマウスにおけるHeLa細胞単独，あるいはマトリゲルとの混合物のTPD_{50}（投与した動物の半数で腫瘍を形成するために必要な細胞数）を検討し，マトリゲルに検体細胞を懸濁してNOGマウスに投与することにより，ヌードマウスを用いた従来の国際ガイドラインにある方法より数千倍高感度で腫瘍細胞を検出することが可能であることを示すデータを得ている．実験動物中央研究所では，このNOGマウスをヘアレス化した系統を樹立しており，NOGマウスと同様の条件において造腫瘍性細胞の検出能力の検討を行った（その際の動物は，体外受精させた胚を仮親に移植して大量生産する手法を用いて作出した80匹以上の同一週齢雄動物を用いた）．雄NOGヘアレスマウスにおけるHeLa細胞のTPD_{50}は，ヌードマウスの1/11（$3.7\times10^4/4.2\times10^5$），マトリゲルを混合した場合は1/2000（$2.1\times10^2/4.2\times10^5$）であり，NOGマウスにおけるそれらよりも高値であった．これは，導入したヘアレス遺伝子がBalb/cマウス由来であり，免疫不全度が僅かながら低下したことによると推測された．NOGヘアレスマウスはNOGマウスと比較し，目視・触診による腫瘍形成の検出が容易であり，造腫瘍性試験の効率化が期待される．

2．幹細胞の*in vitro*培養工程における遺伝子発現の動態解析による品質評価技術の開発

ヒト骨髄由来間葉系幹細胞（hMSC）の*in vitro*培養時の遺伝子発現変化を網羅的に解析し，Ewing肉腫４種類（Hs822.T, Hs863.T, RD-ES, SK-ES-1）を陽性対照として比較検討することにより，細胞のがん化の指標となり得る候補遺伝子として，これまでにCyclin D2, IGF2BP1など９遺伝子を見出している．そこで平成24年度は，hMSCへのCyclin D2，IGF２BP１の過剰発現によるhMSCの増殖能，老化及び染色体数等への影響について検討し，さらにhMSCの遺伝子発現パターンの変化について網羅的に解析した．その結果，Cyclin D2の強制発現によってhMSCの増殖が亢進され，遺伝子発現の網羅的解析により細胞増殖などに関わる遺伝子発現の有意な変化が認められた．Cyclin D2はhMSCでは細胞増殖に正に関与し，さらに細胞老化を遅らせることによって増殖の亢進に寄与した可能性も示された．遺伝子発現の網羅的解析でhMSCと発現パターンが似ていたHs822.T及びHs863.Tはほぼ正常な染色体数だったのに対し，RD-ES及びSK-ES-1はどちらも染色体数が大きく変化し，hMSCの遺伝子発現パターンとの類似性と同様の傾向が見られた．

3．次世代シークエンサーを用いた細胞の遺伝的安定性評価指標の開発

細胞の遺伝的安定性評価を目的としたホールゲノムシークエンスおよびエクソンシークエンスから得られるデータの品質評価および遺伝子変異検出の効率に関して，モデル細胞を用いた実データから検討を行った．その結果，点突然変異および小さな増幅，欠失変異に対する検出率および正確性の高さが確認できた．一方，大きな欠失，増幅ならびに転座などの複雑なリアレンジメントに関しては，通常の解析では検出が難しいことがわかり，これらに特化したデータ解析手法の検討が必要であることがわかった．

4．分化プロペンシティを指標とした細胞特性解析法の開発

無フィーダー培養した未分化状態のヒトiPS細胞９株において網羅的なmRNA発現情報をジーンチップにより取得した．さらに細胞特性評価に用いる候補遺伝子の今後の絞り込みのため，iPS細胞株間で統計学的に有意に発現量の異なる遺伝子群を抽出した．ジーンチップデータを取得したヒトiPS細胞株から低吸着ディッシュ上で胚葉体を形成させRNAの抽出の後に，三胚葉マーカー遺伝子発現を定量的RT-PCRで測定し，各々の細胞株の分化プロペンシティを主成分分析により数値化した．その結果，第１主成分得点は中胚葉分化の正の指標および初期外胚葉分化の負の指標となることが示された．

