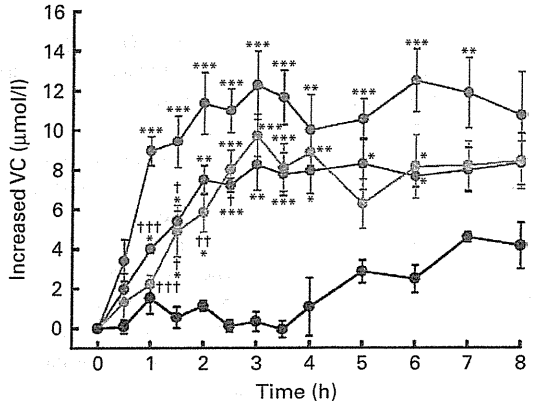

Fig. 2. Increased plasma vitamin C (VC) concentration after consumption of mashed potatoes (●), potato chips (●), VC in water (●) and water (●). Values are means, with their standard errors represented by vertical bars (n 5). Values reached statistical significance for group ($P<0.001$), time ($P<0.0001$) and food group × time interaction ($P<0.0001$) by repeated-measures ANOVA. Mean values were significantly different from water: *$P<0.05$, **$P<0.01$ and ***$P<0.001$ (ANOVA and Tukey's honestly significance test). Mean values were significantly different from VC in water: †$P<0.05$, ††$P<0.01$, †††$P<0.001$ (ANOVA and Tukey's honestly significance test). For mashed potatoes, values at 2, 2·5, 3, 3·5, 4, 5, 6, 7 and 8 h were significantly higher than values at 0, 0·5 and 1 h ($P<0.05$); values at 1·5 h were significantly higher than values at 0 and 0·5 h ($P<0.05$); values at 1 h were significantly higher than values at 0 h ($P<0.05$) (repeated-measures ANOVA and Tukey's honestly significance test). For potato chips, values at 3 h were significantly higher than values at 0, 0·5, 1, 1·5, 2 and 5 h ($P<0.05$); values at 4 and 8 h were significantly higher than values at 0, 0·5, 1 and 1·5 h ($P<0.05$); values at 2, 2·5, 3·5, 5, 6 and 7 h were significantly higher than values at 0, 0·5 and 1 h ($P<0.05$); values at 1·5 h were significantly higher than values at 0 and 0·5 h ($P<0.05$) (repeated-measures ANOVA and Tukey's honestly significance test). For VC in water, values at 1, 1·5, 2, 2·5, 3, 3·5, 4, 5, 6, 7 and 8 h were significantly higher than values at 0 and 0·5 h ($P<0.01$) (repeated-measures ANOVA and Tukey's honestly significance test). For water, values at 5 h were significantly higher than values at 3·5 h ($P<0.05$); values at 7 h were significantly higher than values at 0, 0·5, 1, 1·5, 2, 2·5, 3, 3·5 and 4 h ($P<0.05$); values at 8 h were significantly higher than values at 0, 0·5, 1·5, 2, 2·5, 3, 3·5 and 4 h ($P<0.05$) (repeated-measures ANOVA and Tukey's honestly significance test).

consumption of mashed potatoes, potato chips, VC in water and water, we calculated the time course of increased plasma VC concentrations by subtracting the initial baseline values from the total at each subsequent blood/urine sampling (Fig. 2). In plasma, VC concentrations from mashed potatoes and potato chips almost linearly increased at first in a time-dependent manner after oral consumption and then reached maximal levels at 3 h. At the 3 h mark, the increase in plasma VC concentrations from mashed potatoes was 8·3 (SEM 1·3) μmol/l and from potato chips was 9·7 (SEM 1·1) μmol/l. These increases were higher than those of water (0·3 (SEM 0·5) μmol/l; $P=0.002$ and $P=0.0003$, respectively), but not significantly different from each other ($P=0.8$) or from that of VC in water (12·3 (SEM 1·7) μmol/l). In fact, the only significant change in plasma VC concentration was a decrease for potato chips at 5 h after ingestion. These increases of VC in plasma for mashed potatoes were higher than those for water at 1 h ($P=0.046$), 1·5 h ($P=0.02$), 2 h ($P=0.002$), 2·5 h ($P<0.0001$), 3 h ($P=0.002$), 3·5 h ($P=0.0005$), 4 h ($P=0.02$), 5 h ($P=0.012$) and 6 h ($P=0.04$); for potato chips, they were higher than those for water at 1·5 h ($P=0.03$), 2 h ($P=0.02$), 2·5 h ($P<0.0001$), 3 h ($P=0.0003$), 3·5 h ($P=0.0003$), 4 h ($P=0.006$) and 6 h ($P=0.02$). On the other hand, these increases of VC in plasma for mashed potatoes were lower than those of VC in water at 1 h ($P=0.0001$), 1·5 h ($P=0.048$) and 2·5 h ($P=0.02$); for potato chips, they were lower than those of VC in water at 1 h ($P<0.0001$), 1·5 h ($P=0.02$) and 2 h ($P=0.006$). Overall, at different time points, the values between mashed potatoes and potato chips were not significantly different ($P>0.05$). Additionally, VC concentrations in plasma of all four food groups did not return to baseline until 8 h after consumption.

Next, we calculated the AUC for increased plasma VC concentration at 8 h after administration to estimate the bioavailability of VC in mashed potatoes and potato chips (Fig. 3). The AUC for mashed potatoes (55·1 (SEM 5·7) μmol × h/l) and potato chips (53·6 (SEM 8·1) μmol × h/l) were higher than that of water (14·6 (SEM 3·3) μmol × h/l; $P=0.004$ and $P=0.0048$, respectively). On the other hand, these values tended to be 32% less and were 34% less than that of VC in water (81·5 (SEM 8·9) μmol × h/l; $P=0.06$ and $P=0.048$, respectively). There was no significant difference between the AUC for mashed potatoes and potato chips ($P=0.99$).

Urinary excretion of VC after consumption of mashed potatoes, potato chips, VC in water and water was similarly measured to reveal their differing effect, if any. Total spontaneous urine volumes over 8 h after intake of mashed potatoes, potato chips, VC in water and water were 321 (SEM 15), 331 (SEM 47), 326 (SEM 73) and 286 (SEM 15) ml, respectively, and not significantly different ($P=0.9$). Similarly, the total urine creatinine over that 8 h time span did not significantly differ among mashed potatoes, potato chips, VC in water and water (5·30 (SEM 0·23), 5·48 (SEM 0·24), 5·42 (SEM 0·29) and 4·81 (SEM 0·34) mmol, respectively, $P=0.3$). However, VC in water was excreted at a significantly elevated rate at 3 and 4 h after intake (Fig. 4(a)). Urinary excretion of

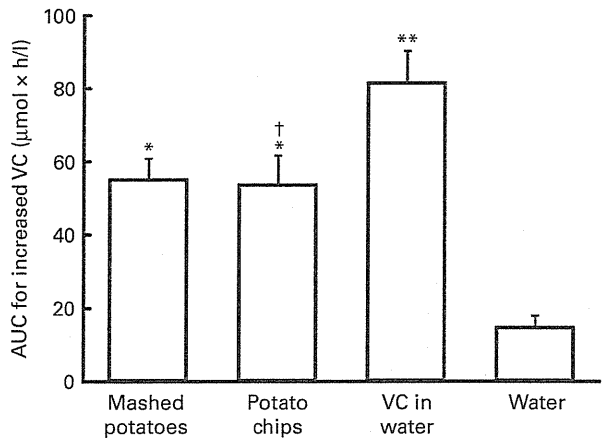

Fig. 3. Area under the curve (AUC) for increased plasma vitamin C (VC) concentration until 8 h after consumption of mashed potatoes, potato chips, VC in water and water. Values are means, with their standard errors represented by vertical bars (n 5). Mean values were significantly different from those of water: *$P<0.005$ and **$P<0.0001$. †Mean value was significantly different from that of VC in water: $P<0.05$.

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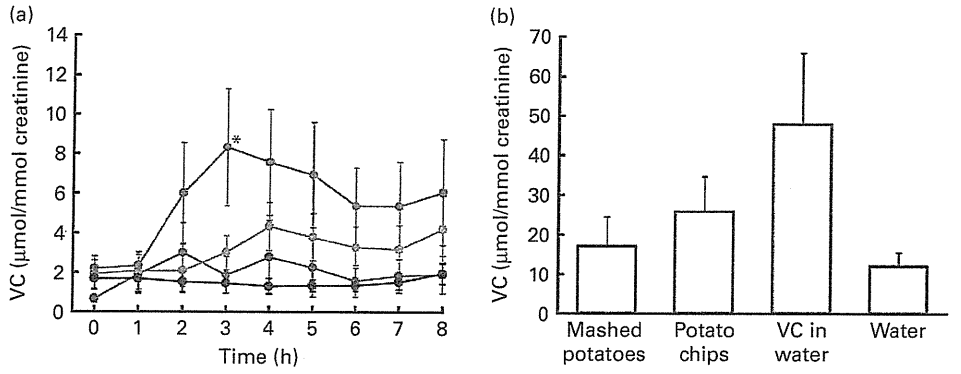

Fig. 4. (a) Urinary excretion of creatinine-corrected vitamin C (VC) after consumption of mashed potatoes (●), potato chips (●), VC in water (●) and water (●). Values are means, with their standard errors represented by vertical bars (n 5). There was no significant main effect of food group (P=0·14); however, the main effect of time reached significance (P<0·0001) as did the food group × time interaction (P=0·0012) by repeated-measures ANOVA. *Mean values were significantly different from water (P<0·05; ANOVA and Tukey's honestly significance test). For mashed potatoes and water, values at different time points did not show a significant difference (P=0·7 and P=0·14, respectively, repeated-measures ANOVA). For potato chips, values at 4 and 8 h were significantly higher than values at 0, 1 and 2 h (P<0·05); values at 5 h were significantly higher than values at 0 h (P<0·05) (repeated-measures ANOVA and Tukey's honestly significance test). For VC in water, values at 3 and 4 h were significantly higher than values at 0 and 1 h (P<0·05) (repeated-measures ANOVA and Tukey's honestly significance test). For water, values at different time points did not show a significant difference (P=0·14, repeated-measures ANOVA). (b) Urinary excretion of creatinine-corrected VC until 8 h after consumption of mashed potatoes, potato chips, VC in water and water. Mean values were not significantly different among all groups (P=0·13; ANOVA).

VC from potato chips was slightly elevated at 4, 5 and 8 h, but not to a significantly different extent from that of VC in mashed potatoes and water until 8 h. Compared with VC in water, urinary excretion of VC at 3 h was 78 and 64% less than that from mashed potatoes (1·9 (SEM 0·9) μmol/mmol creatinine) and from potato chips (3·0 (SEM 0·9) μmol/mmol creatinine) but was not a significant factor (P=0·056 and P=0·14), respectively. Total amounts of urinary VC excreted at 8 h were 64 and 46% less than that from mashed potatoes (17·0 (SEM 7·5) μmol/mmol creatinine, 1·7 (SEM 0·7) mg VC) or potato chips (25·9 (SEM 8·8) μmol/mmol creatinine, 2·9 (SEM 1·1) mg VC) compared with VC in water (47·9 (SEM 17·9) μmol/mmol creatinine, 5·4 (SEM 2·2) mg VC), but again, the difference was not statistically significant (P=0·2 and P=0·5; Fig. 4(b)).

