Table 4 The odds ratios (and 95% confidence intervals) of tertiles of antioxidant intakes on low bone mineral density in post-menopausal Japanese female subjects

Dietary intake		Number	Range (mg/d) or (μg/d)	Model 1			Model 2			Model 3		
				OR	95% CI	*P* for trend	OR	95% CI	*P* for trend	OR	95% CI	*P* for trend
Retinol[a]	Lowest (Q1)	97	(29–213)	1.00			1.00			1.00		
	Middle (Q2)	98	(218–383)	1.30	(0.61–2.75)		1.65	(0.74–3.69)		1.28	(0.56–2.94)	
	Highest (Q3)	98	(386–3531)	2.37	(1.16–4.85)	0.014	3.22	(1.38–7.51)	0.007	2.52	(1.03–6.14)	0.031
Vitamin C	Lowest (Q1)	96	(47–139)	1.00			1.00			1.00		
	Middle (Q2)	99	(140–214)	1.15	(0.55–2.40)		1.02	(0.47–2.22)		1.03	(0.45–2.36)	
	Highest (Q3)	98	(215–625)	0.35	(0.15–0.80)	0.004	0.25	(0.10–0.66)	0.001	0.25	(0.07–0.82)	0.010
Vitamin E	Lowest (Q1)	101	(3.2–7.2)	1.00			1.00			1.00		
	Middle (Q2)	97	(7.3–9.1)	0.61	(0.29–1.27)		0.56	(0.25–1.25)		0.49	(0.21–1.14)	
	Highest (Q3)	95	(9.2–30.9)	0.61	(0.29–1.27)	0.244	0.45	(0.16–1.31)	0.176	0.43	(0.14–1.36)	0.193
Lycopene	Lowest (Q1)	121	(0.00–0.06)	1.00			1.00			1.00		
	Middle (Q2)	76	(0.15–0.15)	1.02	(0.48–2.15)		1.10	(0.51–2.35)		1.06	(0.48–2.34)	
	Highest (Q3)	96	(0.36–1.78)	1.55	(0.79–3.04)	0.177	1.72	(0.85–3.47)	0.117	1.60	(0.75–3.38)	0.201
α-Carotene	Lowest (Q1)	95	(0.03–0.23)	1.00			1.00			1.00		
	Middle (Q2)	97	(0.24–0.37)	0.73	(0.36–1.48)		0.79	(0.38–1.66)		0.90	(0.42–1.95)	
	Highest (Q3)	101	(0.38–1.27)	0.77	(0.38–1.57)	0.522	0.78	(0.36–1.67)	0.551	1.05	(0.45–2.45)	0.882
β-Carotene	Lowest (Q1)	97	(0.34–1.52)	1.00			1.00			1.00		
	Middle (Q2)	99	(1.53–2.36)	0.61	(0.29–1.27)		0.63	(0.29–1.35)		0.74	(0.32–1.70)	
	Highest (Q3)	97	(2.37–8.19)	0.75	(0.37–1.53)	0.586	0.69	(0.31–1.55)	0.487	0.93	(0.33–2.62)	0.981
Lutein	Lowest (Q1)	98	(0.49–1.68)	1.00			1.00			1.00		
	Middle (Q2)	97	(1.70–2.58)	1.56	(0.74–3.28)		1.84	(0.83–4.06)		2.10	(0.89–4.93)	
	Highest (Q3)	98	(2.59–10.01)	1.25	(0.59–2.62)	0.762	1.39	(0.60–3.23)	0.698	1.94	(0.69–5.48)	0.339
β-Cryptoxanthin	Lowest (Q1)	98	(0.00–0.30)	1.00			1.00			1.00		
	Middle (Q2)	101	(0.31–1.21)	0.52	(0.25–1.10)		0.47	(0.22–1.01)		0.49	(0.22–1.10)	
	Highest (Q3)	94	(1.22–7.91)	0.46	(0.21–1.00)	0.099	0.40	(0.17–0.92)	0.068	0.53	(0.22–1.28)	0.295
Zeaxanthin	Lowest (Q1)	95	(0.08–0.46)	1.00			1.00			1.00		
	Middle (Q2)	100	(0.47–0.96)	1.73	(0.82–3.65)		1.95	(0.89–4.27)		1.71	(0.75–3.87)	
	Highest (Q3)	98	(0.97–6.09)	1.96	(0.93–4.13)	0.104	2.65	(1.11–6.31)	0.038	2.51	(0.99–6.33)	0.061

Model 1: Age, weight and height were adjusted. Model 2: Years since menopause, current tobacco use, regular alcohol intake, exercise habits, supplement use, and total energy intake were further adjusted. Model 3: Intakes of calcium, magnesium, potassium, and vitamins D were further adjusted

[a] Preformed retinol

Table 5 The odds ratios (and 95% confidence intervals) of four groups stratified by dietary intakes of vitamin C and β-cryptoxanthin on low bone mineral density in post-menopausal Japanese female subjects

		β-Cryptoxanthin intake					
		Low intake (0–0.96mg/d)			High intake (0.97–7.91mg/d)		
		Number	OR	95%CI	Number	OR	95%CI
Vitamin C intake	Low intake (47–169 mg/d)	113	1.00	(Reference)	34	0.73	(0.27–1.99)
	High intake (170–625 mg/d)	36	0.52	(0.18–1.52)	110	0.42	(0.19–0.93)

Age, weight, height, years since menopause, current tobacco use, regular alcohol intake, exercise habits, supplement use, and total energy intake were adjusted

Discussion

The objective of this study was to investigate the associations of dietary patterns of antioxidant vitamin and carotenoid intake with radial BMD in post-menopausal Japanese female subjects. The results indicate that radial BMD was significantly associated with a dietary pattern heavily loaded on β-cryptoxanthin and vitamin C. Furthermore, we found that a high intake of vitamin C with β-cryptoxanthin was inversely associated with a low radial BMD. This investigation is the first reported cross-sectional study to examine the association of dietary patterns of antioxidant vitamin and carotenoid intake with BMD. Numerous antioxidant vitamins and carotenoids are contained in fruits and vegetables, and several recent epidemiological reports have shown inverse associations of antioxidant vitamin and carotenoid intake or serum level with low BMD, risk of fracture, and/or risk of osteoporosis [16–18]. However, the association of BMD with dietary patterns of antioxidant vitamin and carotenoid intake has not been thoroughly studied. Our findings further support the hypothesis that high intakes of fruits and vegetables rich in antioxidant vitamins and carotenoids, especially vitamin C and β-cryptoxanthin, may be beneficial to bone health in post-menopausal women.

On the other hand, some epidemiological studies have reported that excessive intake of retinol may have adverse effects on BMD [34–36]. In our study, a positive association between the factor score of the "Retinol" pattern and low radial BMD was observed after adjustments for age, years since menopause, weight, height, current tobacco use, regular alcohol intake, exercise habits, use of dietary supplements, and total energy. For dietary antioxidants, a significantly higher odds ratio was observed in the highest tertile of preformed retinol intake against the lowest tertile used for the reference group after multivariate adjustments. The recommended daily intake of retinol activity equivalents is 600 μgRE/day for Japanese women, with a tolerable upper intake of 3,000 μgRE/day [37]. In the highest tertile of preformed retinol intake, all of them consumed more than the recommended dietary allowance for Japanese adult females (600 μgRE/day) although most subjects consumed less than 3,000 μgRE/day. The effect of the dietary amount of preformed retinol on bone metabolism in Japanese female subjects has not been studied in detail, but a high intake of preformed retinol may be associated with the risk for low radial BMD. Further study is required.

In our data analyses, we identified three dietary patterns of antioxidant vitamin and carotenoid intake from the principal component analysis. Although all dietary patterns were heavily loaded on vitamin C intake, the highest positive loading between vitamin C, and dietary pattern was observed in the "β-cryptoxanthin" pattern. On the other hand, an association between β-cryptoxanthin and dietary pattern was observed in only the "β-cryptoxanthin" pattern, which had an extremely high factor loading. Vitamin C and β-cryptoxanthin are especially concentrated in citrus fruits such as Japanese mandarin orange. Therefore, a high intake of citrus fruit may be inversely associated with low BMD. In fact, in our previous study, we found that fruit intake was inversely associated with low radial BMD [25].

In animals, an experimentally induced deficiency of vitamin C led to impairments in bone mass, cartilage, and connective tissues [38, 39]. The protein in the bone matrix is over 90% collagen [40]. Vitamin C is an essential cofactor for the formation of collagen and the synthesis of hydroxyproline and hydroxylysine [41]. Therefore, vitamin C is an important micronutrient for the maintenance of bone health. Furthermore, it is well known that vitamin C reduces oxidative stress by scavenging singlet oxygen and peroxyl radicals. The relationship between oxidative stress and BMD or osteoporosis has recently been reported [16–18]. From the finding of osteopetrosis in mice lacking NF-κB1 and NF-κB2, Iotsova et al. reported that NF-κB proteins are important for osteoclastogenesis [42]. NF-κB is activated by the exposure of cells to oxidative stress [43]. Therefore, it seems that reactive oxygen species enhance osteoclastogenesis and bone resorption. In fact, some studies have implicated reactive oxygen species in bone regulation [44, 45]. Furthermore, in epidemiological studies, it was reported that oxidative stress levels were

negatively associated with BMD and that antioxidant levels were lower in osteoporotic patients [19–24]. These previous findings in epidemiological and experimental studies suggest that antioxidant micronutrients may provide benefits to bone metabolism against oxidative stress. Therefore, it seems that vitamin C is an important micronutrient for the maintenance of bone health through its biological action on cofactors for collagen formation, the synthesis of hydroxyproline and hydroxylysine, and antioxidant activity.

Carotenoids, as antioxidants, may also play an important role in the prevention of oxidative stress-related osteoclastogenesis and bone resorption. Very recently, Yamaguchi et al. reported the beneficial effects of β-cryptoxanthin on bone metabolism [46–48]. Through in vitro and in vivo studies, they found that β-cryptoxanthin stimulated bone formation and inhibited bone resorption. Their results support the idea that β-cryptoxanthin may have a direct stimulatory effect on bone formation and an inhibitory effect on bone resorption. Recent epidemiological studies have shown an association of serum β-cryptoxanthin with bone health. Yang et al. examined serum-carotenoid concentrations in postmenopausal American female subjects and found that the serum concentrations of β-cryptoxanthin and lycopene were significantly lower in osteoporotic subjects than in non-osteoporotic subjects [22]. Furthermore, we found that serum β-cryptoxanthin was significantly but partially associated with radial BMD [25]. The results of these experimental and epidemiological studies strongly support the hypothesis that the development of osteoporosis may be reduced by β-cryptoxanthin intake.