研究の将来展望

これらの成果を更に展開することにより細胞・組織加

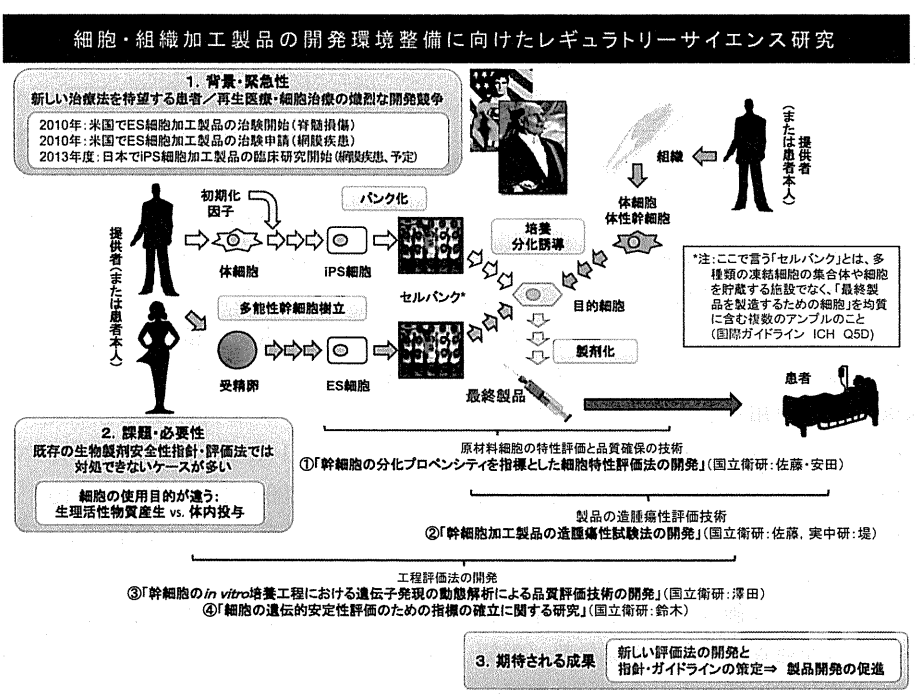

図　研究の背景・緊急性，課題・必要性および期待される成果

工製品の有効性・安全性に関する品質評価に必要な指標・評価法が示され，迅速で適切な製品開発・審査および再生医療の実用化推進に貢献できると考えられる．

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Meeting report

"Scientific Considerations Regarding Radiation Risk" JEMS Open Symposium 2012

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The health effects of low-dose radiation have generated considerable concern after the accident at the Fukushima Daiichi Nuclear Plant. Although the risk of acute direct exposure to high-dose radiation could be avoided, the risk remains for low-level continuous exposure to radiation by long-lived environmental contaminants, such as cesium-137 that is released from the nuclear plant. Scientists have engaged in a contentious debate regarding the actual risk of low-dose radiation. To understand the actual risk of radiation scientifically, the Open Symposium of Japanese Environmental Mutagen Society (JEMS) was held on May 26, 2012 at Keio University in Tokyo. Eight scientists and a special guest from Fukushima were invited to participate in this symposium. We understand that it is difficult to draw a proper conclusion scientifically concerning the actual (absolute) risk of low-dose and low-dose rate radiation from the available data. The risk of radiation exposure can only be estimated in a relative manner if we compare the risk to other confounding risk factors, such as smoking. Being unafraid and controlling risk factors in our lifestyle are important in helping us to cope with the inevitable exposure to low-dose radiation that was caused by the Fukushima accident. It is critical to communicate and to advise people in the nearby environment regarding their risk of radiation exposure and the need to make a rational decision to avoid undue exposure and excess risk concerning radiation emerging from the accident site.