Discussion

In the present study, we show for the first time that the dietary intake of mashed potatoes and potato chips in amounts that contained 50 mg of VC was equally effective at increasing plasma VC concentrations, i.e. both yielded an increase of 24%. This outcome indicates that (1) VC from ingested potatoes is well absorbed in the intestine and transferred to the blood and (2) processing such as steaming/mashing or frying does not affect the bioavailability of VC in potatoes. Furthermore, after the consumption of mashed potatoes or potato chips, the increased VC concentration in plasma up to the maximal level and for 8 h remained greater, and the urinary excretion of creatinine-corrected VC was relatively lower than both values after the intake of VC in water. Overall, the bioavailability of VC from potatoes, either mashed or fried, exceeded that from VC in water.

Human subjects do not usually eat raw potatoes but, instead, consume them in home-processed and commercial preparations. Han *et al.*[14] reported that potatoes home-processed by boiling, pressure-cooking, frying, sautéing, braising, baking or microwaving lose VC to varying degrees. In that study, the VC content of mashed potatoes (17·7 (SEM 0·1) mg/100 g) was at a lower level presumably because the combined processes of steaming, mashing and additional freeze–thawing they applied decreased the VC content. Burg & Fraile[15] showed that steaming at 200°C by using a superheated steam oven reduced VC in potatoes by 73%. Elsewhere, exposing mashed potatoes served to hospitalised patients to a cool-chill-plated catering system resulted in a 76% loss of VC[27]. In contrast, commercial potato chips prepared from the same lot of potatoes contained a much larger amount of VC (57·2 (SEM 0·4) mg/100 g). In good accord with the previous report, frying in oil increased VC content per fresh weight of potatoes by decreasing their water content from about 80 to 2% despite partial loss of VC in the process[16]. Thus, commercial potato chips, because of their substantial content of VC and ready availability, are a more efficient source of VC than home-processed potatoes.

In the present study, subjects consumed orally 282 g mashed potatoes and 87 g potato chips, each portion containing 50 mg VC. This amount is suitable for one daily serving in a meal and snack, and almost all of this VC is absorbed in the intestine[20,21]. In human subjects, the metabolism and utilisation of orally administered, oxidised VC are equivalent to those of reduced VC[22,28–31]. Therefore, to make an appropriate comparison, we evaluated the bioavailability of VC in potatoes by measuring the VC content in plasma and its excretion in urine. Previously, a high dose of oral VC drastically increased VC concentration in the blood[20,21]. Consistently, though, a quantity of VC intake beyond renal tubules' reabsorption capability is readily excreted into urine[20,21]. Levine *et al.*[20,21] reported that little VC was excreted in urine during the 24 h following a single oral dose of <50 mg

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VC, whereas that after >100 mg VC dramatically increased in healthy volunteers. Therefore, such a large dose of oral VC might cause difficulty in examining the bioavailability of VC in food. To overcome this difficulty, we set the amount of orally administered VC in potatoes at 50 mg, which is half the RDA (100 mg) for VC in Japan[32]. However, our highly sensitive method for the measurement of VC[25] enabled us to detect accurately even slight changes of VC in plasma.

As far as we know, few reports describe the bioavailability of VC from food, raw or processed. In one such report, Mangels *et al.*[33] measured VC depletion–repletion during an 8-week period and found that the bioavailability of VC from oranges, orange juice and cooked broccoli is similar to that of synthetic VC; the exception was raw broccoli, which was 20 % lower. Van het Hof *et al.*[34] noted that plasma VC concentrations increased after a 4 d diet of vegetables such as broccoli, green peas, whole-leaf spinach, and chopped spinach in accordance with their VC contents. That group also showed that chopping whole-leaf spinach did not improve the bioavailability of VC. Sánchez-Moreno *et al.*[35,36] stated that drinking orange juice and Mediterranean vegetable soup (gazpacho) increased plasma VC concentrations in a dose–response manner and that daily consumption for 14 d provided a continuously higher plasma VC concentration than that of the baseline. Also, the consumption of pulsed electric fields-processed orange juice with a long shelf-life increased plasma VC concentration to a similar extent as freshly squeezed orange juice, both in one dose and in daily doses for 14 d[37].

Potatoes are consumed worldwide and are the major dietary staple in many countries of Europe and South America. However, this choice is less common in Japan. Interestingly, potatoes and potato chips are an important source of VC (17 mg/d in men and about 7 mg/d in women) for humans, especially those with a low plasma VC status (<11·4 μmol/l). Despite this acknowledgement published after a third Glasgow MONICA population survey[38], it is surprising that there was no evidence about the bioavailability of VC in potatoes. Consequently, the present result in which plasma VC concentration and urinary excretion of VC increased during the 8 h period after the oral intake of mashed potatoes and potato chips is unique and suggests that VC from potatoes is readily absorbed in the intestine and transferred to the blood. Also shown was that the bioavailability of VC from potato chips is closely similar to that from mashed potatoes. Clearly, despite the processing methods of steaming/mashing and frying, VC from potatoes retains adequate bioavailability. Moreover, humans gain more VC from potato chips than from mashed potatoes when comparable proportions are eaten. However, the energy from mashed potatoes and potato chips are 351 kJ and 2318 kJ/100 g, respectively[2]. In the present study, the human subjects took 282 g mashed potatoes and 87 g potato chips, which have 990 kJ and 2017 kJ of total energy respectively. Thus, people, especially obese individuals, should hesitate to consume a large amount of potato foods.

In the present study, increased plasma VC concentrations were less at 1, 1·5, 2, 2·5 h after intake of mashed potatoes and potato chips than that of VC in water. However, after 3 h, which was the time of maximal VC level, the concentrations did not differ significantly among the three food groups. However, less VC was lost in urine after potato ingestion than from VC in water alone, corresponding to a slower increase in VC concentration in plasma.

Water consumption, that is, no intake of VC in subjects did not largely affect the increased VC concentration in plasma, the AUC for increased VC in plasma, and the urinary excretion of VC in subjects except for the slight increase of VC from 5 to 8 h after consumption. In fact, to our knowledge, a study examining VC concentration in plasma after water consumption has not been reported previously. Although it is uncertain as to the reason why VC concentration in plasma after water consumption was slightly increased, we considered that long-time fasting over 17 h might increase the VC concentration in plasma via the leakage of VC from cells in tissues such as liver.

Some limitations here are that mashed potatoes and potato chips are solid foods containing much starch; therefore, digestion might take longer and VC absorption in intestinal lumen could be slower compared with VC in water, which has the fastest absorption time. Our time course was set at 8 h after the administration of potatoes, because of ethical concerns for volunteers who underwent overnight fasting. However, to obtain fully convincing conclusions, the study should be extended until 24 h have elapsed, which was not done here because feeding meals to subjects might affect VC metabolism in their bodies. Another possibility is that the transport of VC into tissues from the blood was enhanced by some nutrient(s) of potatoes, which resulted in an apparently slower increase of VC in plasma. That is, extracellular VC is transported into the cytoplasm by the Na dependent VC transporters 1 and 2 in a reduced form and by the GLUT1, GLUT3 and GLUT4 in an oxidised form[17,18,39]. As almost all VC in the blood exists in a reduced form[20], SVCT1 and SVCT2 are mainly responsible for taking up VC from the blood and into the cytoplasm of multiple tissues. SVCT1, which is expressed in such tissues as the intestine, liver, lung, kidney and skin, is involved in whole-body homeostasis[17]. SVCT2, which is expressed in the brain, eye, liver, kidney, intestine, adrenal gland, bone and skeletal muscle, is a high-affinity VC transporter to maintain high VC concentrations in tissues[17]. As recently reported, SVCT2-mediated VC uptake is quickly enhanced by the translocation of cytoplasmic SVCT2 to plasma membranes from various types of stimulation[40]. Since the regulation of SVCT2-mediated VC uptake is not fully understood, the present results might imply the existence of novel SVCT2-activating nutrient(s) in potatoes.

We have performed this experiment employing only male subjects. As shown in previous papers[20,21], VC metabolism in human subjects is not largely different between males and females. Thus, it is considered that the present data about the bioavailability of VC in mashed potatoes and potato chips could be applicable to females.

In conclusion, the bioavailability of VC in mashed potatoes and potato chips was documented in the present oral, single-dose study. The consumption of mashed potatoes and potato

chips increased the VC concentration in plasma, and less VC tended to be excreted in urine than was consumed. These results explicitly confirm the nutritional value of mashed potatoes and potato chips as a dietary VC source as demonstrated here in a cohort of Japanese males.

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Establishment and characterization of hepatocytes from an Immortomouse/SMP30/GNL knockout mouse hybrid lacking vitamin C to study vitamin C transport

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Senescence marker protein-30 (SMP30) has been identified as the lactone-hydrolysing enzyme gluconolactonase (GNL), which is involved in vitamin C (L-ascorbic acid, AA) biosynthesis. We previously reported the development of SMP30/GNL knockout (KO) mice unable to synthesize AA *in vivo*. For more efficient study of the liver's AA uptake and as yet uncharacterized efflux system, we established an immortal hepatocyte line derived from a hybrid of SMP30/GNL KO mice and Immortomice. Immortomice express the thermolabile simian virus 40 (SV40) large T antigen tsA58. These SMP30/GNL KO immortal hepatocytes proliferate at the permissive temperature of 33°C but degrade rapidly at the non-permissive temperature of 39°C. Additionally, they are SMP30-/GNL-deficient, express SV40 large T antigen and proliferate steadily at 33°C. However, the cells' proliferation is arrested at 39°C. A phase contrast micrograph revealed that the cells are binucleated with an enlarged cytoplasm similar to that of primary cultured hepatocytes from wild-type mice. Dose–response and time-dependent study of AA uptake revealed that the cells, although unable to synthesize AA, took up AA from the culture medium. This property of our SMP30/GNL immortal hepatocytes makes them extremely useful for studying AA uptake and efflux systems in the liver.

Keywords: ascorbic acid/hepatocyte/Immortomouse/senescence marker protein 30/simian virus 40.

Abbreviations: AA, ascorbic acid; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DHA, dehydroascorbic acid; EDTA, ethylenediaminetetraacetic acid; EGF, Epidermal growth factor; EGTA, ethylene glycol-bis (2- aminoethylether) - N,N,N′, N′-tetraacetic acid; FCS, foetal calf serum; FITC, Fluorescent isothiocyanate; GLUT, glucose transporter; GNL, gluconolactonase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid; Hsp, heat shock protein; IFN-γ, interferon gamma; KO, knockout; PBS, phosphate buffered saline; SMP30, senescence marker protein-30; SVCT, sodium-dependent vitamin C transporter; SV40, simian virus 40; Tg, transgenic; WT, wild-type.

Senescence marker protein-30 (SMP30) is a 34-kDa protein whose tissue levels in the liver, kidney and lung decrease with aging (*1*). This protein is expressed most prominently in the liver and kidneys among the various organs (*2*). Its gene is located on the X chromosome (*3*). Recently, we identified SMP30 as the lactone-hydrolysing enzyme gluconolactonase (GNL) (EC 3.1.1.17), which is involved in vitamin C (L-ascorbic acid) biosynthesis and the essential role of SMP30 in this synthetic process was verified by a nutritional study (*4*). That is, SMP30/GNL knockout (KO) mice developed symptoms of scurvy when fed a vitamin C-deficient diet, verifying the pivotal role of SMP30/GNL in vitamin C biosynthesis.