In our data analysis, significantly lower odds ratios in the highest tertiles of vitamin C and β-cryptoxanthin intakes were observed, but these significant associations were not observed after adjusting for β-cryptoxanthin and/or vitamin C intakes, respectively (OR, 0.36; CI, 0.12–1.11 for vitamin C and OR, 0.70; CI, 0.27–1.90 for β-cryptoxanthin). These results indicate that a combined intake of vitamin C and β-cryptoxanthin may be associated with radial BMD. Next, we examined the association of low radial BMD with the combined intake of vitamin C and β-cryptoxanthin. A significantly lower odds ratio was observed in the high-intake group for both of vitamin C and β-cryptoxanthin than in the low-intake group for both nutrients after adjustments for age, years since menopause, weight, height, current tobacco use, regular alcohol intake, exercise habits, use of dietary supplements, and total energy. However, this significantly lower odds ratio became insignificant after further adjustments for intakes of calcium, magnesium, potassium, and vitamin D (data not shown). For this reason, we think that these micronutrients might be more relevant factors for BMD rather than vitamin C and β-cryptoxanthin, or there is no denying the possibility of multicollinearity among these nutrients because these micronutrients were also rich in fruit and vegetables. From these results, we concluded that the intakes of vitamin C and β-cryptoxanthin may be significantly but partially associated with radial BMD, and these associations may be caused by a combination of vitamin C and β-cryptoxanthin. To our knowledge, there has been no experimental or epidemiological study of the combined effect of vitamin C and carotenoid on bone metabolism. It is conceivable that, rather than vitamin C alone, vitamin C intake combined with the intakes of other antioxidants such as carotenoids may yield an important dietary pattern conducive to the maintenance of bone health. Further studies on the complicated interactions of antioxidants on bone metabolism are required.

This study had some limitations. First, the data obtained here cross-sectional; therefore, only limited inferences can be made regarding temporality and causation. Furthermore, the sample size was limited, and thus further large-scale studies are required. Second, in our survey, portion size questions were not included for most items. Absolute nutrient intake could not be estimated from FFQ without portion size questions. Third, we evaluated radial BMD at 1/3 of the forearm length measured from the styloid process on the ulna. Therefore, an analysis of the association of serum carotenoids with BMD in cancellous bone, such as the femoral neck or lumbar spine, is required. Lastly, we could not evaluate the dietary patterns of other antioxidants such as flavonoids. Some studies have shown a beneficial effect of bioactive flavonoids on bone metabolism [49, 50].

In conclusion, dietary patterns heavily loaded on β-cryptoxanthin, and vitamin C are associated with radial BMD in post-menopausal Japanese female subjects. A high intake of vitamin C with β-cryptoxanthin is inversely associated with low radial BMD and may be beneficial to bone health. To determine whether antioxidant vitamins and carotenoids are beneficial to bone health, further cohort or intervention studies are required.

Acknowledgment This work was supported by a grant from the Ministry of Agriculture, Forestry, and Fisheries (MAFF) for a food research project titled "Integrated Research on Safety and Physiological Function of Food" and a grant from the Council for Advancement of Fruit Tree Science. We are grateful to the participants in our survey and to the staff of the health examination program for residents of the town of Mikkabi, Shizuoka, Japan. We are also grateful to the staff of the Seirei Preventive Health Care Center (Shizuoka, Japan).

Conflicts of interest None.

References

1. Ministry of Health, Labor and Welfare. Comprehensive survey of living conditions of the people on Health and Welfare. Section3 2004. Available at: http://www.mhlw.go.jp/toukei/saikin/hw/k-tyosa/k-tyosa04/4-2.html (accessed 1 December 2009)

2. Christodoulou C, Cooper C (2003) What is osteoporosis? Postgrad Med J 79:133–138
3. Gennari C (2001) Calcium and vitamin D nutrition and bone disease of the elderly. Public Health Nutr 4:547–559
4. Prentice A (2004) Diet, nutrition and the prevention of osteoporosis. Public Health Nutr 7:227–243
5. Macdonald HM, New SA, Golden MH, Campbell MK, Reid DM (2004) Nutritional associations with bone loss during the menopausal transition: evidence of a beneficial effect of calcium, alcohol, and fruit and vegetable nutrients and of a detrimental effect of fatty acids. Am J Clin Nutr 79:155–165
6. New SA, Bolton-Smith C, Grubb DA, Reid DM (1997) Nutritional influences on bone mineral density: a cross-sectional study in premenopausal women. Am J Clin Nutr 65:1831–1839
7. Prynne CJ, Mishra GD, O'Connell MA, Muniz G, Laskey MA, Yan L, Prentice A, Ginty F (2006) Fruit and vegetable intakes and bone mineral status: a cross-sectional study in 5 age and sex cohorts. Am J Clin Nutr 83:1420–1428
8. Okubo H, Sasaki S, Horiguchi H, Oguma E, Miyamoto K, Hosoi Y, Kim MK, Kayama F (2006) Dietary patterns associated with bone mineral density in premenopausal Japanese farmwomen. Am J Clin Nutr 83:1185–1192
9. McGartland CP, Robson PJ, Murray LJ, Cran GW, Savage MJ, Watkins DC, Rooney MM, Boreham CA (2004) Fruit and vegetable consumption and bone mineral density: the Northern Ireland Young Hearts Project. Am J Clin Nutr 80:1019–1023
10. Tucker KL, Chen H, Hannan MT, Cupples LA, Wilson PW, Felson D, Kiel DP (2002) Bone mineral density and dietary patterns in older adults: the Framingham Osteoporosis Study. Am J Clin Nutr 76:245–252
11. Gutteridge JM (1994) Biological origin of free radicals, and mechanisms of antioxidant protection. Chem Biol Interact 91:133–140
12. Rock CL, Jacob RA, Bowen PE (1996) Update on the biological characteristics of the antioxidant micronutrients: vitamin C, vitamin E, and the carotenoids. J Am Diet Assoc 96:693–702
13. Almeida M, Han L, Martin-Millan M, O'Brien CA, Manolagas SC (2007) Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor- to forkhead box *O*-mediated transcription. J Biol Chem 282:27298–27305
14. Jilka RL, Weinstein RS, Parfitt AM, Manolagas SC (2007) Quantifying osteoblast and osteocyte apoptosis: challenges and rewards. J Bone Miner Res 22:1492–1501
15. Garrett IR, Boyce BF, Oreffo RO, Bonewald L, Poser J, Mundy GR (1990) Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. J Clin Invest 85:632–639
16. Basu S, Michaelsson K, Olofsson H, Johansson S, Melhus H (2001) Association between oxidative stress and bone mineral density. Biochem Biophys Res Commun 288:275–279
17. Yalin S, Bagis S, Polat G (2005) Is there a role of free oxygen radicals in primary male osteoporosis? Clin Exp Rheumatol 23:689–692
18. Law MR, Hackshaw AK (1997) A meta-analysis of cigarette smoking, bone mineral density and risk of hip fracture: recognition of a major effect. BMJ 315:841–846
19. Melhus H, Michaelsson K, Holmberg L, Wolk A, Ljunghall S (1999) Smoking, antioxidant vitamins, and the risk of hip fracture. J Bone Miner Res 14:129–135
20. Maggio D, Barabani M, Pierandrei M (2003) Marked decrease in plasma antioxidants in aged osteoporotic women: results of a cross-sectional study. J Clin Endocrinol Metab 88:1523–1527
21. Maggio D, Polidori MC, Barabani M, Tufi A, Ruggiero C, Cecchetti R, Aisa MC, Stahl W, Cherubini A (2006) Low levels of carotenoids and retinol in involutional osteoporosis. Bone 38:244–248
22. Yang Z, Zhang Z, Penniston KL, Binkley N, Tanumihardjo SA (2008) Serum carotenoid concentrations in postmenopausal women from the United States with and without osteoporosis. Int J Vitam Nutr Res 78:105–111
23. Sahni S, Hannan MT, Blumberg J, Cupples LA, Kiel DP, Tucker KL (2009) Inverse association of carotenoid intakes with 4-y change in bone mineral density in elderly men and women: the Framingham Osteoporosis Study. Am J Clin Nutr 89:416–424
24. Sahni S, Hannan MT, Gagnon D, Blumberg J, Cupples LA, Kiel DP, Tucker KL (2009) Protective effect of total and supplemental vitamin C intake on the risk of hip fracture—a 17-year follow-up from the Framingham Osteoporosis Study. Osteoporos Int 20:1853–1861
25. Sugiura M, Nakamura M, Ogawa K, Ikoma Y, Ando F, Yano M (2008) Bone mineral density in post-menopausal female subjects is associated with serum antioxidant carotenoids. Osteoporos Int 19:211–219
26. Wakai K, Egami I, Kato K, Lin Y, Kawamura T, Tamakoshi A, Aoki R, Kojima M, Nakayama T, Wada M, Ohno Y (1999) A simple food frequency questionnaire for Japanese diet—Part I. Development of the questionnaire, and reproducibility and validity for food groups. J Epidemiol 9:216–226
27. Egami I, Wakai K, Kato K, Lin Y, Kawamura T, Tamakoshi A, Aoki R, Kojima M, Nakayama T, Wada M, Ohno Y (1999) A simple food frequency questionnaire for Japanese diet—Part II. Reproducibility and validity for nutrient intakes. J Epidemiol 9:227–234
28. Science and Technology Agency (1983) Standard tables of food composition in Japan, 4th edn. Printing Bureau, Ministry of Finance, Tokyo, in Japanese
29. Science and Technology Agency (1997) Standard tables of food composition in Japan. (for new foods], 5th edn. Printing Bureau, Ministry of Finance, Tokyo, in Japanese
30. Yano M, Kato M, Ikoma Y, Kawasaki A, Fukazawa Y, Sugiura M, Matsumoto H, Ohara Y, Nagao A, Ogawa K (2005) Quantitation of carotenoids in raw and processed fruits in Japan. Food Sci Technol Res 11:13–18
31. Aizawa K, Inakuma T (2007) Quantitation of carotenoids in commonly consumed vegetables in Japan. Food Sci Technol Res 13:247–252
32. Sugiura M, Nakamura M, Ogawa K, Ikoma Y, Matsumoto H, Ando F, Shimokata H, Yano M (2009) Synergistic interaction of cigarette smoking and alcohol drinking with serum carotenoid concentrations: findings from a middle-aged Japanese population. Br J Nutr 102:1211–1219
33. Orimo H, Hayashi Y, Fukunaga M et al (2001) Diagnostic criteria for primary osteoporosis. J Bone Miner Metab 19:331–337
34. Feskanich D, Singh V, Willett WC, Colditz GA (2002) Vitamin A intake and hip fractures among postmenopausal women. JAMA 287:47–54
35. Melhus H, Michaëlsson K, Kindmark A, Bergström R, Holmberg L, Mallmin H, Wolk A, Ljunghall S (1998) Excessive dietary intake of vitamin A is associated with reduced bone mineral density and increased risk for hip fracture. Ann Intern Med 129:770–778
36. Promislow JH, Goodman-Gruen D, Slymen DJ, Barrett-Connor E (2002) Retinol intake and bone mineral density in the elderly: the Rancho Bernardo Study. J Bone Miner Res 17:1349–1358
37. Ministry of Health, Labor and Welfare. Recommended dietary allowance for Japanese: dietary reference intakes. Section 2 2009. Available at: http://www.mhlw.go.jp/shingi/2009/05/dl/s0529-4i.pdf (accessed 1 December 2009)