Key words: radiation, risk assessment, nuclear accident, food safety, regulatory science

Introduction

On March 11, 2011, an enormous unexpected tsunami pummeled the northeastern coast of mainland Japan (Tohoku area) after a 9.0 magnitude earthquake. The earthquake itself was not as lethal as the huge tsunami, which killed as many as nineteen thousands people. The tsunami also caused a serious disaster in the Fukushima area by damaging the Fukushima Daiichi Nuclear Plant. Although the nuclear plant was resistant to the earthquake, it was not prepared to withstand the forceful tsunami. Tremendous and powerful waves rolled over the protective wall and penetrated the reactors. Unfortunately, all of the electric power supply had been lost, including the emergency back-up system because the system was not properly designed and situated. The continuous loss of electricity caused the cooling system for the nuclear reactors to cease functioning; the used nuclear fuel pools caused the overheating of these facilities. The plant faced a serious crisis in which the overheating induced a hydrogen gas explosion in multiple reactors, which incurred devastating damage. Consequently, several days after the tsunami, a large amount of radioactive substances, including volatile radioactive iodine and cesium or radioactive water, were released into the surrounding environment. Most of the radioactive substances were released toward the ocean side, but occasional winds from the ocean carried the radioactive substances over the surrounding inland area and caused serious radioactive contamination in the Fukushima area.

In a realistic worst-case scenario, people in the surrounding area were exposed to life-threatening effects caused by the nuclear explosion of the reactor. The Japanese government ordered the residents to evacuate a 20-km area from the nuclear plant. Continuous efforts to cool the reactor and the pool averted additional disaster; however, radioactive contamination from the site, which has remained a central issue, continues to threaten many people in the form of an invisible radiation fear.

From our experiences with the atomic bombs in Hiroshima and Nagasaki and the Chernobyl accident, we know that radiation causes serious health problems in humans. The direct exposure to high-dose radiation has killed many people. We do know about the approximate lethal dose of acute radiation exposure (7 Sv);

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however, we do not have sufficient data to evaluate a risk of low dose/low-dose rate exposure of radiation. In the Chernobyl incident, only radioiodide exposure increased the incidence of thyroid tumor in the young generation by an indirect internal exposure, primarily through contaminated foods. Statistical analysis has shown that the incidence of tumor increased over 100-mSv exposure after the nuclear bomb. Therefore, we do not have a real answer for the health effect by the low-level radiation. After the Fukushima accident, there has been debate between optimistic and pessimistic scientists regarding the actual risk of radiation, which confused residents in the affected area. It may be easy to measure the actual amount of radiation exposure; however, we cannot estimate our risk of exposure.

Radiation is an environmental mutagen; therefore, the JEMS should accept the responsibility of addressing this vital issue. In an initial effort to provide useful information regarding the risk of radiation, we constructed the web site in JEMS HP to provide data on the risk of radiation (http://www.j-ems.org/ray/). Additionally, the radiation risk issue was selected to be the primary theme of the annual JEMS symposium in 2012 to "scientifically" discuss the radiation risk and disseminate the pertinent data.

Eight speakers were invited to present their scientific points of view, and a special guest from the Fukushima area was invited to give a talk from her perspective as both a mother and a teacher.

The primary objective of this symposium was to understand the level of radioactive contamination and to estimate the health risk based on scientific data and, more importantly, to direct a proper approach toward considering effective management of radiation risk.

"It is easy to be scared too much or too less, but it is difficult to be afraid properly."

Torahiko Terada

Opening Address

Hiroshi Kasai, the President of JEMS, delivered the opening remarks and defined the important role that the Society plays in investigating and promoting research on radiation, which is one of the environmental mutagens. From his expert view on the 8-hydroxy guanine, he introduced the contribution of the JEMS to radiation biology and the role of active oxygen species produced by radiation in mutagenesis. He said, "This symposium can be a good opportunity to re-consider a risk of radiation after one year of struggling debate. In addition, this is also a good opportunity for JEMS to show its relevance as a responsible researchers group who can make a scientific judgment on the risk of radiation in relation to those of other mutagens".

Session 1 (Chaired by Hiroshi Kasai, University of Occupational and Environmental Health)

Introduction

The introductory presentation was made by Takayoshi Suzuki (National Institute of Health Sciences), organizer of this symposium and a leader of the working group on radiation risk in JEMS. In the presentation titled "We Have Already Been Exposed to Radiation", he announced the establishment of the new website concerning information on radiation risk at the JEMS site (http://www.j-ems.org/ray/). The symposium was organized by this working group to disseminate information on radiation risk and provide a forum for the scientific discussion of this subject to gain a better understanding of the actual risk of the current Fukushima disaster. In this issue of Genes and Environment, his opinion is described in the commentary paper titled "Unconscious Exposure to Radiation". We must recall an important evidence: We have already been exposed to a much higher nuclear fallout during the 1960s because of the worldwide nuclear bomb experiments; additionally, we are being exposed to potassium-40 radiation derived from our daily food intake. When people think about radiation risk, it is advisable to consider this important evidence before making a rational judgment.