Ascorbic acid (AA) functions as an electron donor and scavenges free radicals such as superoxide radicals (*5*) and hydroxyl radicals (*6*) *in vitro*. Moreover, AA is essential for post-translational proline hydroxylation of collagen molecules (*7*), after which hydroxyproline residues play a critical role in stabilizing the triple helical structure of collagen (*8*). AA and dehydroascorbic acid (DHA), the oxidized form of AA, in tissues regulate the AA transporters, sodium-dependent vitamin C transporters (SVCT) 1 and SVCT2 and the DHA transporters, glucose transporter (GLUT) 1, GLUT3, GLUT4, respectively (*9*). By using primary cultured hepatocytes from SMP30/GNL KO mice, we found that SVCT1 and SVCT2 mRNA expression levels and AA uptake ability were significantly enhanced in the hepatocytes of AA-depleted SMP30/GNL KO mice, indicating that AA exerts a marked impact as a regulatory element of SVCT1 and SVCT2 expression in the liver (*10*).

Primary cultured hepatocytes provide a good experimental system for the cultures as it maintains the body's liver functions. However, primary cultured hepatocytes must be isolated from animals and purified at the time of each experiment, then cultured with great care to maintain the liver's functions and character. Even with the best conditions, the cells' life span is limited in culture. In contrast, immortal cell lines

have an excellent proliferative capacity and long-term stability. Therefore, establishing an immortal hepatocyte line from SMP30/GNL KO mice is an extremely useful way to culture primary hepatocytes without the need to repeat the procedure for each experiment or to provide extraordinary care for preservation of the cultures.

To study further the liver's AA and DHA uptake export system, we sought to establish immortal hepatocyte lines derived from the SMP30/GNL KO mouse crossed with the so-called Immortomouse®. The Immortomouse harbours a transgene that expresses the thermolabile simian virus 40 (SV40) large T antigen tsA58 under control of the interferon (INF)-inducible murine major histocompatibility complex H-2Kb promoter (*11*, *12*). The tsA58 antigen is located on chromosome 16 and degrades rapidly at the non-permissive temperature of 39°C (*13–15*). In other respects, the Immortomouse has an almost normal phenotype at normal body temperature. Thus, the mouse can live healthy until the timing of sexual maturation, and its properties make it possible to crossbreed with other strain mouse. After crossbreeding the Immortomouse with the KO or transgenic (Tg) mice of interest (*16*), the cells harvested from the resulting hybrid have excellent proliferative capacity at the permissive temperature of 33°C. Thus, creating a specialized cell line in this way is more efficient than the traditional method of transfecting a SV40 large T antigen gene to primary cultured cells derived from KO or Tg mice.

In this study, we adapted the SMP30/GNL KO mouse or Immortomouse hybrid technology to establish an immortal hepatic cell line. These hepatocytes cannot synthesize AA, unlike hepatocytes derived from wild-type (WT) mice. This property is extremely advantageous for studying AA and DHA uptake and efflux, systems that involve SVCT, GLUT and other transporters.

Materials and Methods

Materials

Materials came from the locations listed below: L-ascorbic acid, metaphosphoric acid, dexamethasone and Mildform 10N from Wako Pure Chemicals (Osaka, Japan); ethylenediamine tetraacetic acid (EDTA) and 4-(2-hydroxyethyl)-1-piperazineethane sulphonic acid (HEPES) from Dojindo Laboratories (Kumamoto, Japan); ethylene glycol-bis (2-aminoethylether)- N,N,N′,N′-tetraacetic acid (EGTA), collagenase, bovine pancreatic trypsin inhibitor, bovine type I collagen and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) from Sigma-Aldrich (St. Louis, MO, USA). William's medium E was purchased from Invitrogen (Carlsbad, CA, USA) and recombinant mouse interferon-γ (IFN-γ) and the bicinchoninic acid (BCA) protein assay kit from Thermo Fisher Scientific (Waltham, MA, USA). Foetal calf serum (FCS) came from Equitech Bio, Inc (Kerrville, TX, USA) and Epidermal growth factor (EGF) from Biomedical Technologies, Inc. (Stoughton, MA, USA). Rabbit anti-rat SMP30 antibody was purchased from Cosmo Bio (Tokyo, Japan). The supplier of anti-SV40 T antigen antibody was Calbiochem (Darmstadt, Germany), of fluorescent isothiocyanate (FITC) conjugated anti-mouse albumin cross-adsorbed antibody, anti-mouse albumin antibody was Bethyl Laboratories, Inc. (Montgomery, TX, USA) and of anti-p53 antibody was Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit anti-β-actin antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Alexa Fluor® 488 goat anti-rabbit IgG, Alexa Fluor® 488 goat anti-mouse IgG and rhodamine phalloidin came from Molecular Probes (Carlsbad, CA, USA).

Animals

SMP30/GNL KO mice were generated by the gene targeting technique described previously (*2*); the SMP30 gene is located in the p11.3–q11.2 segment of the X chromosome (*3*). The Immortomouse® carrying the thermolabile SV40 large T antigen tsA58 gene was purchased from Charles River Laboratories International, Inc. (Wilmington, MA, USA). We mated female SMP30/GNL KO ($SMP30/GNL^{-/-}$, $SV40^{-/-}$) mice with a male Immortomouse ($SMP30/GNL^{Y/+}$, $SV40^{+/+}$) to produce SMP30/GNL KO mice carrying the SV40 large T antigen gene. In this study, only F1 male SMP30/GNL KO ($SMP30/GNL^{Y/-}$, $SV40^{+/-}$) mice were used. The mice had free access to water containing 1.5 g/l AA and 10 μM EDTA (*4*). Water bottles were changed every 3/4 days until the experiment ended. Male WT ($SMP30/GNL^{Y/+}$, $SV40^{-/-}$) mice were purchased from Japan SLC (Shizuoka, Japan). WT mice had free access to water without AA. All mice were fed an AA-deficient diet (CL-2, CLEA Japan, Tokyo, Japan) *ad libitum*. Throughout the experiments, animals were maintained on a 12-h light/dark cycle in a controlled environment. All experimental procedures using laboratory animals were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology.

Isolation and culture of mouse hepatocytes

Hepatocytes from SMP30/GNL KO mice carrying or not carrying the SV40 large T antigen gene and from WT mice at 6 months of age were isolated by the collagenase perfusion method as described previously (*17*). Briefly, each liver was perfused *in situ* through the vena cava inferior with EGTA solution containing 0.5 mM EGTA, 5 mM glucose, 4.1 mM $NaHCO_3$, 136 mM NaCl, 5.3 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4 and 10 mM HEPES (pH 7.2). Then, the EGTA solution was replaced with collagenase solution containing 0.03% collagenase, 4.8 mM $CaCl_2$, 136 mM NaCl, 5.3 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.006% trypsin inhibitor and 10 mM HEPES (pH 7.2). After the collagenase perfusion, the livers were removed and filtered through nylon mesh (100 μm) and then washed with Hanks' Balanced Salt Solution to remove non-parenchymal cells. Final cell preparations were suspended at 2.5×10^5 cells/ml of defined William's medium E containing 0.4 μg/l dexamethasone, 0.1 μg/ml bovine pancreatic trypsin inhibitor, 2 mM L-glutamine supplemented with 5% FCS and then placed into culture plates coated with bovine type I collagen. Cells were cultured at 33°C under 5% CO_2 in air for 3 h to allow attachment to culture plates, after that, the medium was replaced with William's medium E supplemented with 1 ng/ml IFN-γ, 5% FCS, 2 mM L-glutamine, 10 ng/ml EGF, 10 ng/ml insulin, 5.5 ng/ml transferring, 0.0067 ng/ml sodium selenite, 10 mM nicotinamide, 0.4 μg/l dexamethasone, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell viability was determined by Trypan blue dye exclusion.

Establishment of SMP30/GNL KO immortal hepatocyte cell line

Primary cultured hepatocytes from SMP30/GNL KO mice carrying the SV40 large T antigen gene were grown at 33°C, and the medium exchange was repeated. After 1 month, cells were collected by trypsinization and then diluted to isolate a single cell colony. Each single cell was placed into culture plates coated with bovine type I collagen and grown at 33°C. The SMP30/GNL KO immortal hepatocyte line was established by trypsinization using cloning cylinders on the basis of cell morphology and biochemical characterization.

Fluorescent immunostaining

For fluorescent immunostaining, cells were fixed for 15 min with Mildform 10N and permeabilized for 3 min with 0.5% Triton X-100 in PBS. Cells were stained with rabbit anti-rat SMP30 antibody (1:400 dilution), anti-SV40 large T antigen antibody (1:66 dilution), FITC-conjugated anti-mouse albumin cross-adsorbed antibody (1:100 dilution), rhodamine phalloidin (1:2,500 dilution) for detection of actin fiber or DAPI (1:10,000 dilution). The cells were then incubated with Alexa Fluor® 488 goat anti-rabbit IgG (1:2,500 dilution) or Alexa Fluor® 488 goat anti-mouse IgG (1:3,000 dilution). The immunostained cells were mounted in the presence of Slow-Fade antifade kit (Molecular Probes, Carlsbad,

CA, USA) and examined with a fluorescence microscope OLYMPUS IX70 (Olympus Medical Science Sales CO., LTD., Tokyo, Japan).

Western blot analysis

The collected cells were sonicated by using Vibra Cell (Sonics & Materials, Inc., Newtown, CT, USA) and centrifuged at 9,000*g* for 30 min at 4°C. Each supernatant was electrophoresed on a 10% polyacrylamide gel by the method of Laemmli (*18*), with some modifications. Proteins in the gel were transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, U.S.A.) by the method of Towbin *et al.* (*19*). The membranes were blocked for 30 min with 5% skim milk in 0.01 M Tris–HCl (pH 7.5), 0.14 M NaCl and 0.1% Tween 20. The membrane was incubated with rabbit anti-rat SMP30 antibody (1:1,000 dilution), anti-SV40 T antigen antibody (1:200 dilution), anti-mouse albumin antibody (1:1,000 dilution), anti-p53 antibody (1:200 dilution) or anti-β-actin antibody (1:1,000 dilution). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG. Chemiluminescence signals were detected by LAS-3000 imaging system (Fujifilm, Tokyo, Japan) using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). The band intensity was quantitated with the imaging software of MultiGauge version 3.0 (Fujifilm). Protein concentrations were measured by the BCA protein assay kit (Thermo Fisher Scientific) using bovine serum albumin (BSA) as a standard.

Assessment of temperature-sensitive cell growth

SMP30/GNL KO immortal hepatocytes were plated at 5×10^4 cells/ml and grown at either the permissive temperature (33°C) or non-permissive temperature (39°C). The total cell number on 1, 2, 4 and 7 days after plating was counted with the crystal violet staining method (*20*, *21*) with some modifications. Briefly, cells were collected by trypsinization and centrifuged at 9,730*g* for 5 min. The cells were suspended in a solution containing 0.1 M crystal violet and 0.1% citric acid after which bare nuclei stained with crystal violet were counted by using a Neubauer hemocytometer (Sunlead Glass Corp., Saitama, Japan).

AA uptake study

SMP30/GNL KO immortal hepatocytes were plated at 2×10^5 cells/ml and cultured for 1 day at 33°C. For the dose–response study of AA uptake, cells were incubated with various concentrations of AA (50, 100, 200 or 400 μM) and without AA in medium for 1 h. For a time-course study of AA uptake, cells were incubated with 100 μM AA in medium for 1, 3, 6, 12 or 24 h. After incubation, cells were washed with PBS and collected with 5% metaphosphoric acid to measure the AA content. AA was measured by using a high performance liquid chromatography and electrochemical detector as described previously (*10*, *22*).