38. Poal-Manresa J, Little K, Trueta J (1970) Some observations on the effects of vitamin C deficiency on bone. Br J Exp Pathol 51:372–378
39. Kipp DE, McElvain M, Kimmel DB, Akhter MP, Robinson RG, Lukert BP (1996) Scurvy results in decreased collagen synthesis and bone density in the guinea pig animal model. Bone 18:281–288
40. Termine JD (1990) Cellular activity, matrix proteins, and aging bone. Exp Gerontol 25:217–221
41. Peterkofsky B (1991) Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. Am J Clin Nutr 54:1135S–1140S
42. Iotsova V, Caamano J, Loy J, Yang Y, Lewin A, Bravo R (1997) Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. Nat Med 3:1285–1289
43. Baeuerle PA, Rupec RA, Pahl HL (1996) Reactive oxygen intermediates as second messengers of a general pathogen response. Pathol Biol (Paris) 44:29–35
44. Garrett IR, Boyce BF, Oreffo RO, Bonewald L, Poser J, Mundy GR (1996) Oxygen-derived free radicals stimulate osteoclastic bone resorption in rodent bone in vitro and in vivo. J Clin Invest 85:632–639
45. Bax BE, Alam AS, Banerji B, Bax CM, Bevis PJ, Stevens CR, Moonga BS, Blake DR, Zaidi M (1992) Stimulation of osteoclastic bone resorption by hydrogen peroxide. Biochem Biophys Res Commun 183:1153–1158
46. Yamaguchi M, Uchiyama S (2003) Effect of carotenoid on calcium content and alkaline phosphatase activity in rat femoral tissues in vitro: the unique anabolic effect of beta-cryptoxanthin. Biol Pharm Bull 26:1188–1191
47. Yamaguchi M, Uchiyama S (2004) Beta-cryptoxanthin stimulates bone formation and inhibits bone resorption in tissue culture in vitro. Mol Cell Biochem 258:137–144
48. Uchiyama S, Yamaguchi M (2004) Oral administration of beta-cryptoxanthin induces anabolic effects on bone components in the femoral tissues of rats in vivo. Biol Pharm Bull 27:232–235
49. Hosseinimehr SJ, Nemati A (2006) Radioprotective effects of hesperidin against gamma irradiation in mouse bone marrow cells. Br J Radiol 79:415–418
50. Chiba H, Uehara M, Wu J, Wang X, Masuyama R, Suzuki K, Kanazawa K, Ishimi Y (2003) Hesperidin, a citrus flavonoid, inhibits bone loss and decreases serum and hepatic lipids in ovariectomized mice. J Nutr 133:1892–1897

2．サルコペニアの疫学

下方　浩史*　安藤　富士子**
(しもかた　ひろし　あんどう　ふじこ)

● サルコペニアは40歳以上の地域在住男性の25.0%，女性の24.2%に認められる．
● 加齢とともに男女で筋力は年間約1%低下する．筋量は男性のみで低下し，女性では筋量の変化は少なく，筋肉の質的な変化があるものと思われる．
● 一般の高齢者では，筋力や筋量には遺伝子多型よりも生活習慣などの影響のほうが大きい．
● サルコペニアの主な危険因子は運動不足と低栄養，特に蛋白摂取の不足，カロテノイドやビタミンDの不足である．
● 適度な運動と適切な栄養摂取に心がけることで筋量や筋力の低下を防ぐことは十分可能であると考えられる．

Key Words　サルコペニア，疫学研究，カロテノイド，ビタミンD，DXA．

サルコペニアは高齢者のADLを低下させ，健康長寿実現の大きな障害となる．しかし，老化に伴う筋量減少の実態は明らかでなく，また日常生活機能との関連もはっきりはしていない．特に一般地域住民での日常生活機能と骨格筋量，筋力との関連についての疫学研究は日本ではほとんどない．高齢者のサルコペニアや脆弱を予防していくためには，日本におけるサルコペニアの実態を明らかにするとともに，サルコペニアの危険因子を明らかにするような観察研究や介入研究などの疫学的研究が重要である．

□ サルコペニアの実態

サルコペニアの頻度はその定義により異なるが，米国でのNew Mexico Elderly Health Studyからの報告[1]では，二重エネルギーX線吸収法（DXA）を用いた診断で，70歳未満では20%程度であるが，80歳以上になると50%以上がサルコペニアとなるとしている．

われわれは平成9年から，無作為抽出された地域住民を対象としたコホート研究「国立長寿医療研究センター・老化に関する長期縦断疫学研究（NILS-LSA）」を実施しており[2]，第5次調査の参加者，40～88歳の中高年者2,419名（男性1,200名，女性1,219名）を対象としてサルコペニアの頻度について検討を行った．DXA（QDR 4500，Hologic）装置をと用いて四肢除脂肪・除骨重量測定し，これを四肢筋量とした．前述のNew Mexico Elderly Health Studyの方法[1]に準じ，四肢筋量（kg）を身長（m）の二乗で除した値をskeletal muscle index（SMI）とし，サルコペニアの指標とした．その判定基準には同じDXAのHologic QDR 4500で測定したSanadaら[3]によるYAM（young adult mean：18～40歳の若年成人平均値）から標準偏差の2倍を引いた男性6.87 kg/m^2，女性5.46 kg/m^{2}を基準値としてサルコペニアの有無を判定した．その結果，この基準値から40歳以上の男性の25.0%が，女性の24.2%がサルコペニアに分類された（**図1**）．

運動神経線維のうち，筋線維を支配して実際の筋収縮に関与するα運動ニューロンは，加齢とともに50%も低下するといわれる．特に下肢では軸索が長くなって障害を受けやすい．また筋の増殖に必要な骨格筋組織特異的幹細胞であるサテライト細胞も数が減少することが知られている．食欲の低下や運動不足，性ホルモンの分泌低下，炎症反応の増大などサルコペニアを引き起こすさまざまな要因が，加齢に伴って増加する[4,5]．

SMIは男性年間約0.3%低下するが，女性ではSMIの低下はほとんどない．一方，筋力は男女

*国立長寿医療研究センター予防開発部　**愛知淑徳大学健康医療科学部

とも 40 歳以降，握力も下肢筋力も年間約 1%ずつ減少する．このことは女性では筋肉の量的な変化よりも質的な変化が問題になっていることを示している．男性ではどの年代においても女性よりも筋力は強く，80 代の男性の筋力は 40 代の女性の筋力にほぼ等しい（**図 2**）．もともと女性は男性よりも筋力が弱いために，加齢による筋力の低下は女性により大きな影響を与える．

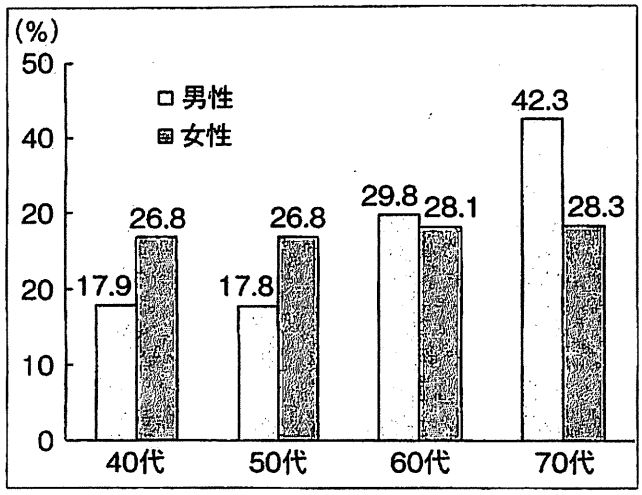


図 1　年齢・性別にみたサルコペニアの頻度

DXA 法により性別の若年成人平均値（YAM）の −2 SD を基準としてサルコペニアの判定を行った．女性では年齢による変化はなかったが，男性では年代上昇で割合が有意に上昇していた（Cochran-Mantel-Haenszel 検定で p trend<0.001）．

□ サルコペニアの危険因子

ヨーロッパにおける老年学，栄養学などの学会によるワーキンググループである The European Working Group on Sarcopenia in Older People (EWGSOP)[6] ではサルコペニアを，加齢以外に明らかな誘因がない原発性サルコペニア（primary sarcopenia）と，加齢以外の何らかの要因がサルコペニアを引き起こす二次性サルコペニア（secondary sarcopenia）に分類している．さらに二次性サルコペニアを，ベッド上安静，運動しない生活スタイル，廃用，無重力状態などが原因となる身体活動性サルコペニア（activity-related sarcopenia），高度な臓器障害，炎症性疾患，悪性腫瘍に伴う悪液質，内分泌疾患などによる疾患性サルコペニア（disease-related sarcopenia），吸収不良，胃腸疾患，食思不振を引き起こす薬剤の使用に伴うエネルギーおよび蛋白質摂取不足などによる栄養性サルコペニア（nutrition-related sarcopenia）の 3 つに分けている（**表 1**）．

これまでに報告されているサルコペニアの危険因子には遺伝的素因，性別，加齢，身長，体重，BMI，閉経，エストロゲン，テストステロン，

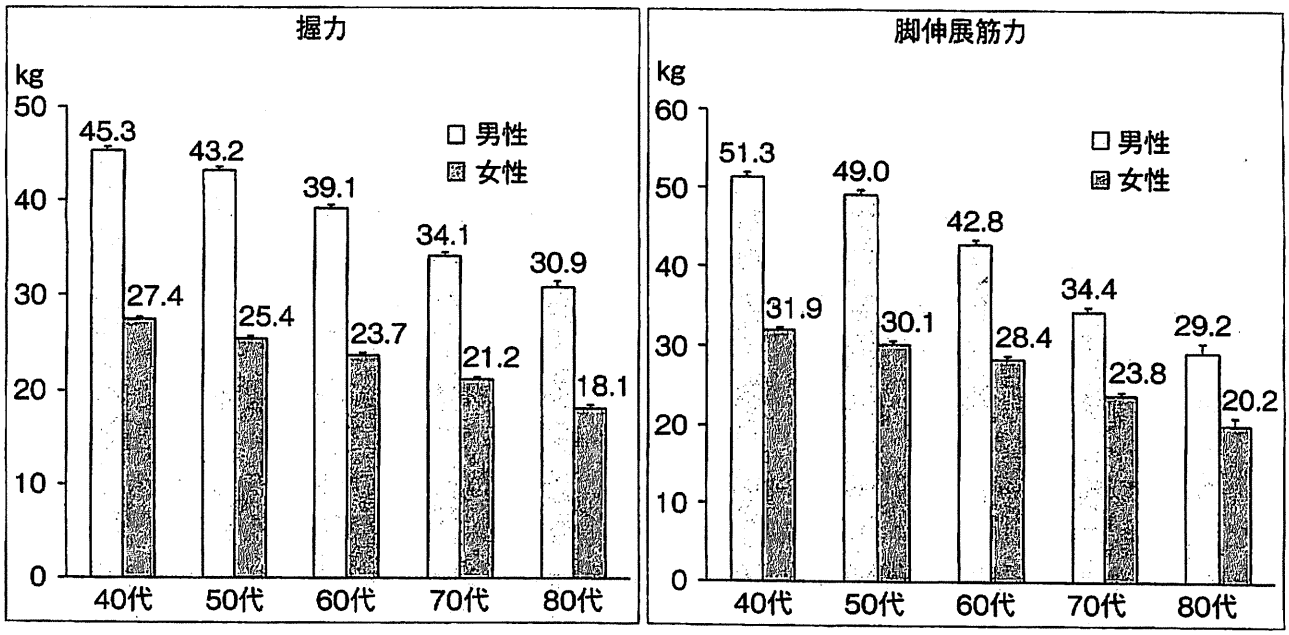


図 2　年齢・性別にみた筋力

利き手の握力および脚伸展筋力（大腿四頭筋筋力）の年代別平均値および標準誤差．握力，脚伸展筋力は男女ともに，年代上昇に伴い減少していた（p trend<0.001）．しかし，男性では握力は 80 代でも女性の 40 代よりも大きく，また男性の脚伸展筋力は 80 代でも女性の 40 代とほぼ同じ値であった．