Special Lecture

Yasushi Yamazoe (Food Safety Commissions), the former president of JEMS, presented a special lecture on risk assessment and radioactive nuclides in food. As chairperson of the working group in the Food Safty Commisions, which was established just after the Fukushima nuclear accident, Dr. Yamazoe was able to assess whether radioactive nuclides in food can impact one's health. The risk-assessment report on radioactive nuclides in food was released after an extensive survey of available data and a series of discussions in the working group. An abstract of this report is available in English at (http://www.fsc.go.jp/english/emerg/abstract_risk_assessment_report.pdf). A complete document in Japanese is available at (http://www.fsc.go.jp/sonota/emerg/radio_hyoka_detail.pdf).

Upon a large release of radioactive materials into the environment from the Fukushima Daiichi Nuclear Plant, the Japanese government requested that the Food Safety Commissions conduct an assessment of the situation. An emergency report was quickly released in March 2011 using available information for the evaluation; a continuous effort was made to collect and review 3300 manuscripts to finalize a report that was released in October 2011. The report was evaluated from the following points of view that are described below.

An important principle for evaluation was dealing with both the internal and external exposure to deter-

mine the standard limit of exposure because the majority of available data were based on a mixed exposure. The radioactive nuclides of concern were iodide, cesium, uranium, plutonium, americium, curium, and strontium. Epidemiological data based on previous experiences were considered to be more important than animal experiments.

The biological effect of radiation was categorized into the stochastic (without threshold) and the deterministic (with threshold) effects. It was difficult to judge the existence of threshold for the low-level radiation effect based on the current scientific knowledge; therefore, an argument on threshold was avoided. It was important to evaluate the exact level of exposure; in most cases, exposure should be calculated as long-term exposure (both internal and external).

In addition to radiation exposure, confounding factors, such as smoking, made the evaluation difficult. Among many reports, the most reliable data were derived from the atomic bomb exposure in Hiroshima and Nagasaki (1,2). The lowest effective dose of radiation that can increase the incidence of cancer in Hiroshima and Nagasaki was reportedly 200 mSv. Preston *et al.* (1) reported a significant increase of total tumor deaths at 125 mSv but no effect below 100 mSv. Based on these data, the working group set the limit for the level of radiation exposure in a person's lifetime as 100 mSV, although no effect was reported by a lifetime exposure at 500 mSv in the high background area in India.

At the end of his talk, Dr. Yamazoe gave an important message that "exposure to 100 mSV does not directly mean an increase of cancer incidence and an overall risk for carcinogenesis can be reduced by controlling other confounding factors in life style".

Session 2 (Chaired by Kazuo Fujikawa, Kinki University)

Nori Nakamura presented data on "Lessons Learned from Atomic Bomb Survivors in Hiroshima/Nagasaki." from the research experience at the Radiation Effects Research Foundation in Hiroshima and Nagasaki. His talk focused on the following points:

- An epidemiological research on approximately 120,000 atomic bomb survivors revealed an early onset of leukemia after the exposure; however, it took more than 20 years to observe an increase in the incidence of solid tumors.
- The relative risk for leukemia increased approximately 5 times after radioactive exposure at 1 Gy.
- In contrast, a relative risk of solid cancers was approximately 1.5 at 1 Gy (1) and the dose response was linear. However, the linearity at the lower dose (<200 mSv) was not clear.
- There is no relationship between increased cancer risks by radiation and incidence of spontaneous cancer of the organ.
- One of the approaches to evaluate the risk of low-dose radiation takes a so-called "linear non-threshold (LNT) model," which extrapolates a linear relationship into lower dose range. This type of safety-margin approach does not underestimate the possible risk.
- There is no evidence of heritable genetic effects in the offspring of the survivors.