Statistical analysis

Results are expressed as means ± standard error of the mean (SEM). The probability of statistical differences between experimental groups was determined by one-way analysis of variance (ANOVA) followed by post-hoc Tukey's honestly significant difference test. ANOVAs were performed using Kareida Graph software (Synergy Softoware, Reading, PA, USA). Statistical differences were considered significant at $P < 0.05$.

Results

Morphology of SMP30/GNL KO immortal hepatocytes

A micrograph of the SMP30/GNL KO immortal hepatocytes appears in Fig. 1A. These hepatocytes were derived from a hybrid of a SMP30/GNL KO mouse and an Immortomouse carrying the SV40 large T antigen gene under control of the IFN-inducible murine H-2Kb promoter (*11*, *12*, *15*). Morphologically, the cells cultured at 33°C with AA-free William's medium E supplemented with IFN-γ in culture plates coated with bovine type I collagen were flat and had a relatively small amount of cytoplasm at confluent stages. Therefore, these hybrid cells were similar to the primary cultured hepatocytes from WT mice (Fig. 1B), SMP30/GNL KO mice (Fig. 1C) and other established immortal hepatocyte lines derived from a hybrid between Immortomice and other KO or Tg mice of interest (*23–25*). The morphology of the SMP30/GNL KO immortal hepatocytes in AA-free medium at 33°C have not changed over 15 passages.

Immunostaining of SMP30/GNL KO immortal hepatocytes

By fluorescent immunostaining analysis, our SMP30/GNL KO immortal hepatocytes were positive for the expression of SV40 large T antigen within the nuclei (Fig. 2A), but that antigen was not detectable in primary cultured hepatocytes from WT mice (Fig. 2B). Conversely, the SMP30/GNL KO immortal hepatocytes did not express SMP30/GNL (Fig. 2C), which was detected in the cytoplasm and nuclei of primary cultured hepatocytes from WT mice (Fig. 2D). Moreover, the cytoplasms of both SMP30/GNL KO immortal hepatocytes and primary cultured hepatocytes from WT mice were positively stained with albumin, which is a hepatocyte-specific marker (Fig. 2E and F).

Characterization of SMP30/GNL KO immortal hepatocytes

To confirm the characterization of SMP30/GNL KO immortal hepatocytes, the expression of SV40 large T antigen, SMP30/GNL and albumin was analysed by western blot analysis. SV40 large T antigen was detected only in SMP30/GNL KO immortal hepatocytes (Fig. 3, lane 1), whereas, no SMP30/GNL was detected in SMP30/GNL KO immortal hepatocytes. SMP30/GNL was clearly present in primary cultured hepatocytes from WT mice (Fig. 3, lane 2), but not in primary cultured hepatocytes from SMP30/GNL KO mice (Fig. 3, lane 3). Moreover, albumin was detectable in all three cell types, i.e. SMP30/GNL KO immortal hepatocytes, as well as primary cultured hepatocytes from WT mice and SMP30/GNL KO mice (Fig. 3).

Temperature-sensitive cell growth

SV40 large T antigen tsA58 has the property of rapid degradation at the non-permissive temperature of 39°C (*13*, *14*). To confirm the temperature-sensitivity of SMP30/GNL KO immortal hepatocytes bearing the SV40 large T antigen tsA58, cells were cultured at either 33 or 39°C and counted on Days 1, 2, 4 and 7 of culture. SMP30/GNL KO immortal hepatocytes grew continuously at 33°C, and the total cell number increased throughout the 7 days of culture (Fig. 4A). However, cells cultured at 39°C showed a far slower proliferation rate. That is, the total number of cells on Day 7 in cultures maintained at 33°C was 3.5 times as much as that cultured at 39°C. Thus, the established SMP30/GNL KO immortal hepatocytes showed markedly temperature-sensitive cell growth.

Moreover, the protein level of SV40 large T antigen for cells cultured at 39°C for 1 day was 44% lower than that of the same cells cultured at 33°C

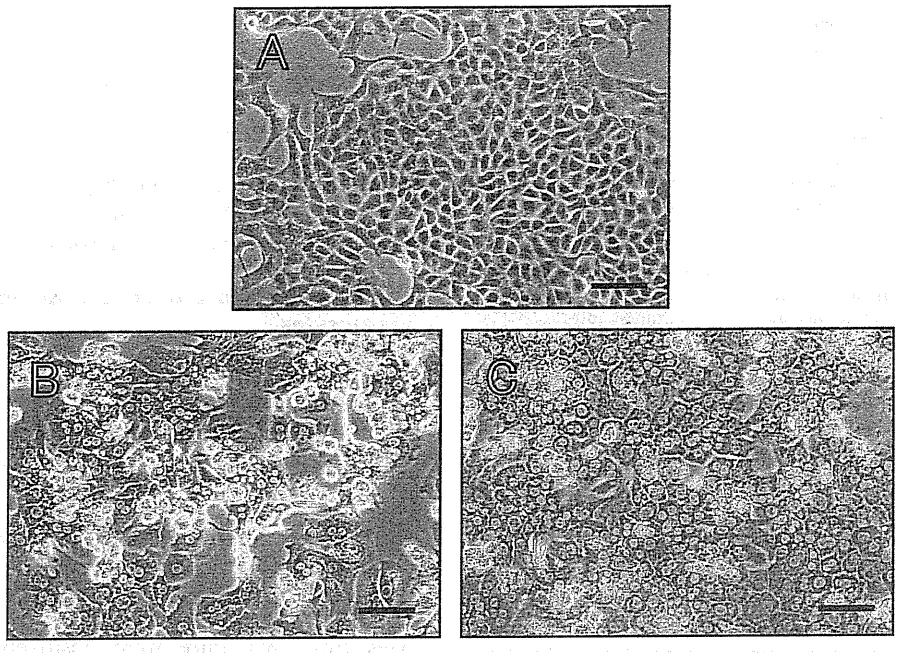

Fig. 1 Phase contrast micrograph of SMP30/GNL KO immortal hepatocytes cultured at 33°C (A), primary cultured hepatocytes from WT (B) and SMP30/GNL KO mice (C). Scale bar, 100 μm.

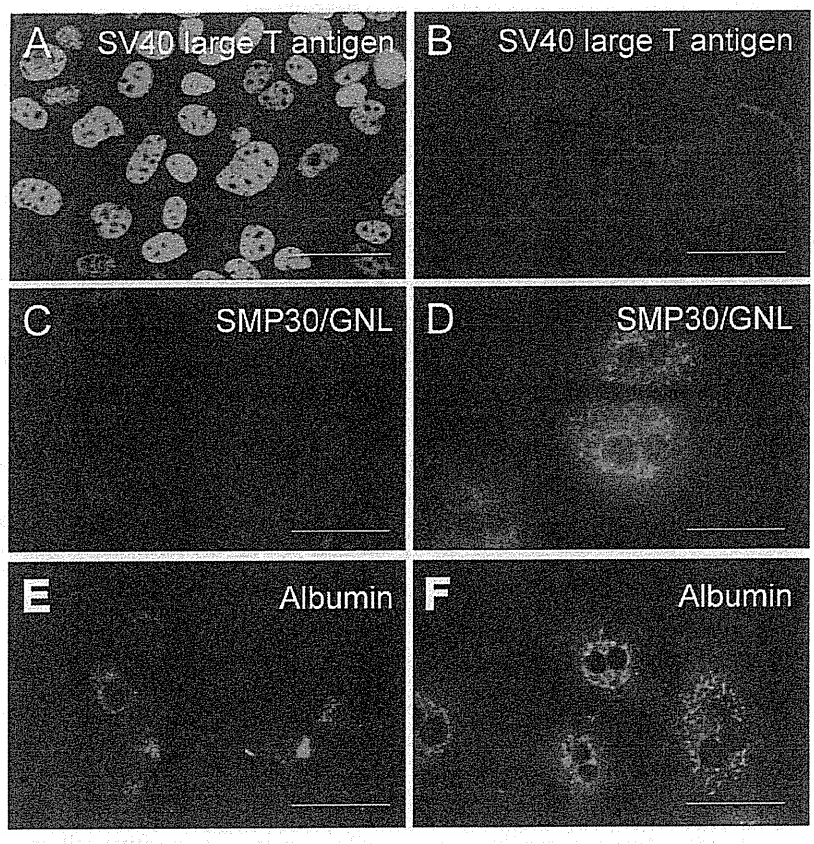

Fig. 2 Fluorescent immunostaining images of SMP30/GNL KO immortal hepatocytes (A, C, E) and primary cultured hepatocytes from WT mice (B, D, F). Cells were stained with anti-SV40 large T antigen antibody and rhodamine phalloidin for detection of actin fiber (A, B), rabbit anti-rat SMP30/GNL antibody and DAPI (C, D) or FITC-conjugated anti-mouse albumin cross-adsorbed antibody (E, F) as described in 'Materials and Methods' section. Scale bar, 50 μm.

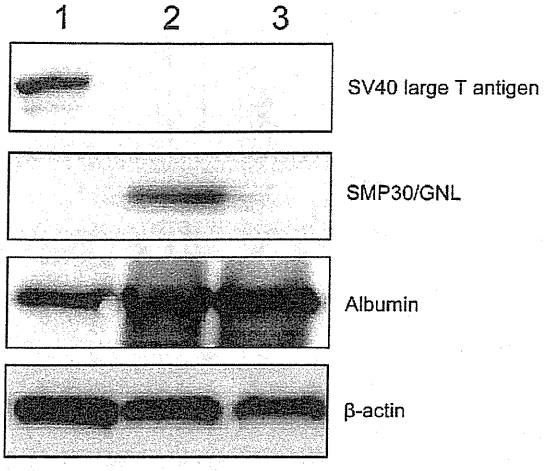

Fig. 3 Western blot analysis of SV40 large T antigen, SMP30/GNL, albumin and β-actin in SMP30/GNL KO immortal hepatocytes (lane 1), primary cultured hepatocytes from WT mice (lane 2) and primary cultured hepatocytes from SMP30/GNL KO mice (lane 3). Proteins (2 μg for SMP30/GNL and β-actin, and 10 μg for SV40 large T antigen and albumin) were electrophoresed on a 10% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. The membranes were incubated with anti-SV40 large T antigen antibody, rabbit anti-rat SMP30 antibody, anti-mouse albumin or rabbit anti-β-actin antibody. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG. Chemiluminescence signals were detected by LAS-3000 imaging system.

(Fig. 4B). The concentration of p53 was then evaluated, because p53 is a known tumour-suppressor gene that forms complexes with SV40 large T antigen on immortalized cells cultured at 33°C (*23*). When examined by western blot analysis, the p53 protein was readily detected when cells were cultured at 33°C for 1 day (Fig. 4B). However, no p53 protein was apparent on identical cells cultured at 39°C. In contrast, albumin was present when cells were cultured at both 33 and 39°C for 1 day (Fig. 4B).

Phase contrast micrograph of SMP30/GNL KO immortal hepatocytes cultured at 39°C for 2 days revealed many more bi-nucleated cells with a larger cytoplasm content than that of the cells cultured at 33°C (Fig. 4C). Morphologically, the cells cultured at 39°C closely resembled primary cultured hepatocytes from SMP30/GNL KO and WT mice (Fig. 1B and C).