表1　サルコペニアの分類

分類	原因
原発性サルコペニア	
加齢性サルコペニア	加齢以外の原因がない
二次性サルコペニア	
身体活動性サルコペニア	ベッド上安静，運動しない生活スタイル，廃用，無重力状態
疾患性サルコペニア	高度な臓器障害（心臓，肺，肝臓，腎臓，脳），炎症性疾患，悪性腫瘍，内分泌疾患
栄養性サルコペニア	吸収不良，胃腸疾患，食思不振を引き起こす薬剤の使用に伴うエネルギー，蛋白質摂取不足

(Cruz-Jentoft AJ, et al.：Age Ageing 39, 412-423, 2010. より引用)[6]

総体脂肪量，身体活動，カロテノイド，ビタミンD，分岐鎖アミノ酸および蛋白摂取量などがある．遺伝的な素因としては，myostainのLys153Arg多型，alpha-actinin 3のR577X多型が筋量や筋力に関連しているとの報告がある[7,8]．しかし，レジスタンストレーニングを行うスポーツ選手ではこうした遺伝子多型の影響があるとしても，一般の高齢者では遺伝子多型よりもむしろ生活習慣のほうが筋力や筋量への影響が大きいと思われる．

□ 身体活動とサルコペニア

運動不足による筋量や筋力の低下はどの年代にも起きうる．しかし高齢者では筋の再生・増殖機能が低下しており，いったん減少した筋量は回復が難しい．筋量が低下し筋力が低下すれば，運動が困難になり，さらに筋量が低下するという悪循環に陥りやすい[1]．一方，筋力トレーニングを中心とした運動介入により高齢者でも筋力や筋量が増加するという報告は多く[4,6]，身体活動はサルコペニア予防の重要な要素であるともいえる．

□ 性ホルモンとサルコペニア

閉経により内臓脂肪は増加し，骨密度が低下し，筋量および筋力が低下する．一方，エストロゲンの投与はこれらの変化を予防する効果があるとされる．テストステロンの筋増殖効果はよく知られている[9]．高齢男性のテストステロンの低下と筋量，筋力の低下が報告されている．加齢に伴い，性ホルモン結合グロブリン（SHBG）が増加し，生体作用を持つ遊離テストステロンが大きく低下する．テストステロンは蛋白合成を促進する．テストステロンの低下は蛋白合成能の低下をきたし，筋肉を萎縮させる．さらにテストステロンの低下は筋サテライト細胞数の低下を引き起こし，筋肉の再生・増殖能を低下させるといわれている[10]．

□ カロテノイドとサルコペニア

高齢者の筋力低下，身体機能低下はフリーラジカルによる酸化ストレスが原因の1つとなっている可能性が指摘されている．酸化ストレスは骨格筋のDNAを傷つけ，蛋白質や脂質に障害を与える[11,12]．

抗酸化作用を持つカロテノイドが不足すると，高齢者では筋力低下や歩行障害をきたすことが，いくつかの疫学的研究で報告されている．米国のWomen's Health and Aging Studiesでは，年齢，人種，喫煙，心血管性疾患，関節炎，血清IL-6を調整して検討したところ，血清総カロテノイドの低下は握力，腰や脚の筋力の低下と有意に関連していた[13]．イタリア，トスカーナ州キャンティ地区の地域在住高齢者での研究でも，βカロテン摂取量が高齢者の脚伸展筋力と相関していた[14]．

NILS-LSAのデータでは血清カロテノイドと体力・運動やADLとの関係が示されている．外出に不安がある人，階段の昇降や長距離の歩行が困難である人では，血清カロテノイドが低値を示した．一方，筋力や余暇活動時間，一日平均歩数は血清カロテノイドと正の相関を示し，特に日常活動量を示す一日平均歩数はすべての血清カロテノイドで正の関連を示していた．摂取エネルギー

で調整したβカロテン摂取量も正の関連を示したことから，単に「元気な人がたくさん食べている」のではなく，多く摂取する人が体力的にも健康であり，また，ADLの低下している人ではカロテノイドが不足している状況が明らかになった．

□ ビタミンDとサルコペニア

血中の25-OHビタミンDレベルは経口摂取，あるいは皮膚で産生されたビタミンDの量を反映する指標である．25-OHビタミンDは老化とともに低下することが知られている．ビタミンDはカルシウム代謝に関連するビタミンであり，摂取量の不足は骨粗鬆症などの骨疾患の要因となる．このビタミンDが筋肉とも関連することが明らかになってきた．

ビタミンD受容体は筋肉中に存在し，ビタミンDが低下することで筋肉の蛋白同化作用が下がってしまう．またビタミンD受容体の遺伝子多型が高齢者のサルコペニアの要因の1つであることも報告されている[15]．ビタミンDの低下が，高齢者の転倒や身体機能障害の要因であるとの報告がある[16,17]．アムステルダム縦断加齢研究（Longitudinal Aging Study Amsterdam）での3年間の追跡研究ではベースラインの25-OHビタミンDが低値の場合には，高値の場合に比べて3年後にサルコペニアとなるオッズ比は2.57（95%信頼区間1.40～4.70）であった．このような結果からビタミンDの摂取の不足が，高齢者のサルコペニアを引き起こす可能性があると思われる[18]．

□ 蛋白質，アミノ酸とサルコペニア

筋肉は蛋白質からなっており，蛋白質摂取量，アミノ酸摂取量が低下すれば，筋量は当然低下する．1食あたりの蛋白質量が20～25 gであるときが筋蛋白質の合成がもっとも高いとされている[19,20]．食事摂取基準では，健康な70歳以上者に必要な蛋白質量は1.06 g/体重kgであり，男性60 g，女性50 g以上が必要とされている．しかし，エネルギー消費量が減少し，食事量全体が少なくなってしまう高齢者の食事では，必要な蛋白質摂取を維持することが難しい場合もある．

体内で合成できない必須アミノ酸のうちロイシン，イソロイシン，バリンは炭素骨格が分岐した構造をもつことから分岐鎖アミノ酸と呼ばれる．これらの分岐鎖アミノ酸は筋肉をつくる主な蛋白質であるアクチンとミオシンの主成分である．十分な分岐鎖アミノ酸を摂取することで筋肉の消耗を防ぐことができる可能性がある[21,22]．分岐鎖アミノ酸は肉類，乳製品，レバーなどに多く含まれるが，高齢者ではこうした食品は敬遠されることが多いことも，高齢者のサルコペニアの要因となっている可能性がある．

まとめ

サルコペニアは40歳以上の地域在住男性の25.0%，女性の24.2%に認められ，多くの人がサルコペニアの状態にある．サルコペニアは特に高齢者に多くみられ，健康長寿達成の大きな障害となる．サルコペニアの予防が，高齢化がさらに進んでいく今後の日本にとって重要な課題である．サルコペニアの主な危険因子は運動不足と低栄養，特に蛋白質摂取の不足，カロテノイドやビタミンDの不足である．筋力や筋量の低下には，老化による避けがたい生理的な要因もあるが，適度な運動と適切な栄養摂取に心がけることで，筋量や筋力の低下を防ぐことは十分可能である．

文 献

1) Baumgartner RN, Koehler KM, Gallagher D, et al.: Epidemiology of sarcopenia among the elderly in New Mexico. Am J Epidemiol 147: 755-763, 1998
2) Shimokata H, Ando F, Niino N: A new comprehensive study on aging-the National Institute for Longevity Sciences, Longitudinal Study of Aging (NILS-LSA). J Epidemiol 10: S1-S9, 2000
3) Sanada K, Miyachi M, Tanimoto M, et al.: A cross-sectional study of sarcopenia in Japanese men and women: reference values and association with cardiovascular risk factors. Eur J Appl Physiol 110: 57-65, 2010
4) Volpi E, Nazemi R, Fujita S: Muscle tissue changes with aging. Curr Opin Clin Nutr Metab Care 7: 405-410, 2004
5) Doherty TJ: Aging and sarcopenia. J Appl Physiol 95: 1717-1727, 2003
6) Cruz-Jentoft AJ, Baeyens JP, Bauer JM, et al.: Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in

Older People. Age Ageing 39 : 412-423, 2010
7) Clarkson PM, Devaney JM, Gordish-Dressman H, et al. : ACTN3 genotype is associated with increases in muscle strength in response to resistance training in women. J Appl Physiol 99(1) : 154-163, 2005 Jul
8) Ivey FM, Roth SM, Ferrell RE, et al. : Effects of age, gender, and myostatin genotype on the hypertrophic response to heavy resistance strength training. J Gerontol A Biol Sci Med Sci 55 : M641-648, 2000
9) Maltais ML, Desroches J, Dionne IJ : Changes in muscle mass and strength after menopause. J Musculoskelet Neuronal Interact 9 : 186-197, 2009
10) Solomon AM, Bouloux PM : Modifying muscle mass-the endocrine perspective. J Endocrinol 191 : 349-360, 2006
11) Semba RD, Lauretani F, Ferrucci L : Carotenoids as protection against sarcopenia in older adults. Arch Biochem Biophys 458 : 141-145, 2007
12) Weindruch R : Interventions based on the possibility that oxidative stress contributes to sarcopenia. J Gerontol A Biol Sci Med Sci 50 : 157-161, 1995
13) Semba RD, Blaum C, Guralnik JM, et al. : Carotenoid and vitamin E status are associated with indicators of sarcopenia among older women living in the community. Aging Clin Exp Res 15 : 482-487, 2003
14) Cesari M, Pahor M, Bartali B, et al. : Antioxidants and physical performance in elderly persons : the Invecchiare in Chianti (InCHIANTI) study. Am J Clin Nutr 79 : 289-294, 2004
15) Roth SM, Zmuda JM, Cauley JA, et al. : Vitamin D receptor genotype is associated with fat-free mass and sarcopenia in elderly men. J Gerontol A Biol Sci Med Sci 59 : 10-15, 2004
16) Suzuki T, Kwon J, Kim H, et al. : Low serum 25-hydroxyvitamin D levels associated with falls among Japanese community-dwelling elderly. J Bone Miner Res 23 : 1309-1317, 2008
17) Kwon J, Suzuki T, Yoshida H, et al. : Concomitant lower serum albumin and vitamin D levels are associated with decreased objective physical performance among Japanese community-dwelling elderly. Gerontology 53 : 322-328, 2007
18) Visser M, Deeg DJ, Lips P : Low vitamin D and high parathyroid hormone levels as determinants of loss of muscle strength and muscle mass (sarcopenia) : the Longitudinal Aging Study Amsterdam. J Clin Endocrinol Metab 88 : 5766-5772, 2003
19) Paddon-Jones D, Short KR, Campbell WW, et al. : Role of dietary protein in the sarcopenia of aging. Am J Clin Nutr 87 : 1562S-1566S, 2008
20) Paddon-Jones D, Rasmussen BB : Dietary protein recommendations and the prevention of sarcopenia. Curr Opin Clin Nutr Metab Care 12 : 86-90, 2009
21) Dreyer HC, Volpi E : Role of protein and amino acids in the pathophysiology and treatment of sarcopenia. J Am Coll Nutr 24 : 140S-145S, 2005
22) Fujita S, Volpi E : Amino acids and muscle loss with aging. J Nutr 136(Suppl) : 277S-280S, 2006

Geriat. Med. 49(3)：303～306, 2011

Seminar

2．虚弱の危険因子

下方 浩史　安藤富士子

KEY WORD

- 虚弱
- 高齢者
- 危険因子
- 体力
- 老化

SUMMARY

高齢者の虚弱は年齢が高いほど割合が高くなり，また男性よりも女性で割合が高い．虚弱の要因としては，低栄養，喫煙，慢性疾患への罹患，慢性炎症，性ホルモンの減少などの身体的要因に加えて，世帯の年収や教育歴，人種，生活空間など社会的な因子も重要である．高齢者ではこれらの多くの要因が重積し，虚弱を引き起こすものと考えられる．高齢者の虚弱に対しての運動や栄養による介入研究が数多く行われているが，その効果ははっきりしていない．運動介入や栄養の単独の介入では虚弱の予防は難しく，生活全般のサポートでの対応が望まれる．