In conclusion, Dr. Nakamura proposed a desired operation after the accident as follows: "Rather than draw a line of evacuation zone on a map, the Government could provide information to the residents on their possible exposure levels and the estimated risks, and let them choose either stay at their home or evacuate. And the maximum support had to be given to their decision.

Jun Takada (Sapporo Medical University), a specialist of radiation protection and hygiene, evaluated individual dose on affected populations and surveyed environments from Sapporo to Tokyo, including the entrance gate of the Fukushima Daiichi Nuclear Plant soon after the accident (April 6–10, 2011). He also performed a recent survey in Namie-cho, which is located within 20 km of the nuclear plant. He reported the results of these radiation hygiene surveys in relation to the Chernobyl accident. Detailed data, as well as his opinions, appeared in the following article, titled "Low Dose and No Health Risk in Fukushima in Contrast to Chernobyl", in this issue. His critical view on the political operations in Fukushima after the accidents is also included.

Shizuyo Sutou (Shujitsu University) participated in volunteer work in the radiation monitoring in Fukushima in July 2011, pursuant to a request from the Ministry of Education, Sports, Science and Technology to all Japanese universities. He presented his experience as a volunteer in Minamisouma, Fukushima, in addition to the survey results. He measured the radiation level in the residents who returned to their houses temporarily with permission. As a reference, he discussed measurements of radioactivity in other places. His report, "The Fukushima Daiichi Nuclear Power Plant Disaster: A Report on Volunteer Activity for Radioactivity Screening of Temporary Returnees to the Evacuation Zone," is available in this issue.

Session 3 (Chaired by Chie Furihata, Aoyama Gakuin University/National Institute of Health Sciences)

Takeshi Morita (National Institute of Health Sciences) discussed his paper, "Radiation Risk in Relation to Risk Evaluation of Chemicals." He explained the basic strategy for risk evaluation on chemicals from his expert experiences.

The risk is determined by the hazard level and the incidence (dose of exposure). Outputs from the risk evaluation of chemicals appear as the regulatory standard

values, such as the acceptable daily intake (ADI) and the acute reference dose (ARfD). In this process, important factors to be considered are quality of the data, extrapolation to humans, dose-response, and weight of evidence. ADI is calculated by the NOAEL (no observed adverse effect level) in animal experiments, which is multiplied by 1/100 as a safety margin for an extrapolation to humans. The virtual safety dose (VSD) is set for chemicals without the NOAEL (i.e., non-threshold response), such as genotoxic carcinogens. The carcinogenicity of radiation is also considered to be a non-threshold response.

Dr. Morita explained the risks of ethanol, NaCl, and arsenic in foods. People are exposed daily to those chemicals in food; however, they are not afraid of those risks as much as they fear risks of radiation, food additives, or pesticides. However, the former generally tend to bear a higher risk than the latter. Ethanol and NaCl have no regulatory standard values because they are categorized in food, but there is substantial evidence to indicate an association with carcinogenesis. The estimated ADI of ethanol from animal data will be 24 mg/kg, which corresponds to only 30 ml for adults (50-kg). For NaCl, it will be 20 mg/kg, which corresponds to 1 g for adults. An average daily intake of NaCl (10 g) exceeds the estimated ADI. Arsenic is toxic to multiple organs and is considered to be a genotoxic carcinogen; however, it is contained in many foods, such as drinking water, seaweed, and rice at relatively high levels.

MOE (margin of exposure) is calculated by toxicological values, such as NOAEL, divided by the exposure levels in humans. MOE is important for the risk control and communication. Tobacco smoking and diet reportedly correspond to 2/3 of the total risk factors for carcinogenesis and radiation contributes only 2% of the risk factors. The National Cancer Center reported that the carcinogenic risk factor of 100–200 mSv of radiation is estimated to be 1.08-fold, which is lower than smoking (1.6-fold).

Dr. Morita emphasized the importance of the risk trade-off. It happened after the Fukushima accident when people drank a bottle of mineral water, instead of tap water, to avoid the cesium intake. The high arsenic contents in the mineral water became a concern for carcinogenesis. Avoiding a certain risk produced another risk (trade-off).