AA uptake of SMP30/GNL KO immortal hepatocytes

To ensure that the established SMP30/GNL KO immortal hepatocytes were unable to synthesize AA but adept at AA uptake, we performed a dose–response and time-dependent study of the culture medium. First, cells were incubated for 1 h without AA after which no AA was noted (Fig. 5A), thereby affirming that SMP30/GNL KO immortal hepatocytes themselves are unable to synthesize AA. In contrast, after the cells were incubated with concentrations of

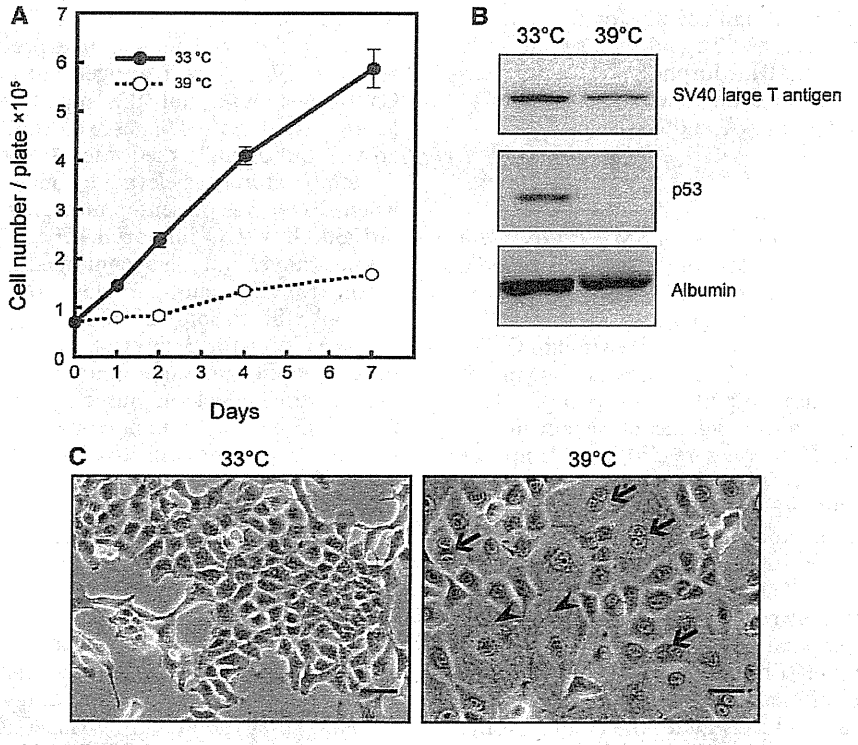

Fig. 4 Temperature-sensitive cell growth and morphological change of SMP30/GNL KO immortal hepatocytes. (A) SMP30/GNL KO immortal hepatocytes were plated at 5×10^4 cells/ml and grown at 33°C (solid circle) and 39°C (open circle). Total cell numbers on 1, 2, 4 and 7 days after plating were counted after crystal violet staining. Values are expressed as means ± SEM of 3 plates. (B) Western blot analysis of SV40 large T antigen, p53 and albumin in SMP30/GNL KO immortal hepatocytes cultured at 33 and 39°C for 1 day after plating. (C) Phase contrast micrograph of SMP30/GNL KO immortal hepatocytes cultured at 33 and 39°C for 2 days after plating. Arrows indicate bi-nucleated cells, and arrow heads indicate cells' enlarged cytoplasm. Scale bar, 100 μm.

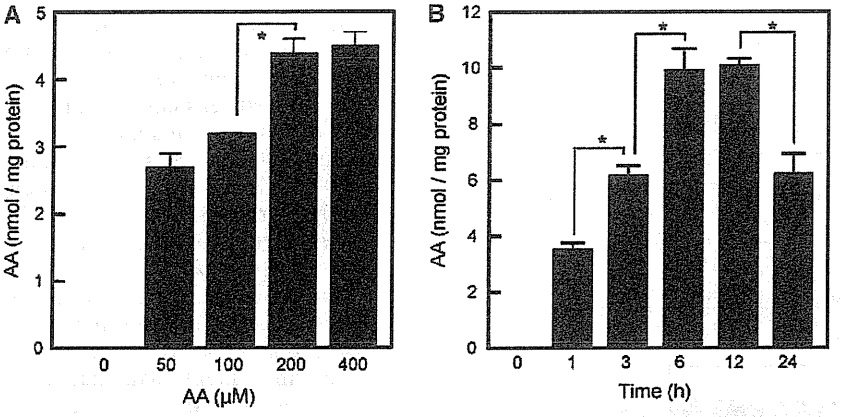

Fig. 5 Dose–response and time-dependent study of AA uptake into SMP30/GNL KO immortal hepatocytes. Cells were plated at 2×10^5 cells/ml and cultured for 1 day at 33°C. (A) For the dose–response study, cells were then incubated with 50, 100, 200, 400 μM AA and without AA in medium for 1 h at 33°C. After incubation, cells were collected with 5% metaphosphoric acid and measured for AA contents by using high performance liquid chromatography and an electrochemical detector as described in 'Materials and Methods' section. (B) For the time-course study, cells were then incubated with 100 μM AA in medium for 1, 3, 6, 12 and 24 h at 33°C and assessed for AA content. Values are expressed as means ± SEM of 3 plates. $*P < 0.05$.

AA from 50 to 100 μM, 200 and 400 μM in medium for 1 h, AA was clearly detectable. AA content in the cells increased dose-dependently from 50 μM to 200 μM AA, but no difference in uptake was noted at amounts from 200 to 400 μM AA. For time-dependent study of AA uptake, cells were incubated with 100 μM AA in medium for 1, 3, 6, 12 and 24 h. Thereafter, the AA content of cells increased significantly for the first 6 h, remained constant until the 12 h and then significantly decreased at 24 h (Fig. 5B). Morphologically, the cells didn't show remarkable changes within 24 hours at 33°C in the medium with AA and AA free.

Discussion

We have established a population of SMP30/GNL KO immortal hepatocytes derived from a hybrid between a SMP30/GNL KO mouse and an Immortomouse carrying the SV40 large T antigen gene. SMP30/GNL KO immortal hepatocytes cannot synthesize vitamin C, because they lack SMP30/GNL, an essential enzyme of the vitamin C biosynthetic pathway *in vivo* (*4*). This property provides us with a significant benefit in studies designed to resolve issues regarding AA uptake into and, particularly, AA efflux from the liver. Moreover, SMP30/GNL KO immortal hepatocytes will facilitate research to discover other functions of SMP30/GNL in humans.

The SMP30/GNL KO immortal hepatocytes developed here were grown and proliferated steadily at the permissive temperature of 33°C in William's medium E containing IFN-γ, but not without IFN-γ. The cause of this difference is that the expression of SV40 large T antigen tsA58 is under the control of the IFN-inducible murine major histocompatibility complex H-2Kb promoter (*11*, *12*, *15*). This outcome coincides with those of other immortal cell lines derived from hybrid mice (*16*, *23*, *24*). Moreover, SMP30/GNL KO immortal hepatocytes did not proliferate efficiently at the non-permissive temperature of 39°C, because the SV40 large T antigen tsA58 degrades rapidly at that temperature (*13*, *14*). In fact, the protein level of SV40 large T antigen in the cells cultured at 39°C for 1 day was <44% than that in the cells cultured at 33°C (Fig. 4B). These results indicated that the decrease of SV40 large T antigen at 39°C interfered with cell proliferative functions. Results were similar for other liver cell lines that expressed temperature-sensitive SV40 large T antigen but originating from a C57BL/6 mouse, not the Immortomouse (*26*, *27*). Moreover, those cell lines were not only arrested in growth but actually died after 2 days of culture after the temperature was elevated from 33°C to 39°C, even when IFN-γ was present in medium (*23*). Notably, our SMP30/GNL KO immortal hepatocytes not only remained alive, but also continued to grow to some extent for the entire 7 days of culture at 39°C (Fig. 4A). Since some SV40 large T antigen remained in the culture for 3 days at 39°C (data not shown), possibly even that small amount remaining exerted a proliferation effect on our cell line from a SMP30/GNL KO mouse or Immortomouse hybrid.

The temperature-sensitive SV40 large T antigen tsA58 forms complexes with p53 at 33°C, but at 39°C, the conformation of those complexes rapidly changes and they release p53 and then degrade (*28*, *29*). The temperature elevation from 33°C to 39°C simultaneously induces the expression of several chaperone proteins including the heat shock proteins (Hsp) 70 and Hsp40 (*30*). Hsps are highly conserved proteins, and Hsp70 is a central chaperone molecule involved in such functions as folding and prevention of protein aggregation (*30*, *31*). Hsp40 is known to interact and cooperate with Hsp70 (*30*, *31*). Hsp70 is generally located in the cytoplasm, although it is rapidly translocated to the nucleus in response to heat shock (*30*, *32*). As previously reported, translocated Hsp70 and/or Hsp40 seems likely to interact with

mis-folded SV40 large T antigen tsA58 and refold it in the nuclei at 39°C (*30*). The SMP30/GNL KO immortal hepatocytes expressed p53 at 33°C, but that expression was marginal after only 1 day of culture at 39°C (Fig. 4B).

In a phase contrast micrograph, SMP30/GNL KO immortal hepatocytes cultured at 39°C appear as distinct bi-nucleated cells with an enlarged cytoplasm (Fig. 4C). These features of a bi-nucleated form and enlarged cytoplasm are known to typify primary cultured hepatocytes from WT mice (Fig. 1B) (*33*). Decreases of p53 and SV40 large T antigen proteins in these cells when cultured at 39°C must have led to the morphological changes of SMP30/GNL KO immortal hepatocytes that caused their shape to so closely simulate that of primary cultured hepatocytes. Moreover, the albumin, that is a hepatocyte-specific marker, was clearly apparent when cells were cultured at both 33 and 39°C (Figs 2 and 4B). Thus, SMP30/GNL KO immortal hepatocytes have the same liver-specific function as primary cultured hepatocytes from WT mice.

As expected, SMP30/GNL KO immortal hepatocytes failed to synthesize vitamin C, because they completely lacked AA unless incubated with AA (Fig. 5). However, when the medium did contain AA, its content in the cells increased dose-dependently from 50 to 200 μM. Moreover, the AA content of these cells increased time-dependently for 6 h when cells were incubated with 100 μM AA in the medium, remained constant for 12 h, then decreased at 24 h. In our previous report, we examined the AA uptake by using primary cultured hepatocytes from SMP30/GNL KO and WT mice (*10*). We found that AA uptake ability were significantly enhanced in the AA-depleted hepatocytes from SMP30/GNL KO mice compared with the hepatocytes from WT mice which did not have depleted AA (*10*). After incubation with 100 μM AA in medium for 1 h at 37°C, the AA content of primary cultured hepatocytes from SMP/GNL KO and WT mice were 5.54 ± 0.35 nmol/mg protein and 3.25 ± 0.06 nmol/mg protein, respectively (*10*). In SMP/GNL immortal hepatocytes, AA contents were 3.82 ± 0.35 nmol/mg protein after incubation with 100 μM AA in medium for 1 h at 33°C (Fig. 5B). Although SMP/GNL immortal hepatocytes differ in culture condition such as medium and temperatures from primary cultured hepatocytes from SMP/GNL KO and WT mice, AA uptake seems to be higher in AA-depleted SMP/GNL immortal hepatocytes than in primary cultured hepatocytes from WT mice. The latter decrease of AA in the cells might have resulted from its consumption and/or export into the medium. Thus, SMP30/GNL KO immortal hepatocytes are a valuable model for studying AA uptake and the still undeciphered mechanism of AA efflux systems.