はじめに

日本人の平均寿命は年々長くなり，高齢者，特に後期高齢者の人口が急増している．しかし，高齢になるほど虚弱な高齢者は増加する．一方で少子化が進み，今後は若い労働力が不足していくことが予想される．そのような状況で，介護のために若い人材の労力が費やされるようでは，日本の国が成り立っていかなくなってしまうだろう．高齢化する日本の社会で，介護や支援を要するような虚弱な高齢者を減らし，健康長寿を達成することは急務となっている．本稿では，高齢者の虚弱の要因を明らかにし，その予防法を探る．

性別，加齢と虚弱

虚弱は男性よりも女性に多い．日本では平均寿命は女性の方が男性よりも7歳近く長い．寝たきりの期間も女性の方が長く，虚弱の女性の数は男性よりも多い．しかし年齢を調整しても虚弱のリスクは男性よりも女性の方が高い[1]．また，虚弱な女性の死亡率は男性よりも高いという報告もある[2]．

虚弱の定義にもよるが，虚弱は75歳以上の20～30％に認められ，高齢になるほどその割合は高くなる[3]．多くの研究で，加齢は虚弱の最も強い危険因子の1つに挙げられている．しかし，加齢そのものが虚弱の要因なのか，加齢に伴って生じる様々な障害や疾病が虚弱の要因であって，これらの要因をすべて除いても加齢が虚弱の要因であるかどうかについては，まだ十分には明らかにされていない．

生活習慣と虚弱

高齢者では一般に身体活動量が減り，また歯の脱落，嗅覚や味覚の低下，消化機能の低下など生理学的な要因に加えて，抑うつなどの精神的な要因のため食欲が低下する．こうした生活習慣の変化が高齢者の虚弱を引き起こす可能性が高い．虚弱の栄養学的要因として低栄養，痩

しもかた ひろし（国立長寿医療研究センター予防開発部長）／あんどう ふじこ（愛知淑徳大学健康医療科学部教授）

せが重要である．特に摂取エネルギー，蛋白質や必須アミノ酸摂取の低下，ビタミンやミネラル，特にビタミンD，カロテン，ビタミンB_{12}，葉酸の摂取不足は高齢者の虚弱と関連が深いといわれている[4]．

虚弱における栄養の指標としてアルブミン，コレステロールが使われてきた．横断的な解析では，低アルブミン血症（血清アルブミン3.5 g/dL未満）は地域在住高齢者の身体機能やADL障害に関連していた[5,6]．縦断的研究では，3.8 g/dL以下の低アルブミン血症が3年後の身体機能低下と関連していたが，7年後の身体機能低下とは関連をしていなかった．170 mg/dL未満の低コレステロール血症は死亡のリスクにはなっていたが，虚弱のリスクにはなっていなかった[7]．コレステロールとアルブミンを組み合わせた縦断的な検討では，血清総コレステロールが5.2 mmol/L（201 mg/dL）以下で女性での虚弱危険因子となっていたが，血清アルブミンが4.3 g/dL以下での判定では男女とも虚弱危険因子とはならなかった．しかし，コレステロールとアルブミンの両方を組み合わせたところ，男性での虚弱危険因子となった[8]．HDLコレステロールについても施設入所の高齢者の2年間の追跡で，身体機能低下の重要なリスクファクターになっていることが示されている[9]．虚弱の栄養指標は，単独では虚弱を見逃してしまうこともある．いくつかの指標を組み合わせて判断することも重要であろう．

高齢者の虚弱についての大規模な縦断研究として，米国の40,657人の65～79歳の女性を対象とした3年間の追跡研究Women's Health Initiative Observational Study（WHI-OS）がある[10]．WHI-OSではベースライン調査で16.3%が虚弱と判断され，さらに3年間の追跡で14.8%が新たに虚弱となった．虚弱の要因として生活習慣についても詳細な調査が行われているが，その結果では喫煙は虚弱の危険因子であるが，飲酒は少量ならばむしろ虚弱を予防するという結果が出ている．また，体重は低体重も肥満もともに正常体重に比べて虚弱の要因となっていた．

慢性疾患と虚弱

WHI-OSの報告では慢性疾患やうつ症状が虚弱の要因であり，一方，自覚的健康度が高いことは虚弱を防ぐ要因であった．虚弱との関連が認められた慢性疾患は，冠動脈疾患，脳血管障害，糖尿病，高血圧症，大腿骨頸部骨折，慢性閉塞性肺疾患（COPD），転倒，抑うつ，関節炎であった[10]．さらに認知症や認知機能障害が，高齢者の虚弱と関連しているとする報告もある[11,12]．

慢性の炎症も虚弱の要因となる．IL-6が3.8 pg/mLを超える場合，CRPが2.65 mg/Lを超える場合には，3年間の追跡で有意に身体機能が低下していた[7]．男性ホルモンの低下についても，高齢男性の虚弱の要因であるとの報告がある．米国での1,469名の65歳以上高齢男性の検討では，血清テストステロン濃度が低いほど虚弱の割合が多く，4年間の縦断的追跡でも血清テストステロン濃度が低いほど虚弱となるリスクが高かった[13]．男性高齢者の場合，アンドロポーズと呼ばれる加齢に伴う男性ホルモンの低下が，虚弱の要因として重要である．副腎や性腺で産生される男性ホルモンの1種であるデヒドロエピアンドロステロン（DHEA）も低値であることが，高齢男女で虚弱と関連していた[14]．これら様々な慢性疾患や病態が重積することで，さらに虚弱の危険が増加する．

社会経済的要因と虚弱

同じ定義を用いても，虚弱高齢者の分布には地域差があるといわれている．ヨーロッパ10カ国の調査では，65歳以上の虚弱高齢者の割合はスイスの5.8%からスペインの27.3%までと異なっており，同じヨーロッパでも概して南欧は北欧よりも虚弱な高齢者が多いと結果であった[15]．この地域差には教育など社会経済的な要因が関与しているという．

米国のWHI-OSでは社会経済的要因として，世帯年収が高いほど，教育が長いほど，白人に比べむしろ黒人やアジア人でリスクが低かっ

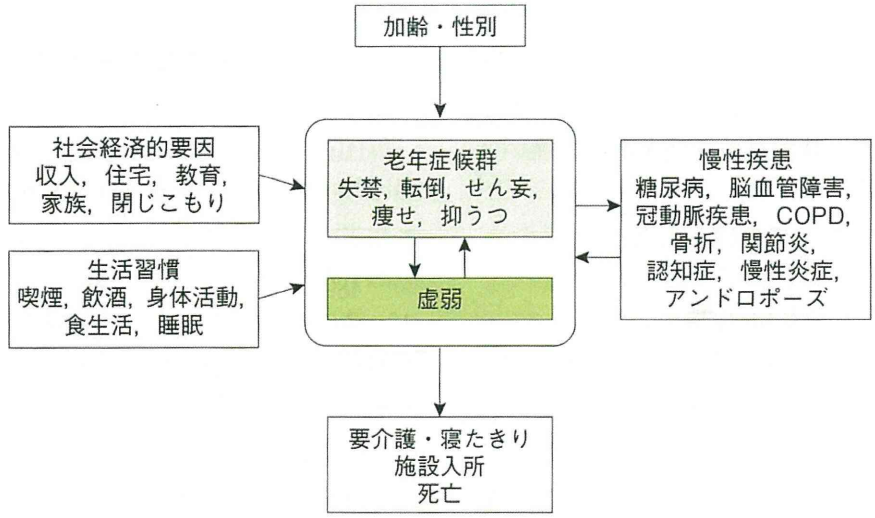


図1　高齢者の虚弱の要因と老年症候群

た[10]．また，一人暮らしは虚弱となるリスクを20%下げていた．一人暮らしは，他の家族に依存できず自立が必要なためと思われる．一方で，3年間にわたる縦断的研究で，外出頻度が少ない，いわゆる「閉じこもり」で虚弱の発生率が高かったとの報告もある[16]．

虚弱高齢者への介入研究

虚弱の予防を目指しての介入研究が繰り返し行われている．1994年に『New England Journal of Medicine』に掲載されたFiataroneらによる虚弱高齢者への古典的な介入研究がある[17]．施設入所中の高齢者に対する無作為割付研究で，筋肉トレーニングにより虚弱の有意な改善が認められている．運動による介入の虚弱の改善効果については，ほかのよくデザインされた研究でも認められているが[18]，否定的な結果の研究もある[19]．

栄養での介入でも虚弱の改善効果ははっきりしない．Fiataroneらによる無作為割付研究でのビタミン，ミネラル，蛋白質，脂質，炭水化物による栄養介入では，虚弱の改善効果は認められなかった[17]．必須アミノ酸であるバリン，ロイシン，イソロイシンの3つを分岐鎖アミノ酸という．筋肉を構成している必須アミノ酸の約35～40%がこの分岐鎖アミノ酸であり，筋肉の蛋白質分解を抑制する．高齢者の筋量維持，増加にこの分岐鎖アミノ酸が有効だとする報告は多い[20]．しかし，実際に無作為割付研究を行っても，ロイシンをサプリメントとして3カ月間にわたって高齢男性に投与した介入試験では，筋肉量や筋力への影響はなかったという[21]．このほかにもビタミンDの投与による栄養介入の研究などもあるが[19]，虚弱の改善効果は認められていない．

おわりに

老年症候群は高齢者に特有のあるいは高頻度にみられる諸症状であり，高齢者のADLやQOLを阻害する．老年症候群には，めまい，息切れ，痩せ，食欲不振，抑うつ，転倒，関節痛，視力低下，聴力低下などが含まれる．これらの老年症候群の諸症状は，高齢者虚弱との関わりが強い．**図1**に示すように，加齢，性別は高齢者の虚弱の要因として重要であるが，世帯年収や教育，住宅環境，家族構成などの社会経済的要因，喫煙，飲酒，身体活動，食生活などの生活習慣，糖尿病，脳血管障害，冠動脈疾患，COPD，骨折，関節炎，認知症，慢性炎症，アンドロポーズなどの慢性疾患や慢性的な病態が，高齢者の虚弱を引き起こす．これらの要因は同時に様々な老年症候群の要因にもなる．さらに

高齢者の虚弱が老年症候群の原因ともなる．そして要介護や寝たきり，施設入所，最終的には死に至る．このように，多くの要因が重積し虚弱を引き起こす．運動介入や栄養の単独の介入では虚弱の予防は難しい．高齢者に対しての生活全般のサポートによる対応が望まれる．