Akihiro Shima (Institute for Environmental Sciences) presented his data from animal experiments titled "Considerations on the Risk of the Low Dose-Rate Radiation Based on Experimental Data in Mice." He focused on the biological effect of low dose-rate/low dose γ-ray in mice. This presentation is an interim report from the study with 4,000 mice at his institute. As a definition by UNSCEAR (United Nations Scientific Committee on the Effects of Atomic Radiation) (2010), the low-dose rate is <0.1 mGy/min and the low dose is <200 mGy. Dose-rates of 0.05 mGy/22 h/d, 1.1 mGy/22 h/d, and 21 mGy/22 h/d for 400 days were used; the total dose went up to 20 mGy, 400 mGy, and 8,000 mGy. A total of 500 male and female B6C3F1 mice per group had been exposed to cesium-137 radiation. A statistically significant shortening of the lifespan was observed at 21 mGy/day in males and 1.1 mGy/day and 21 mGy/day in females. An increased incidence of hemangiosarcoma and myeloid leukemia in males and ovarian neoplasm in females were observed (3). However, the lifetime shortening in mice continuously exposed to a low-dose-rate γ-ray is considered to be attributed to early death from a variety of neoplasms (4).

No change by the lower-dose radiation was observed, which suggested that the effect is too small to be detected even by this size of experiment. Brenner *et al.* (5) suggested that the cohort size required to detect statistically significant increases in overall cancer incidence after 150 mGy of radiation is 10,000.

In the experiment with the *gpt*-delta transgenic mice (6), total doses of 2–8 Gy were exposed at different dose-rates of 0.0125–920 mGy/min. A dose-dependent increase of mutation frequency (MF) in the red/gam gene (primarily by a large deletion) was observed in the spleen and liver. The spleen was more sensitive than the liver. A clear dose-rate effect was observed with the higher MF by the higher dose-rate. It is very important that the biological effect, such as an increased cancer or mutation incidence, was induced by the low dose-rate radiation although the total dose should be high. This finding suggests a likelihood that the lower-dose effect can be detected using a larger number of mice; however, this study seems to be an endless trial.

Suminori Akiba (Kagoshima University) introduced the human epidemiological data in his talk, "Considerations on the Risk of the Low Dose Radiation from Epidemiological Data in Human."

Dr. Akiba stated that there are several areas where there is a high level of natural background radiation; e.g., Karunagapally in Kerala, India; Yangjiang in Guangdong, China; and Talesh Mahalleh in Ramsar, Iran. Among these regions, useful epidemiological data were retrieved from Kerala where radioactive monazite sands in the coast released approximately 5–10 mSv gamma ray per year. Dr. Akiba surveyed the radiation level of this area and performed epidemiological analysis in nearly 400,000 residents. There was a small increase in the relative risk of lymphoma; however, this amount was not statistically significant. The results also suggested that the excess relative risk per dose for solid cancer after chronic radiation exposure is significantly lower than that observed among the atomic bomb survivors. There was a statistically significant increase in

the incidence of cancer among persons who were exposed to an acute high level of radiation, such as near the Techa River. An increase of mammary tumors was evident among female patients with tuberculosis who were exposed frequently to diagnostic chest radiographs. Therefore, an importance of radiation dose-rate was suggested, which can be confirmed by analysis with workers in nuclear plants. In conclusion, there are no epidemiological data that demonstrate an increase in the incidence of cancer or heritable genetic effects by the low-dose radiation exposure (no evidence does not necessarily mean no effect).

Dr. Akiba's talk was summarized as a review paper titled "Cancer Risk Associated with Low Dose and Low Dose-Rate Ionizing Radiation Exposure" in this issue.

General Discussion (Chaired by Takayoshi Suzuki and Masanobu Kawanishi, Osaka Prefecture University)

A general discussion was held at the end of the symposium. Before starting the discussion, a special talk was presented by Maki Momose, a mother who works as a high school teacher in Fukushima (Hanawa Technical High School). It was a good opportunity for scientists to listen directly to a resident from the affected area.