SMP30/GNL is best known as a protein that is expressed most prominently in the liver and kidney and that decreases with aging (*1*). Although mice normally synthesize vitamin C in the liver *in vivo*, humans cannot make vitamin C *in vivo* because, during evolution, many mutations altered the gluconolactone oxidase gene, which is essential for the AA biosynthetic pathway. However, humans have SMP30/GNL in various tissues such as the liver, kidney, pancreas and adrenals (*1*). These facts strongly indicate that SMP30/GNL has functions other than AA synthesis. In our previous reports, primary cultured hepatocytes from SMP30/GNL KO mice were more susceptible to apoptosis induced by tumour necrosis factor-α plus actinomycin D, than hepatocytes from WT mice (*2*). In addition, aged SMP30/GNL KO mice had unusually prominent deposits of lipofuscin and SA-β-GAL, which are regarded as senescence markers, in their renal tubular epithelia (*34*). Since these observations suggest that SMP30/GNL has multiple functions, not AA synthesis alone, SMP30/GNL KO immortal hepatocytes become an especially valuable tool for identifying those functions and malfunctions in humans.

In conclusion, with SMP30/GNL KO immortal hepatocytes, we have established a long-surviving and abundantly proliferating cell line. These SMP30/GNL KO immortal hepatocytes are extremely useful for studying AA and DHA uptake and unknown export systems, which involves multiple transporters including SVCTs and GLUTs. Moreover, SMP30/GNL KO immortal hepatocytes are a promising model for studying other probable but still obscure functions of SMP30/GNL.

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Conflict of interest

None declared.

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Absorption and Excretion of Ascorbic Acid Alone and in Acerola (*Malpighia emarginata*) Juice: Comparison in Healthy Japanese Subjects

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It has been suggested that some food components, such as bioflavonoids, affect the bioavailability of ascorbic acid in humans. Since little is known in Japan about the effective intake of this dietary requirement, we tested young Japanese males after the ingestion of commercial ascorbic acid or acerola (*Malpighia emarginata* DC.) juice to compare the quantities absorbed and excreted. Healthy Japanese subjects received a single oral dose of ascorbic acid solution (50, 100, 200 or 500 mg) and received distilled water as a reference at intervals of 14 d or longer. All subjects were collected blood and urine until 6 h after ingestion and evaluated for time-dependent changes in plasma and urinary ascorbic acid levels. Predictably, the area under the curve (*AUC*) values in plasma and urine after ingestion increased dose-dependently. Next, each subject received diluted acerola juice containing 50 mg ascorbic acid. Likewise, their plasma and urinary ascorbic acid concentrations were measured. In plasma, the *AUC* value of ascorbic acid after ingestion of acerola juice tended to be higher than that from ascorbic acid alone. In contrast, the urinary excretion of ascorbic acid at 1, 2 and 5 h after ingestion of acerola juice were significantly less than that of ascorbic acid. These results indicate that some component of acerola juice favorably affected the absorption and excretion of ascorbic acid.

Key words ascorbic acid; acerola; vitamin C

Ascorbic acid (vitamin C) is vitally important for human health, as widely reported and reviewed by Weber *et al.*[1] Among its nutritive attributes, ascorbic acid has numerous metabolic functions that are largely dependent on its potent reducing properties.[2] Additionally, ascorbic acid acts as a co-factor in reactions catalyzed by several metal-dependent oxygenases, *e.g.*, Cu^+-dependent mono-oxygenases including peptidylglycine α-amidating mono-oxygenase involved in peptide hormone synthesis,[3,4] dopamine β-hydroxylase involved in norepinephrine synthesis,[5,6] and Fe^{2+}/α-ketoglutarate-dependent dioxygenases including prolyl and lysyl hydroxylases involved in collagen synthesis.[7] Others include 6-*N*-trimethyllysine dioxygenase and γ-butyrobetaine dioxygenase involved in carnitine synthesis,[8] and asparaginyl hydroxylase, which modifies hypoxia-inducible factor 1.[9] Ascorbic acid depletion induces scurvy with such symptoms as dry skin, fatigue and bleeding. Moreover, ascorbic acid has non-enzymatic reductive activity in chemical reactions. That is, ascorbic acid's strong anti-oxidant function is evident as its ability to scavenge superoxide radicals in intracellular and extracellular reactions.[10] Many animals can synthesize ascorbic acid *in vivo*; however, others such as humans and guinea pigs have lost the ability to make ascorbic acid because of mutations in the L-gulono-γ-lactone oxidase gene, which is essential for ascorbic acid synthesis *in vivo*.[11] Therefore, animals without the enzyme activity of L-gulono-γ-lactone oxidase must obtain ascorbic acid from dietary sources.

Acerola (*Malpighia emarginata* DC.) is a fruit found throughout Central America and within the northern part of South America. This fruit is well known to be one of the best natural sources of ascorbic acid and has become extremely popular among health-conscious people. Besides ascorbic acid, acerola contains functional ingredients such as carotenoids,[12] γ-amino butyric acid (GABA)[13] and polyphenols.[14,15] As for polyphenols, acerola was found to contain cyanidin-3-α-*O*-rhamnoside and pelargonidin-3-α-*O*-rhamnoside as anthocyanins, quercitrin (quercetin-3-α-*O*-rhamnoside), hyperoside (quercetin-3-β-*O*-galactoside) and kaempferol glycosides as flavonols, and astilbin and proanthocyanidin.[15]

In this study, we measured the time-dependent changes of the plasma and urinary ascorbic acid levels after a single oral ingestion by healthy Japanese males, because little information about ascorbic acid bioavailability is available in this population. Moreover, we compared the pharmacokinetics in healthy subjects given ascorbic acid alone to that from acerola juice, one of its natural sources.

MATERIALS AND METHODS

Acerola Juice Acerola juice was prepared from the frozen mature fruit procured from Nichirei do Brazil (Recife, Brazil). The frozen acerola fruit was defrosted and squeezed by using a juice extractor (GP-E1503, GREEN POWER Co., Ltd., South Korea) and then filtered (No. 5C, Toyo Advantec Co., Tokyo, Japan).

Study Design This study protocol was approved by the Human Subjects Committee of the Tokyo Metropolitan Institute of Gerontology. All subjects gave their written informed consent.

Healthy Japanese males volunteered as subjects and ranged in age from 22 to 26 years with an average of 24±1 years. Only subjects who were non-smokers and did not take high-dose vitamin C supplements were included. All subjects

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received the same limited diet for three days before each test to control their blood level of ascorbic acid.

To measure the absorption and excretion of ascorbic acid alone, all subjects fasted overnight before ingesting a single dose of either 50, 100, 200 or 500 mg and distilled water as a reference. For this crossover experimental design, the ascorbic acid solution of all doses contained 100 ml distilled water. Each experiment was carried out at intervals of 14 d or longer. The blood was collected from each subject in tubes containing ethylenediaminetetraacetic acid (EDTA) at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5 and 6 h after the oral dose. The blood was immediately centrifuged at 1700 *g* for 15 min at 4 °C and the supernatant obtained was used as plasma. Urine samples were collected and their volume recorded every 1 or 2 h after the oral dose. The overall collection time was 6 h.

The same and more subjects volunteered for the acerola juice ingestion study. After an overnight fast, each subject ingested 100 ml acerola juice diluted with water containing 50 mg ascorbic acid. Plasma and urine collecting schedules were the same as above.

Measurement of Ascorbic Acid Total ascorbic acid was measured by using high-performance liquid chromatography (HPLC)-electrochemical detection as described previously.[16] The plasma was mixed with 4.5 volumes of 3% metaphosphoric acid (MPA) (Wako Pure Chemical, Osaka, Japan) and centrifuged at 21000 *g* for 10 min at 4 °C. The supernatants obtained were stored at −80 °C until use. Urine samples were also diluted with 10% MPA and stored at −80 °C until use. Samples were analyzed by HPLC using Atlantis dC18 5 μm columns (4.6×150 mm, Nihon Waters, Tokyo, Japan). The mobile phase was 50 mM phosphate buffer (pH 2.8), 0.2 g/l EDTA, 2% methanol at a flow rate of 1.3 ml/min, and electrical signals were recorded by using an electrochemical detector with a glassy carbon electrode at 0.6 V.

Creatinine levels in urine were measured with a Creatinine Test Wako kit (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instructions, and ascorbic acid levels in urine were normalized by creatinine value.

Statistical Analysis The experimental results are displayed as means±S.E.M. The significant differences were calculated by Student's *t* test.

RESULTS

Subjects' Characteristics The initial anthropometric and hematological characteristics of subjects in this study appear in Table 1. All hematological components were within normal levels.

Absorption of Ascorbic Acid in Plasma Oral ascorbic acid ingestion was measured in the plasma of all fasting subjects, as shown in Fig. 1A. The means of fasting plasma ascorbic acid concentrations were 32.9±1.1 μM. We measured the ascorbic acid amounts of the three day's prescribed diets. These were 34.3, 14.3 and 11.3 mg, respectively. Dietary control for 3 d and overnight fasting provided enough conditioning to ascertain accurate ascorbic acid levels in plasma.

Thereafter, upon oral ingestion of 50, 100, 200 and 500 mg, maximal concentrations of ascorbic acid (C_{max}) increased dose-dependently, as expected, to 41.1±4.7, 53.9±4.8, 55.2±5.8 and 63.1±4.0 μM, respectively. By contrast, in case of not receiving an ascorbic acid, the plasma ascorbic acid concentration had no notable change (Fig. 1). The time intervals to reach maximal ascorbic acid concentrations (T_{max}) were also longer dose-dependently as the interval lengthened from 1.5 to 3 h. Values for area under the curve (*AUC*) are shown in Fig. 1B. The slope rose most sharply at between 50 and

Table 1. Anthropometric and Hematological Characteristics of Subjects

	Japanese references	Means±S.D.
Age (year)	—	24.4±1.5
Height (cm)	—	172.4±2.7
Weight (kg)	—	63.6±6.7
TP (g/dl)	6.5—8.2	7.48±0.2
ALB (g/dl)	4.1—5.1	4.52±0.2
GLU (mg/dl)	70—110	90.68±2.2
AST (IU/l)	8—40	19.5±2.2
ALT (IU/l)	5—35	22.3±9.6
TG (mg/dl)	50—150	79.3±36.2
TC (mg/dl)	150—220	173.08±35.2
UN (mg/dl)	8—20	12.52±3.5
CRE (mg/dl)	0.7—1.2	0.84±0.1

TP: total protein; ALB: albumin; GLU: glucose; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TG: triglyceride; TC: total cholesterol; UN: urea nitrogen; CRE: creatinine.