文　献

1) Mor V et al：Functional transitions among the elderly：patterns, predictors, and related hospital use. Am J Public Health **84**：1274-1280, 1994.
2) Puts MT et al：Sex differences in the risk of frailty for mortality independent of disability and chronic diseases. J Am Geriatr Soc **53**：40-47, 2005.
3) Topinkova E：Aging, disability and frailty. Ann Nutr Metab **52**(Suppl 1)：6-11, 2008.
4) Bartali B et al：Low nutrient intake is an essential component of frailty in older persons. J Gerontol A Biol Sci Med Sci **61**：589-593, 2006.
5) Salive ME et al：Serum albumin in older persons：relationship with age and health status. J Clin Epidemiol **45**：213-221, 1992.
6) Jensen GL et al：Nutrition risk screening characteristics of rural older persons：relation to functional limitations and health care charges. Am J Clin Nutr **66**：819-828, 1997.
7) Reuben DB et al：Peripheral blood markers of in inflammation predict mortality and functional decline in high-functioning community-dwelling older persons. J Am Geriatr Soc **50**：638-644, 2002.
8) Schalk BW et al：Lower levels of serum albumin and total cholesterol and future decline in functional performance in older persons：the Longitudinal Aging Study Amsterdam. Age Ageing **33**：266-272, 2004.
9) Zuliani G et al：Low levels of high-density lipoprotein cholesterol are a marker of disability in the elderly. Gerontology **45**：317-322, 1999.
10) Woods NF et al；Women's Health Initiative：Frailty：emergence and consequences in women aged 65 and older in the Women's Health Initiative Observational Study. J Am Geriatr Soc **53**：1321-1330, 2005.
11) Black SA and Rush RD：Cognitive and functional decline in adults aged 75 and older. J Am Geriatr Soc **50**：1978-1986, 2002.
12) Buchman AS et al：Frailty is associated with incident Alzheimer's disease and cognitive decline in the elderly. Psychosom Med **69**：483-489, 2007.
13) Cawthon PM et al；Osteoporotic Fractures in Men (MrOS) Research Group：Sex hormones and frailty in older men：the osteoporotic fractures in men (MrOS) study. J Clin Endocrinol Metab **94**：3806-3815, 2009.
14) Voznesensky M et al：The association between dehydroepiandosterone and frailty in older men and women. Age Ageing **38**：401-406, 2009.
15) Santos-Eggimann B et al：Prevalence of frailty in middle-aged and older community-dwelling Europeans living in 10 countries. J Gerontol A Biol Sci Med Sci **64**：675-681, 2009.
16) Xue QL et al：Life-space constriction, development of frailty, and the competing risk of mortality：the Women's Health And Aging Study I. Am J Epidemiol **167**：240-248, 2008.
17) Fiatarone MA et al：Exercise training and nutritional supplementation for physical frailty in very elderly people. N Engl J Med **330**：1769-1775, 1994.
18) Gill TM et al：A program to prevent functional decline in physically frail, elderly persons who live at home. N Engl J Med **347**：1068-1074, 2002.
19) Latham NK et al：A randomized, controlled trial of quadriceps resistance exercise and vitamin D in frail older people：the Frailty Interventions Trial in Elderly Subjects (FITNESS). J Am Geriatr Soc **51**：291-299, 2003.
20) Fujita S and Volpi E：Amino acids and muscle loss with aging. J Nutr **136**(Suppl)：277S-280S, 2006.
21) Verhoeven S et al：Long-term leucine supplementation does not increase muscle mass or strength in healthy elderly men. Am J Clin Nutr **89**：1468-1475, 2003.

(執筆者連絡先)　下方浩史　〒474-8511　愛知県大府市森岡町源吾35番地　国立長寿医療研究センター予防開発部

Gene Therapy (2011) 18, 857–866

www.nature.com/gt

ENABLING TECHNOLOGIES

CDK4 and cyclin D1 allow human myogenic cells to recapture growth property without compromising differentiation potential

K Shiomi[1], T Kiyono[2], K Okamura[3], M Uezumi[1], Y Goto[4], S Yasumoto[5], S Shimizu[6] and N Hashimoto[1]

In vitro culture systems of human myogenic cells contribute greatly to elucidation of the molecular mechanisms underlying terminal myogenic differentiation and symptoms of neuromuscular diseases. However, human myogenic cells have limited ability to proliferate in culture. We have established an improved immortalization protocol for human myogenic cells derived from healthy and diseased muscles; constitutive expression of mutated cyclin-dependent kinase 4, cyclin D1 and telomerase immortalized human myogenic cells. Normal diploid chromosomes were preserved after immortalization. The immortalized human myogenic cells divided as rapidly as primary human myogenic cells during the early passages, and underwent myogenic, osteogenic and adipogenic differentiation under appropriate culture conditions. The immortalized cells contributed to muscle differentiation upon xenotransplantation to immunodeficient mice under conditions of regeneration following muscle injury. We also succeeded in immortalizing cryopreserved human myogenic cells derived from Leigh disease patients following primary culture. Forced expression of the three genes shortened their cell cycle to <30 h, which is similar to the doubling time of primary cultured human myogenic cells during early passages. The immortalization protocol described here allowed human myogenic cells to recapture high proliferation activity without compromising their differentiation potential and normal diploidy.
Gene Therapy (2011) **18,** 857–866; doi:10.1038/gt.2011.44; published online 14 April 2011

Keywords: muscle satellite cell; CDK4; telomerase; immortalization; replicative senescence; growth arrest

INTRODUCTION

Skeletal muscle stem cells of adult muscle are known as muscle satellite cells because they are located adjacent to the plasma membrane of myofibers beneath the basement membrane. The postnatal growth, repair and maintenance of skeletal muscle rely on muscle satellite cells that proliferate and then fuse together to form myotubes. Actually, phenotypic analysis of Pax7-deficient mice strongly suggests that the loss of satellite cells abolishes the regenerative capacity of skeletal muscle.[1,2] The decrease of regenerative capacity of muscle results in muscle dysfunction during both normal aging and progression of muscle-regenerative diseases, such as muscular dystrophies.

Most of the data on the regulation of proliferation and differentiation of muscle satellite cells and their descendant progenitor cells have been obtained from primary cultured chick myogenic cells or mouse myogenic cell lines.[3–5] However, several previous studies strongly suggest that animal myogenic cells do not always use the same pathways to control proliferation and differentiation as human myogenic cells.[6,7] Although animal cell models certainly contribute to understanding the mechanisms of human myogenesis and muscle diseases, the precise and detailed analysis of human myogenic cells is essential for fundamental and therapeutic investigation. Unfortunately, progressively compromised differentiation potential, as well as proliferation potential, is seen in cultured human myogenic cells.[8,9] The limited proliferation capacity and progressive alterations of characteristics of human myogenic cells do not allow us to carry out both qualitative and quantitative analyses with high reproducibility.

Previous attempts have been made to extend the replication capacity of human myogenic cells using viral oncogenes such as simian virus 40 large T antigen and/or the reverse transcriptase component of human telomerase (hTERT).[10] However, no reliable model of immortalized human myogenic cells that exhibit differentiation potential had been established until our previous study.[9] We previously reported that constitutive expression of hTERT and human papillomavirus type 16 gene E7 immortalizes a primary normal human myogenic cell clone designated Hu5. The immortalized human myogenic cell clone Hu5/E18 largely preserves the myogenic phenotype represented by parental Hu5 cells, but their doubling time is approximately 12 h longer than that of primary human myogenic cells during early passages. E7 is an oncogene that inactivates the retinoblastoma protein pRb,[11] and does not transform human myogenic cells. However, we cannot exclude a possibility that E7 also affects other biological functions, including transformation-related pathways.

[1]Department of Regenerative Medicine, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Oobu, Japan; [2]Virology Division, National Cancer Center Research Institute, Tokyo, Japan; [3]Department of Urology, National Center for Geriatrics and Gerontology, Oobu, Japan; [4]Department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience, Nervous, and Muscular Disorders, National Center of Neurology and Psychiatry, Tokyo, Japan; [5]Laboratory of Molecular Cell Biology and Oncology, Kanagawa Cancer Center Research Institute, Yokohama, Japan and [6]Department of Plastic Surgery, Kanagawa Cancer Center Research Institute, Yokohama, Japan

Correspondence: Dr T Kiyono, Virology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
E-mail: tkiyono@ncc.go.jp
or Dr N Hashimoto, Department of Regenerative Medicine, National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, 35 Gengo, Morioka, Oobu, Aichi 474-8522, Japan.
E-mail: nao@ncgg.go.jp
Received 10 June 2010; revised 16 September 2010; accepted 28 September 2010; published online 14 April 2011

Cellular stress activates a pathway of the cyclin-dependent kinase inhibitor $p16^{INK4a}$, resulting in premature cell cycle arrest before telomere attrition,[12] probably due to the activation of Rb. The forced expression of wild-type cyclin-dependent kinase 4 (CDK4) enabled hTERT to immortalize primary human myogenic cells, presumably because cdk4 sequesters the increased p16 exclusively when stimulated with dexamethasone and hepatocyte growth factor.[13] In addition, the co-expression of hTERT and Bmi-1, which suppresses $p16^{INK4a}$ expression, failed to immortalize human myogenic cells.[9,14] These results indicate that combining the expression of hTERT and sequestration of p16 ^{INK4a} is insufficient to immortalize human myogenic cells, or that the p16 ^{INK4a} pathway is incompletely suppressed under these conditions.

In the present study, to block the $p16^{INK4a}$–Rb pathway and enhance cell cycle progression, without the use of oncoprotein E7, expressions of hTERT and both mutant CDK4 (CDK4R24C) and cyclin D1 were induced in human myogenic cells. Combined expression of the three genes efficiently immortalized normal human myogenic cells. The immortalized cells still retained multipotentiality and a doubling time similar to that of primary cultured human myogenic cells. The established normal human myogenic cell clones in the present study are the human equivalents of mouse cell lines such as C2 (ref. 3) and Ric10.[5,15] In addition, we succeeded in immortalization of diseased muscle-derived primary human myogenic cells that showed the prolonged doubling time. The newly established method for immortalization of primary human myogenic cells will open new avenues for mechanistic and therapeutic research on human muscle diseases.

RESULTS

p16 ^{INK4a}–Rb pathway is activated upon growth arrest of primary cultured human myogenic cells

Proliferation capacity of primary cultured human myogenic cells severely declined during serial passages under the present culture condition (Figure 1a). The doubling time of the cells became longer as they were serially succeeded (Supplementary Figure 1). Constant or high level expression of cyclin D1, CDK4, cyclin-dependent kinase inhibitor $p21^{cip1}$ and p53 was observed in primary human myogenic cells even upon growth arrest (Figure 1b). In contrast, the amount of the cell cycle inhibitor $p16^{INK4a}$ increased along with the culture period, whereas the amount of hyperphosphorylated form of Rb declined. The amount of another cell cycle-driving kinase CDK2 decreased following the disappearance of hyperphosphorylated form of Rb. The results indicate that the p16 ^{INK4a}–Rb pathway is activated before growth arrest of primary cultured human myogenic cells, suggesting that their cell cycle arrest is due to the activation of Rb. The disappearance of hyperphosphorylated Rb seems unlikely to depend on the downregulation of either CDK2 or CDK4 that are kinases relevant for phosphorylation of Rb.

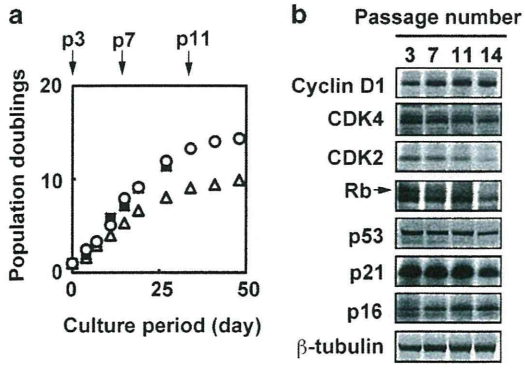


Figure 1 Growth properties of primary cultured human myogenic cells. (**a**) Life span plots of primary cultured human myogenic cells Hu20 (filled square), Hu23 (triangle) and Hu26 (circle) between passages 3 and 13. Arrows show the timing of passages 3, 7 and 11. Day 0 of culture period represents the day when the cells were plated for passage 3. (**b**) Expression patterns of growth-related proteins in primary cultured human myogenic cell H23 during serial passages. Fifteen micrograms of total proteins were subjected to immunoblotting analysis with antibodies against proteins shown in the left panels. Similar expression patterns of the proteins were obtained in Hu20 and Hu26. An arrow represents the position of hyperphosphorylated Rb protein.