Mrs. Momose suffered essentially from a lack of information on radioactive contamination level and safety. Because of this lack of information, she felt insecure and temporarily evacuated her house with her small children, leaving her husband at home. It had been very stressful for her family; finally, she returned with her children to their home. They live in Nasushiobara (Tochigi Prefecture), where hot spots of radioactive nuclear fallout are found, although the city is not close to the nuclear plant. They were informed about this fact three months after the accident. As a teacher, she reconsidered the risk of the surrounding materials and realized that a similar level of risk of radiation existed, which are considered to be "not immediately harmful". She said "it is important to teach students how to cope with the surrounding risk". She emphasized that it is critical to make your own decision and to assess the situation based on sufficient data and a long-term vision of the future.

As a mother, she worried that the children's health might be adversely affected by the radioactive contamination, and she felt guilty about staying in that environment if there would be ill side effects in the future.

At the end of her talk, she posed the following questions to challenge scientists:

- How effective is the human defense system against DNA damage?
- Does the low-level radiation cause mental defects, such as a general malaise?
- How different is an individual's susceptibility to radiation?

Additionally, she expected scientists to contribute to the following tasks:

- Evaluation of the radiation effects in combination with other risk factors;
- Collection of data on the health effects induced by a long-term exposure to the low-level radiation; and
- Communication about a health risk of radiation or chemicals in a comprehensible fashion.

These tasks that she proposed are very difficult. Nevertheless, as scientists, we should endeavor to provide useful information with regard to these critical issues.

After her talk, a general discussion with all of the presenters ensued. At the beginning, Dr. Kasai (former ICRP and UNSCER member) explained about a principle of the ICRP report and of the LNT model, which is occasionally misunderstood by researchers. He asked for a proper understanding on the meaning of the reports and a transfer of the correct information.

A question was raised regarding the recent change in food regulation in which the level of maximum acceptable radioactivity had decreased, therefore getting severer. Dr. Yamazoe answered that "the food regulation is applied for a whole population, therefore the safety level should be higher as much as possible within an achievable range. It is necessarily to avoid the risk, at least immediately after accidents, but it should be reconsidered later from a long term view."

Finally, in closing, each speaker addressed their issues and proposals for the future risk management of low-level radiation as follows:

- Transparency of information is important for an unbiased risk communication and understanding. (Dr. Yamazoe)
- We should remember the evidence that a forced evacuation of the aged people from their care facility caused death, although no death was reported by a direct exposure to radiation. (Dr. Nakamura)
- Political operations were not properly conducted after the nuclear accident, particularly with respect to monitoring the actual radiation level and human exposure in Fukushima. A lack of information caused confusion among the residents in Fukushima. (Dr. Takada)
- It is important to know the background radiation is not zero on earth. Excess fear caused unnecessary stress, which occasionally leads to a suicide. Therefore, an appropriate understanding about the actual risk is mandatory. (Dr. Sutou)
- Our risk-communication ability should be improved by understanding the actual feelings of the affected residents to better contribute toward a future risk management in Fukushima. (Dr. Morita)
- Experiences in Aomori for an environmental

monitoring of background radiation performed by the Institute for Environmental Sciences, which was built according to an establishment of the Rokkasho Nuclear Reprocessing Plant (not yet operated), before and after the accident in Fukushima, can contribute to a better understanding of the health effect by radiation in Fukushima. (Dr. Shima)

- Verification of operations after the accident is necessary. For example, it would be useful to know why the monitoring of thyroid exposure to radiation in children could not be performed soon after the accident. The Japanese government should contribute to the evaluation of the health effect by low-dose exposure to radiation. (Dr. Akiba)

Take-Home Messages from the Symposium

- It is difficult to draw a conclusion scientifically on a real risk of low-dose radiation.
- The current regulatory standards were set without scientific evidence for the increased risk, including a safety margin.
- The risk of radiation should be considered in conjunction with other confounding factors.
- The increased risk of carcinogenesis by radiation in Fukushima can be a trade-off by controlling the other confounding risk factors in lifestyle.
- The risk communication (education) is important to understand a real risk of low-level radiation exposure and to avoid unnecessary fear among the affected individuals.
- The decision should be made personally whether to accept or avoid the risk of low-level radiation because the level of risk varies depending on the person and his or her situation.

I believe this symposium could provide useful information for the participants. To disseminate this information publicly, all original presentations will be made available as online movies at the radiation risk site in the JEMS homepage.

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