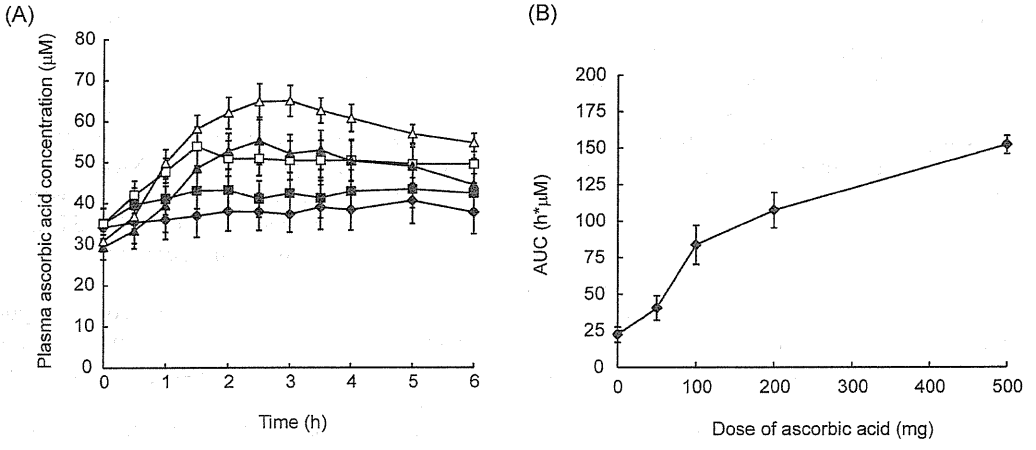

Fig. 1. (A) Plasma Time–Concentration Curves for Fasting Subjects Ingesting 0 to 500 mg of Ascorbic Acid

Symbols represent ingestion of ascorbic acid amount as follows: 0 mg; ◆, 50 mg; ▣, 100 mg; □, 200 mg; ▲, 500 mg; △. (B) The areas under the plasma ascorbic acid time–concentration curves after oral ingestion. The values are means±S.E.M., n=5.

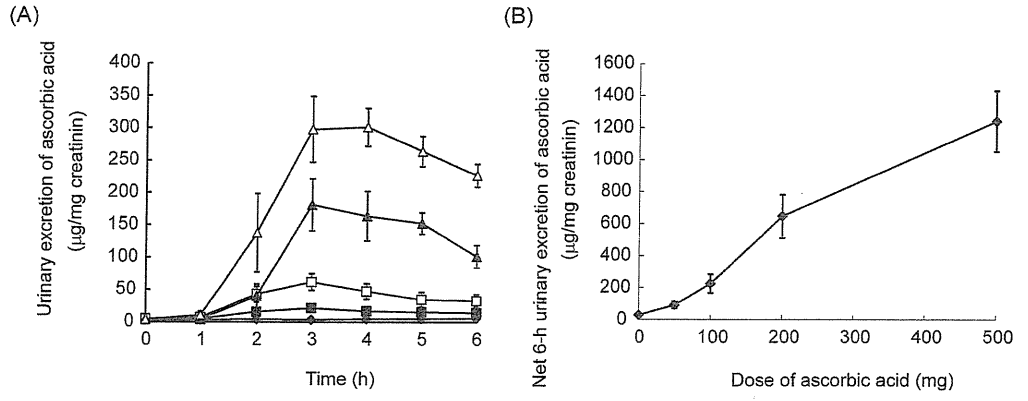

Fig. 2. (A) Urinary Excretion of Ascorbic Acid for Fasting Subjects Ingesting 0 to 500 mg of Ascorbic Acid

Symbols represent resting ascorbic acid amount as follows: 0 mg; ◆, 50 mg; ■, 100 mg; □, 200 mg; ▲, 500 mg; △. (B) The areas under the urinary ascorbic acid time–concentration curves after oral ingestion. The values are means±S.E.M., $n=5$.

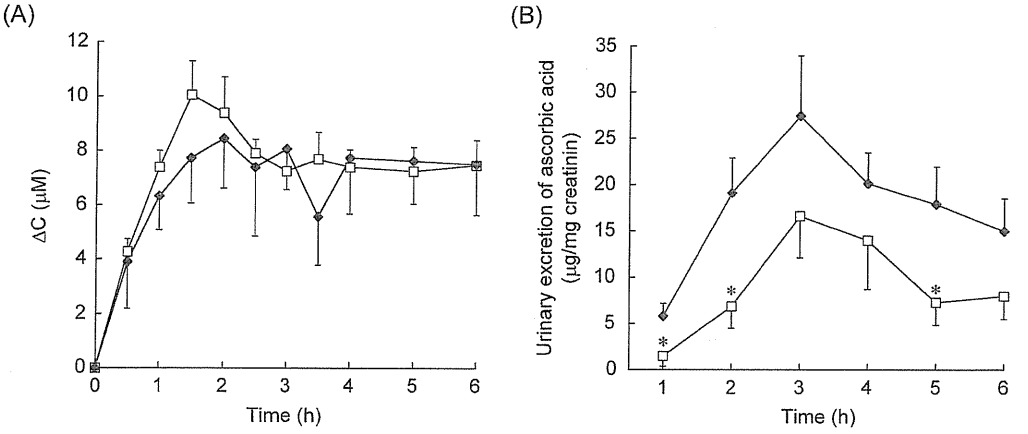

Fig. 3. (A) Plasma Time–Concentration Curves for Fasting Subjects Ingesting 50 mg Ascorbic Acid Alone; ◆, and That in Acerola juice; □ and (B) Urinary Excretion of Ascorbic Acid for Fasting Subjects Ingesting 50 mg Ascorbic Acid Alone; ◆, and That in Acerola Juice; □

The values are means±S.E.M., $n=6$. $*p<0.05$.

100 mg of ascorbic acid and then continued to increase but at a lesser rate.

Excretion of Ascorbic Acid from Urine The time to excretion of urinary ascorbic acid after oral ingestion is graphed in Fig. 2A. The y-axis indicates fractional excretion with creatinine collection. The peak times of ascorbic acid excretion were 3 to 4 h after oral ingestion of 50, 100, 200 and 500 mg. The total amount excreted for 6 h after oral ingestion increased dose-dependently (Fig. 2B).

Absorption and Excretion of Ascorbic Acid in Acerola Juice Considering the above results, we determined the most effective ascorbic acid dose for this comparative study was 50 mg. Accordingly, the subjects drank acerola juice containing 50 mg of ascorbic acid with the results shown in Fig. 3. The y-axis of Fig. 3A indicates ΔC calculated by the plasma ascorbic acid concentration, as follows; ΔC=concentration at each time−initial concentration. The ΔC_{max} of acerola juice exceeded that of ascorbic acid alone. In contrast, the urinary excretion of ascorbic acid at 1, 2 and 5 h after ingestion of acerola juice were significantly less than that of ascorbic acid (Fig. 3B). The ΔAUC value of ascorbic acid in plasma after acerola juice ingestion was a little greater than that of ascorbic acid alone (Table 2). Moreover, the net 6-h urinary excretion of ascorbic acid after acerola juice ingestion was less than that of ascorbic acid alone.

Table 2. Average ΔAUC of Plasma and Urinary of Ascorbic Acid

Source	ΔAUC of plasma ascorbic acid (h·μM)	Net 6-h urinary excretion of ascorbic acid (μg/mg creatinin)
Ascorbic acid	40.8±8.9	105.3±16.4
Acerola juice	43.4±3.6	54.0±16.4

Values are means±S.E.M.

DISCUSSION

In the present study, we measured the time-dependent changes of the plasma and urinary ascorbic acid levels after a single oral ingestion by healthy Japanese males, because little information about ascorbic acid bioavailability is available in this population. Levine *et al.*[17] measured the *AUC* value in plasma after oral or intravenous doses of 200, 500 and 1250 mg ascorbic acid. Bioavailability was complete for the 200 mg dose, but much lower for the higher doses. Our data were almost similar to theirs suggesting that there are no differences between the population groups in this aspect of ascorbic acid bioavailability.

To investigate the possibility that some components of food affect the absorption and excretion of ascorbic acid differently from that in ascorbic acid alone, we compared the bioavailability of ascorbic acid alone and that naturally present in acerola juice. As seen in Fig. 3 and Table 2, the acerola juice tended to promote the absorption of ascorbic acid in plasma and suppressed its excretion in urine better than the ascorbic acid alone.

Similarly, Vinson and Bose[18] reported that the ascorbic acid in citrus extract was more bioavailable than ascorbic acid alone in human subjects. They studied under two conditions. Their subjects were either saturated with or deprived of ascorbic acid in the body, then tested for the absorption and excretion of ascorbic acid in citrus extract *versus* ascorbic acid alone. In the subjects who were deprived with ascorbic acid in the body, the *AUC* value for ascorbic acid containing 500 mg citrus extract was greater than for ascorbic acid alone. In addition, significantly less ascorbic acid containing citrus extract was excreted. In contrast to the subjects who were saturated with ascorbic acid had a completely opposite outcome. These results suggest that the effect of some food components depends on ascorbic acid fractional saturation of subjects.

Bates *et al.*[19] also investigated this issue by using stable isotope-labeled ascorbic acid. There, grape juice containing anthocyanins, flavanols and flavonols tended to reduce absorption compared with ascorbic acid alone. We presume that their negative results were caused by using subjects who were saturated with ascorbic acid in the body. They concluded that the flavonoid in grape juice inhibited the ascorbic acid transporter, sodium-dependent vitamin C transporter 1 (SVCT1) in their report. Song *et al.*[20] reported that the most potent inhibitor class of flavonols, for example, myricetin, quercetin and anthocyanin, also had inhibitory capacity for ascorbic acid transport in a cell culture model. However, they noted that inhibition was eliminated if glycosylated residues were present at the C3 position of benzopyran. Acerola juice, which was used in the present study, contains cyanidin-3-α-*O*-rhamnoside and pelargonidin-3-α-*O*-rhamnoside. The C3 positions of these two polyphenols are glycosylated by rhamnose. Apparently, then, these two polyphenols in acerola juice are not inhibitors of SVCT1. In addition, the content of these polyphenols in diluted acerola juice was 6.5 μM and 1.0 μM, respectively. According to the inhibition study, the concentration giving 50% inhibition (IC_{50}) of cyanindin was 84 μM. Therefore, even if the anthocyanin in acerola juice is hydrolyzed in the ileum and intestines, it is considered that its presence does not affect SVCT1, because the content is less than the IC_{50} value of cyanindin.

In conclusion, the data in this study indicate that some component(s) of acerola juice affects the absorption of ascorbic acid into plasma and minimizes its excretion *via* urine. Clarification of not only those effects but also their mechanisms will assure the Japanese population's most effective intake of vitamin C.