E7 promotes nuclear progression in terminally differentiated myotubes

The primary human myogenic progenitor cell clone Hu5 was obtained from a healthy muscle of a non-dystrophic woman.[4] Hu5 cells have limited ability to proliferate but can be immortalized by constitutive expression of both telomerase and the *E7* gene from human papillomavirus type 16.[9] E7 inactivates Rb but is also suspected to affect other cellular functions. To determine whether constitutive expression of E7 transforms human myogenic cells, the Hu5-derived myogenic cell clone Hu5/E18 (ref. 9), immortalized by constitutive expression of both hTERT and E7, was transplanted into cardiotoxin-injected TA muscles of immunodeficient NOD/Scid mice (Figures 2a and b). Before transplantation, E18 cells were infected with a lentivirus vector encoding green fluorescent protein Venus (kindly provided by Dr Miyoshi). Transplanted cells were identified by the fluorescence of Venus and antibodies specific for green fluorescent protein. Transplanted E18 cells (2.5×10^6 cells per TA) gave rise to myofibers labeled with green fluorescence. No tumor was formed in the transplanted TA muscles. Soft agar assays also showed that E18 was unable to grow in an anchorage-independent way (Supplementary Figure 2). The results indicate that E18 cells did not show any oncogenic potential either *in vivo* or *in vitro*.

In the next experiment, effects of the immortalization on cell cycle exit during terminal muscle differentiation were analyzed *in vitro*. E18 cells undergo myogenic terminal differentiation under the myogenic differentiation-inducing condition.[9] Primary cultured human myogenic progenitor cells exited the cell cycle and gave rise to terminally differentiated myotubes (Figures 2c–f). In contrast, the nuclei of E18 myotubes synthesized DNA and also contained the proliferation marker protein Ki-67, although neither nuclear nor cellular division was observed in the myotubes (Figures 2g–j). The results suggest that E7 promotes nuclear progression in terminally differentiated myotubes that have lost mitogenic potential. In addition, the doubling time of the Hu5-derived immortalized cells is approximately 35 h,[9] whereas primary cultured human myogenic cells divided at 20–29 h intervals (Supplementary Figure 3). Taken together with the results here, the expression of hTERT and E7 immortalizes human myogenic cells without the loss of their differentiation potential but also affects their cell cycle properties during the terminal myogenic differentiation.

Cell cycle drivers efficiently immortalize primary cultured human myogenic cells

E7 promotes nuclear progression in myotubes, perhaps, because it accelerates the degradation of Rb family proteins including Rb, p130 and p107. To inactivate Rb directly and avoid unusual promotion of nuclear progression in myotubes, Hu5 cells were infected with recombinant lentiviruses encoding hTERT, CDK4R24C and cyclin D1.

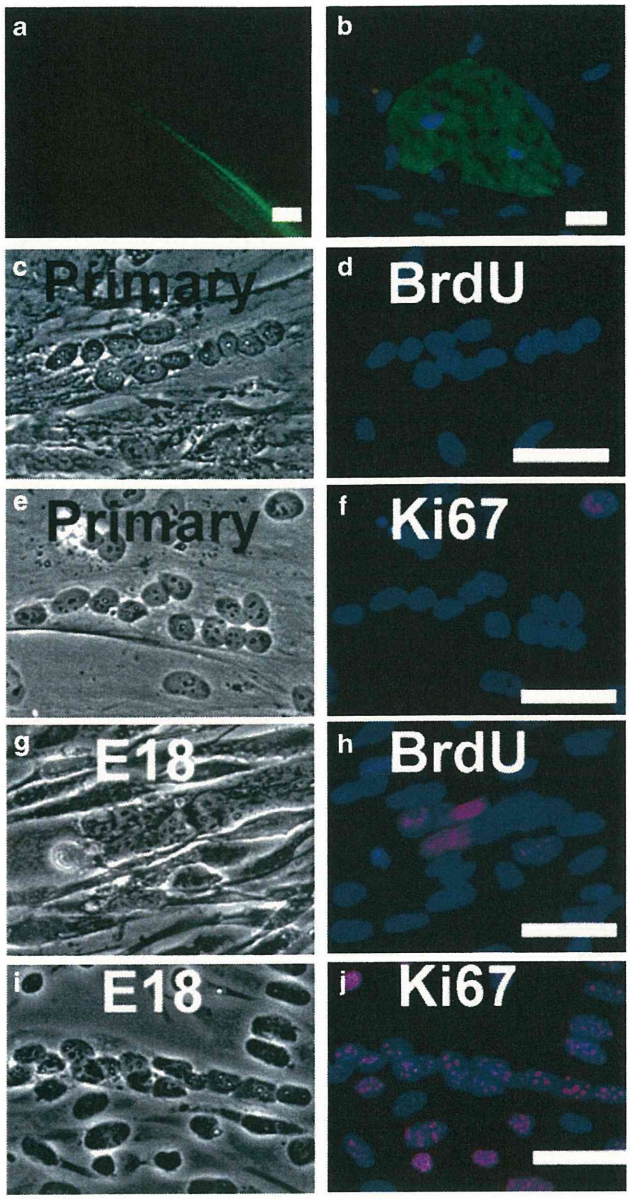


Figure 2 Nuclear progression in terminally differentiated immortalized human myogenic cells expressing telomerase and E7. (**a**, **b**) E18 cells were labeled with modified green fluorescent protein, and then 2.5×10^6 cells were transplanted into the TA muscles of NOD/Scid mice. (**a**) Whole TA muscles were recovered at 4 week after transplantation. Scale bar, 1 mm. (**b**) Pathological view of a TA muscle. Modified green fluorescent protein (green) was detected by immunofluorescence. Nuclei were stained with 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate. Scale bar, 50 μm. (**c**–**j**) Primary cultured human myogenic cell Hu26 (**c**–**f**) and immortalized human myogenic cell clone E18 expressing telomerase and E7 (**g**–**j**) were cultured for up to 78 h in primary cultured myocyte differentiation medium. For the detection of DNA synthesis, cells were incubated with 10 μM 5-bromo-2′-deoxyuridine for the last 6 h of a 78-h differentiation culture (**c**, **d**, **g**, **h**). Phase contrast images (**c**, **e**, **g**, **i**) and immunofluorescence analysis with anti-5-bromo-2′-deoxyuridine antibody (red in **d**, **h**), or Ki67 (red in **f**, **j**) of the same fields are shown in each row. Nuclei were stained with 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (blue in **d**, **f**, **h**, **j**). Scale bars, 50 μm.

The single amino acid change in CDK4 prevented a cyclin-dependent kinase inhibitor, $p16^{INK4a}$, from inhibiting kinase activity of CDK4. Forced expression of CDK4R24C, cyclin D1 and hTERT efficiently expanded the lifespan of Hu5 cells and virtually immortalized Hu5 cells. Immortalized Hu5 derivatives expressing CDK4R24C and cyclin D1 under control of the human cytomegalovirus immediate early promoter were designated as Hu5/KD, whereas the cells expressing them under the control of the Tet-Off system were designated as Hu5/TKD. The pooled populations, Hu5/KD and Hu5/TKD, and their derivative clones, KD3 and TKD1, divided rapidly at a similar interval as primary myogenic cells did (Figures 3a–d). The expression of hTERT, CDK4R24C and cyclin D1 culminated in continuous cell proliferation for more than 200 population doublings (Figures 3e and f). In contrast to E7, the cell cycle drivers did not promote nuclear progression in terminally differentiated myotubes nor interfere with the cell cycle exit of myogenic progenitor cells under the differentiation-inducing condition (Figures 3g–k; Supplementary Figure 4). Hu5 derivatives transduced with recombinant lentiviruses encoding hTERT and CDK4R24C proliferated continuously but relatively slowly. Forced expression of hTERT and cyclin D1 did not immortalize Hu5 cells. We therefore concluded that the combined expression of the three genes immortalized human myogenic progenitor cells, resulting in restoration of their growth properties similar to that of primary cultured human myogenic cells.

Immortalized human myogenic cells preserve myogenic phenotype

To determine the karyotype of immortalized human myogenic cells at passages 18–30, about 22–32 metaphase spreads of each cell type were analyzed. The results show the cells maintained a normal 46XX diploid karyotype in both the immortalized populations and the immortalized clones (Figure 4).

High-level expression of CDK4 and cyclin D1 was observed in the immortalized cells (Figure 5a). pRb was highly phosphorylated under the growing condition. The cell cycle inhibitor $p16^{INK4a}$ remained at an extremely high level in the immortalized cells (Figure 5a). However, hypophosphorylated form of pRb was accumulated under the myogenic differentiation-inducing condition. Both the immortalized populations and the immortalized clones fused together and gave rise to myotubes. In addition, MyoD was highly expressed in the nuclei of myotubes (Figures 5b and c). The results here indicate that the immortalized clones KD3 and TKD1 preserved the myogenic phenotype represented by the previously immortalized Hu5 derivatives.[9]

Immortalized human myogenic cells retain differentiation potential both *in vivo* and *in vitro*

The cells immortalized by the forced expression of hTERT and E7 preserved the phenotypic characteristics of their parental Hu5 cells, including multipotentiality; one of the E7-expressing immortalized Hu5 cell clones, E18, retained the ability to undergo myogenic, osteogenic and adipogenic terminal differentiation.[7,9] The CDK4R24C and cyclin D1-expressing immortalized clones, KD3 and TKD1, also underwent myogenic, osteogenic and adipogenic terminal differentiation under the appropriate culture conditions (Figures 6a–c and f–h), although adipogenic differentiation was induced at relatively low efficiency.