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連載講座　老化を考える-6

加齢による臓器障害

加賀美弥生　丸山直記

老化が重大な関心事となったのは，生物学的な役割を終えた繁殖期より後の長期生存が多数の人間にとって可能になった結果である。生体を維持するためのシステム，すなわち酸素呼吸による代謝や免疫などは，正しく制御されなければ同時に自身を損なう害をもたらす存在でもある。野生における進化の過程では，集団の維持に必要な繁殖期までは有害性が有益性を超えないよう制御されてきたのであろう。しかし，その繁殖期を過ぎると，加齢に伴い制御が十分に行われないことにより蓄積された有害性が有益性を凌ぐことになる(図1)。このことが加齢による臓器障害の概観である。様々な事柄が加齢に伴う臓器障害に関与しているが，本稿ではこのような観点から，これまでのわれわれの研究を例に，活性酸素や分子修飾の有益性と有害性による臓器障害について解説したい。

Ⅰ．活性酸素と臓器障害

好気性生物は酸素を用いてエネルギー産生をするが，その代謝過程で反応性に富むラジカル(不対電子を持つ化学物質)が生じる。これが生体内の分子を傷つけ機能を低下させることが老化の原因とする老化のフリーラジカル説[1]，あるいはフリーラジカルを含め，酸素に由来する反応性の高い分子種を老化の原因とする酸化ストレス説は，様々な老化の原因を一元的に説明する有力な学説として認知されている。

酸素に由来する反応性の高い分子(活性酸素)は，生体内ではおもにミトコンドリアの電子伝達系で生じると考えられている。ほかにも細胞質にあるキサンチンオキダーゼやNADPHオキシダーゼなどによっても産生される[2-4]。活性酸素は細胞や組織を構成する生体分子，脂質，DNA，タンパク質などと反応し[5-7]，タンパク質の変性，酵素の不活性化，DNAの切断や塩基の修飾，細胞膜の変性などの障害を引き起こす毒性の高い分子である[8]。一方で，重要な生理的役割も有している。たとえば，NADPHオキシダーゼによって産生された活性酸素は，白血球による殺菌などの生体防御に用いられる[9]。また，近年，シグナル伝達物質としての作用も広く報告されている[10]。タンパク質のシステイン残基に対する活性酸素による酸化修飾は生体機能を調節するスイッチとして作用し，シグナル伝達に関連する脱リン酸化酵素，リン酸化酵素，代謝酵素，抗酸化酵素，NF-

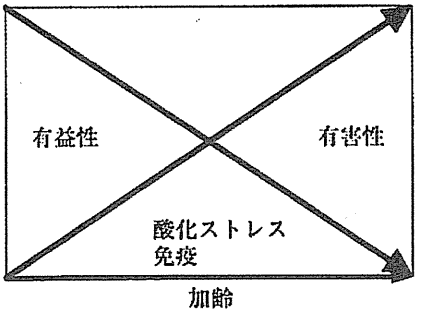

図1　加齢に伴う有害性の増加と有益性の減少の概念

Aging and organ disorder

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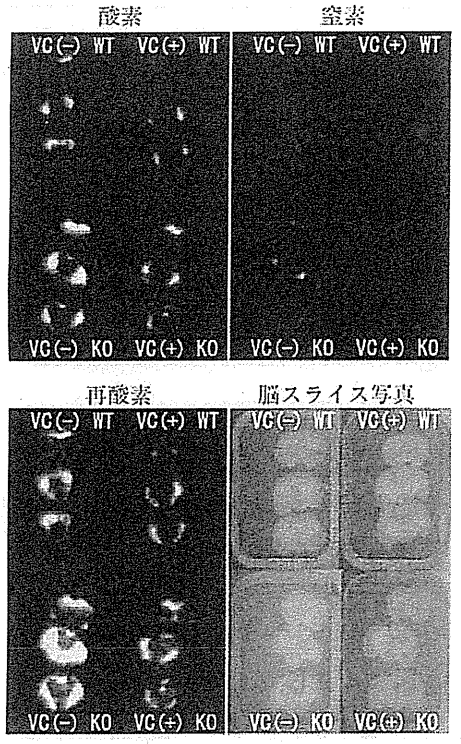

図 2　ビタミンC欠乏脳における活性酸素の生成増加
SMP30-KOマウス(KO)と野生型(WT)のマウスについて，それぞれビタミンC投与群VC(+)と非投与群VC(−)で低酸素(窒素)-再酸素処理を行った脳スライスのスーパーオキサイドによる化学発光像を示す。再酸素処理後VC(-)KOの脳において発光が増加している。(文献24より引用して改変)

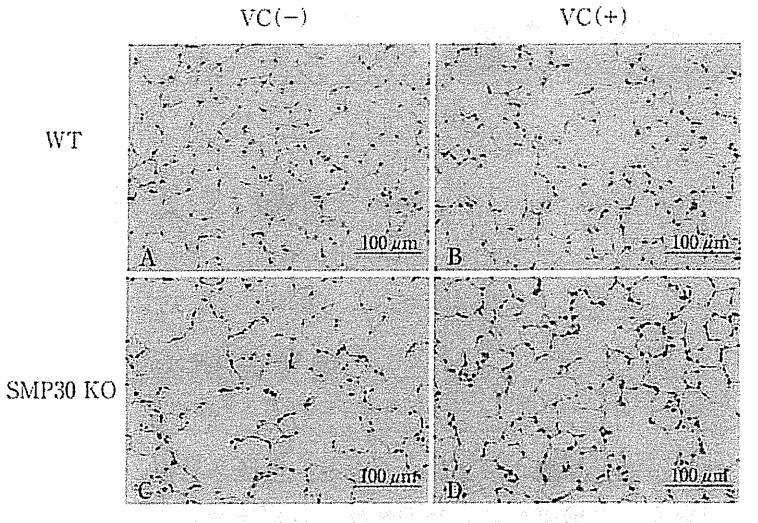

図 3　ビタミンC欠乏による肺の形態変化
SMP30-KOマウス(SMP30 KO)と野生型(WT)のビタミンC投与群VC(+)と非投与群VC(−)における肺組織(HE染色)。(文献26より引用)

κBやCrebなどの転写因子が制御されている[11-13]。

活性酸素は生体には必要不可欠な，しかし毒性を持つ分子であり，適切に管理される必要がある。このため細胞内には活性酸素の毒性を制御するための抗酸化酵素スーパーオキシドジスムターゼ(SOD)をはじめ，カタラーゼ，グルタチオンペルオキシダーゼや，グルタチオン，アスコルビン酸(ビタミンC)，ポリフェノールなどのフラボノイド類，カロテノイド類などの抗酸化物質が存在する[2]。また，酸化によって傷害を受けた分子をプロテアーゼやDNA修復酵素，ホスホリパーゼなどで除去，修復している[8]。

加齢に伴い，細胞内での活性酸素の産生と消去を制御する機構のバランスが偏ると，酸化ストレスが生じることになり，これによる老化や老化関連疾患との関連が研究されている。たとえば，アルツハイマー型認知症や筋萎縮性側索硬化症(ALS)，パーキンソン病などの神経変性疾患では，脳や神経細胞への酸化物質の蓄積や抗酸化物質の減少などの酸化ストレスの亢進が報告されている[14-16]。また，慢性閉塞性肺疾患(COPD)においても酸化ストレスは主要な因子であることが示唆されている[17,18]。当然のことながら，老化ばかりではなく生活習慣病にも重要なかかわりを持っている現象である。それゆえに中年期から初老期にかけて生活習慣病を管理することは高齢期における健康に重大な影響を与える。

本稿では，抗酸化物質であるアスコルビン酸(ビタミンC)欠乏による加齢臓器障害をわれわれが開発したモデル系を用いて紹介する。加齢に伴い発現が減少するタンパク質として発見された加齢指標タンパク質30(SMP30)[19]は，哺乳類のビタミンC合成酵素に必須のグルコノラクトナーゼとしての活性を有する[20]。SMP30/GNLノックアウト(-KO)マウスはビタミンCを合成できず，様々な臓器障害を呈する[21]。

ビタミンCが欠乏した臓器ではどのようなことが起こるのであろうか。脳は重量あたりの酸素消費量がほかに比べて非常に高い臓器である[22]。したがって，ミトコンドリアでのエネルギー産生量が大きく，必然的に多くの活性酸素が産生されて酸化ストレスに曝されやすい。われわれは生体脳組織の活性酸素レベルをリアルタイムバイオグ

ラフィー法[23]を用いて解析した[24]。ビタミンCを含む食餌と含まない食餌でそれぞれ飼育したSMP30/GNL-KOマウスと野生型マウスの脳スライスを比較すると，ビタミンCを含まない食餌で飼育したSMP30/GNL-KOマウス脳では活性酸素による化学発光が広範に強く検出された(図2：酸素)。次に虚血-再灌流のモデルとして，低酸素状態(図2：窒素)にしてから酸素を再添加すると(図2：再酸素)，ビタミンCを含まない餌で飼育されたSMP30/GNL-KOマウスでは活性酸素が多く検出された。このことから，ビタミンCの欠乏は脳での活性酸素の生成を増加させることが示された。ビタミンCは脳における活性酸素を消去することで，神経を酸化ストレスから保護していると考えられる。加齢による酸化ストレスの増大は種々の神経変性疾患の背景とみなすことができる。

高齢者の呼吸器疾患で重要なものとして慢性閉塞性肺疾患(chronic obstructive pulmonary disease：COPD)がある。喫煙による酸化ストレスが発症に関与していると考えられている[17]。これまでにわれわれは，SMP30/GNL-KOマウスをビタミンCの少ない食餌で飼育すると，野生型のマウスに比べて，肺での酸化ストレスマーカーであるカルボニル化タンパクが増加することを報告した[25]。さらに，ビタミンCを含む食餌と含まない食餌でそれぞれ2ヵ月間飼育したSMP30/GNL-KOマウスの肺を観察し，ビタミンCを含む食餌で飼育されたマウスは野生型の肺と同様の形状で違いはみられないが，ビタミンCが欠乏したマウスでは肺胞壁が破壊され，肺気腫を起こしていることを明らかにした(図3)。これはビタミンC欠乏による酸化ストレスの亢進と，ビタミンCがコラーゲンの合成に重要な役割を持つため，コラーゲンの合成の低下によって引き起こされたものと考えられる[26]。この肺胞壁の破壊は喫煙によりさらに増幅される。このような酸化ストレスの亢進は加齢に伴い全身で生じており，そのような背景を持つ個々の臓器に特有な誘因が加わることにより加齢性の臓器障害が発生する。

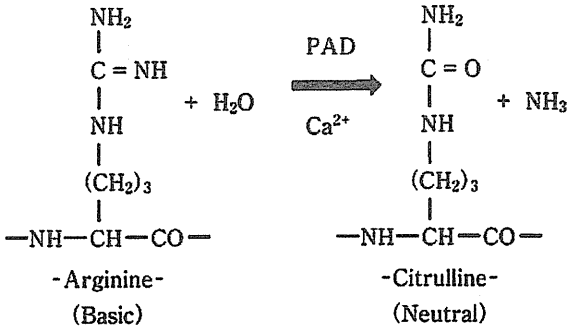

図4　PADによるタンパク質のシトルリン化
PADはカルシウムイオン存在下でタンパク質中のアルギニン残基をシトルリン残基へ転換する。

Ⅱ．分子修飾の異常と臓器障害

老齢動物の組織には異常なタンパク質が蓄積する。異常なタンパク質とは活性を失った酵素や立体構造が変化した酵素，異常な分子修飾を受けたタンパク質である。異常タンパク質が蓄積すると，細胞は正常な代謝機能を行えなくなる[27]。以下に，異常タンパク質の成因の一つである分子修飾，特にシトルリン化と加齢臓器障害について紹介したい。

分子修飾には活性酸素による酸化修飾，ブドウ糖による糖化修飾，酵素による翻訳後修飾などが挙げられる。カルボニル化タンパク質や終末糖化産物(AGE)などは酸化修飾により生じるタンパク質で，加齢に伴い増加することが知られている[8]。翻訳後修飾の一つであるシトルリン化は，カルシウム依存的に触媒する酵素ペプチジルアルギニンデイミナーゼ(PAD)によりペプチド中のアルギニン(塩基性)がシトルリン(中性)に変化し，タンパク質の構造と機能が変化する現象である(図4)[28-30]。

タンパク質のシトルリン化は生理的および病理的側面を有する事象である。生理的機能の例としては皮膚の保水性維持が挙げられる。表皮形成において，ケラチンを束ねる親水性アミノ酸を多く含むタンパク質のフィラグリンがシトルリン化タンパク質となり分解されることや，角化細胞の正常な分化に必要となっている[31,32]。また，好中球による病原体からの防御機構の一つであるneutrophil extracellular trapsの形成には，PADによ