To determine whether the immortalized human myogenic cells contributed to muscle regeneration *in vivo*, KD3 and TKD1 cells were transplanted into cardiotoxin-injected TA muscles of immunodeficient NOD/Scid mice. Before transplantation, KD3 and TKD1 cells were infected with a lentivirus vector encoding green fluorescent protein Venus. Transplanted cells were identified by the fluorescence of Venus and antibodies specific for green fluorescent protein. Transplanted KD3 and TKD1 cells (1×10^6 cells per TA) gave rise to many myofibers labeled with strong green fluorescence (8.6 ± 4.3 and

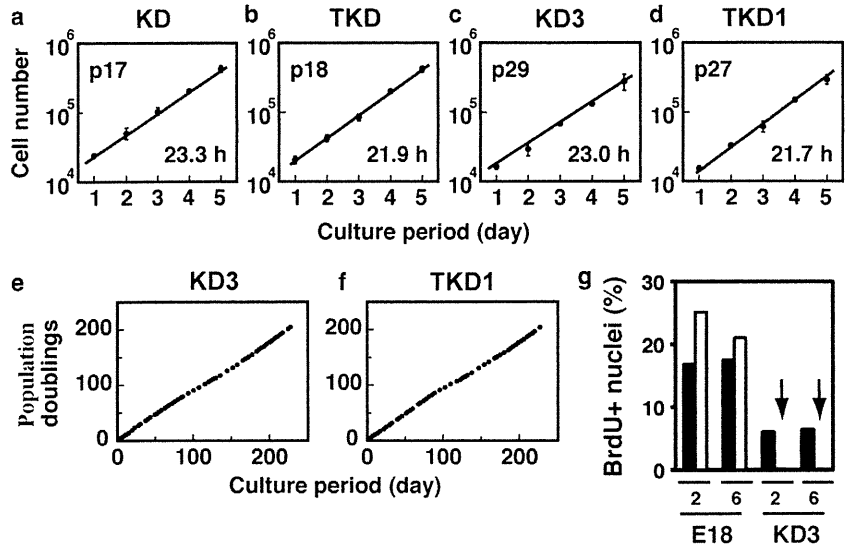


Figure 3 Proliferation of immortalized human myogenic cells. (**a–d**) Growth properties of a multiclonal population named KD, expressing hTERT, CDK4R24C and cyclin D1 under the control of a cytomegalovirus promoter (**a**), a multiclonal population named TKD, expressing hTERT, CDK4R24C and cyclin D1 under the control of a Tet-off system (**b**), a clone named KD3 isolated from KD (**c**) and a clone named TKD1 isolated from TKD (**d**). Passage numbers and doubling times are shown in the panels. (**e**, **f**) Life span plots of immortalized clones KD3 (**e**) and TKD1 (**f**). (**g**) E18 and KD3 cells were incubated with 10 μM 5-bromo-2′-deoxyuridine for the last 2 or 6 h of a 78-h culture in primary cultured myocyte differentiation medium. Ratios of 5-bromo-2′-deoxyuridine-positive nuclei in mononucleated progenitors (filled column) and myotubes (open column) were estimated from 1466–3196 nuclei of mononucleated progenitors and 404–1223 nuclei of myotubes, respectively. Numbers under the column represent the incubation time with 5-bromo-2′-deoxyuridine. Arrows represent the positions of open columns.

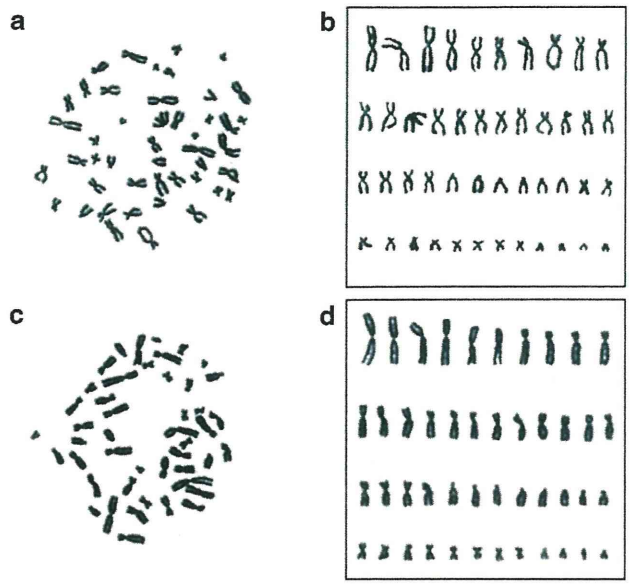
10.2 ± 9.1% of total TA myofibers, respectively) (Figures 6d and i). The relatively large s.d. in the present results was because of the low numbers of positive myofibers in the two specimens, probably due to leakage of the transferred cells to the injected TA muscle. Venus-positive myofibers were regenerated myofibers because they contained central nuclei (Figures 6e and j). No tumor was observed in the transplanted TA muscles. *In vitro* soft agar assay also showed that KD3 cells did not grow in an anchorage-independent way (Supplementary Figure 2). The results suggest that KD3 cells do not possess oncogenic potential. The ability of the immortalized human myogenic cells to regenerate muscle *in vivo* indicates that the immortalized cells established here represent a good model cell system for the fundamental and therapeutic study of human muscle development and disease.

Human myogenic cells recaptured proliferation capacity in cell-cycle driver-dependent manner

Both CDK4R24C and cyclin D1 were expressed under the control of the Tet-Off system in TKD1 cells. To determine the role of cell cycle drivers in the continuous proliferation of human myogenic cells, the expressions of CDK4R24C and cyclin D1 were suppressed by administration of doxycycline. Expression levels of CDK4 and cyclin D1 in TKD1 cells markedly declined during 5 days of incubation with doxcycline (Figure 7a). Doxcycline itself impaired neither the protein levels of either CDK4 or cyclin D1 in KD3 cells (Supplementary Figure 5) nor their DNA synthesis (Figures 7b–g). The number of proliferating TKD1 cells reduced following the decline in CDK4 and cyclin D1 proteins (Figures 7i, l). The morphology of doxycycline-treated TKD1 cells also became more flattened like senescent cells, and the nuclei looked thin during the cessation of proliferation (Figures 7h, j, k, m). In contrast, when doxycycline was removed from the culture, CDK4 and cyclin D1 were restored, and the proliferation capacity was completely recaptured by TKD1 cells (Figures 7a lane 4 and n–p). The results suggest that the proliferation capacity of human myogenic cells expressing hTERT is fully dependent on CDK4R24C and cyclin D1, and that before cellular senescence accompanied by telomeric attrition, human myogenic cells are capable of recapturing proliferation capacity.

Cryopreserved human myogenic cells derived from a disease muscle recapture proliferating activity by immortalization

Primary cultured human myogenic cells lose the ability to proliferate by degrees during culture *in vitro*. Cryopreserved primary cultured human myogenic cells obtained from Leigh disease muscle suffered from growth impairment accompanied by a prolonged cell cycle. One of the mortal cell clones from the primary cultured Leigh disease myogenic cells, HM2-5, which had a cell cycle of 73.5 h at passage 10 (Figure 8a), was infected with recombinant lentiviruses. Forced expression of hTERT, CDK4R24C and cyclin D1 had the cells dividing rapidly with a doubling time of 27.7 h (HM255, Figure 8b). A combination of hTERT and E7 also rescued the cells from growth impairment, but their doubling time (36.6 h) (HM253, Figure 8c) was longer than that of the clone immortalized by hTERT, CDK4R24C and cyclin D1. Both immortalized multiclonal populations HM253 and HM255 retained the ability to undergo terminal myogenic, osteogenic and adipogenic differentiation (Figures 8d–i). A cryopreserved mortal cell clone from muscle of another Leigh disease patient also recaptured its proliferation capacity and multipotentiality through immortalization by the combined expression of hTERT, CDK4R24C and cyclin D1 (Supplementary Figure 6). These results suggest that transduction of the three genes renders growth-impaired human myogenic cells proliferative and immortalized without loss of their differentiation potentialities.



e

	Diploid / Total	% Diploid
KD (p.18) (multiclonal)	30 / 32	93.8
KD3 (p.27) (monoclonal)	19 / 22	86.4
TKD (p.19) (multiclonal)	25 / 28	89.3
TKD1 (p.30) (monoclonal)	29 / 32	90.6

Figure 4 Karyotype analysis of immortalized human myogenic cells. Cells were treated with colcemid (2 μM) for 9 h. Metaphase chromosomes were visualized by Giemsa staining (**a**, **c**) and then aligned (**b**, **d**). Immortalized clones, KD3 (**a**, **b**) and TKD1 (**c**, **d**) and multiclones, KD and TKD (**e**), were analyzed.

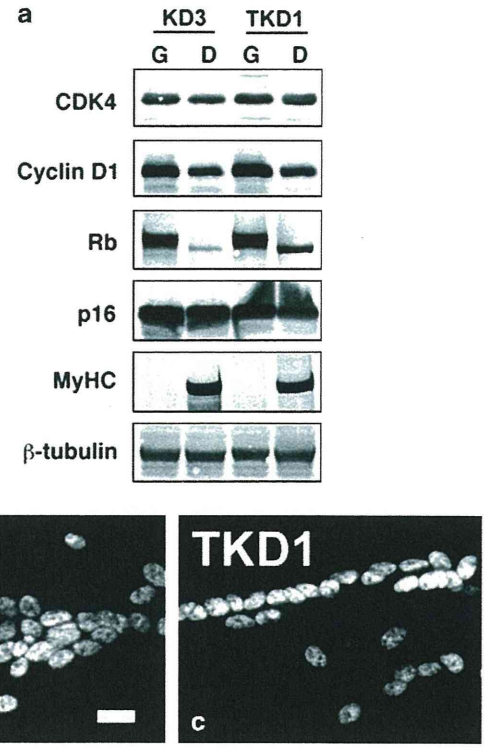


Figure 5 Expression patterns of growth- and differentiation-related proteins in immortalized human myogenic cells. (**a**) KD3 and TKD 1 cells were cultured in pmGM (g) or in primary cultured myocyte differentiation medium for 5 days (d). Fifteen micrograms of total proteins were subjected to immunobloting analysis with antibodies against CDK4, cyclin D1, Rb, $p16^{INK4a}$, myosin heavy chain and β-tubulin. (**b**, **c**) KD3 (**b**) and TKD1 (**c**) were cultured for 6 days in primary cultured myocyte differentiation medium and then subjected to immunofluorescence analysis with antibodies to MyoD. Scale bar, 20 μm.

DISCUSSION

Sarcopenia is an age-related loss of muscle mass leading to muscle weakness and atrophy. The slower regenerative capacity of aging muscle may be attributed to a decrease in the number and/or proliferation and differentiation capacities of muscle satellite cells. Actually, the number of satellite cells declines with age in humans.[16,17] In addition, the proliferation potential of human muscle satellite cells is limited by cellular senescence induced by progressive telomere shortening.[16,18] When the telomere length becomes less than about 5 kb, the Rb and p53 pathways are activated and culminate in irreversible growth arrest.[11,19,20] Cells also enter a state designated as stress or aberrant signal-induced senescence [18,20] (STASIS) or stress-induced premature senescence[21] (SIPS) that closely resembles replicative senescence when subjected to sub-lethal stress or oncogenic signals. The major characteristics of cells undergoing STASIS/SIPS are similar to those of replicatively senescent cells: the Rb and/or p53 pathways are activated and the cells stop proliferation. STASIS/SIPS can be induced in a telomere-independent way in human epithelial cells[11] and even in human fibroblasts,[12] although acceleration of telomere shortening is associated with STASIS/SIPS. Under conventional culture conditions, many types of human cells are likely to undergo precocious growth arrest before replicative senescence induced by telomere shortening,[22] though some types of human cells appear to be immortalized by the expression of hTERT alone without transformation of cell properties.[11,23,24] In fact, our previous and present studies strongly suggest that both inactivation of the Rb pathway and restoration of telomerase activity are required for efficient immortalization of human myogenic cells (Figure 9A). The growth arrest of primary cultured human myogenic cells may be attributable to an inadequate cellular context including culture conditions that stimulate the stress signaling pathway.[25]

Several previous studies emphasized that the age-related dysfunction of muscle is attributed to the age-related changes in environmental factors that attenuate the potential of muscle satellite cells. Transplantation of whole muscles between old and young rats shows that the regenerative capacity of aged muscle is enhanced when grafted into young muscle.[26] The decrease of circulating growth factors[27] and the number of motor units[28] are candidates for the responsible environmental factors or age-related changes in skeletal muscle. In addition, primary cultured human myogenic cells derived from skeletal muscles of aged persons (>75 years old) show growth properties similar to those of the myogenic cells obtained from younger persons under the appropriate culture conditions (Supplementary Figure 3A) (Hashimoto and Okamura, unpublished data). On the other hand, a previous study showed that myogenic cells from

aged muscle demonstrated less ability to proliferate in primary cultures.[6] Given that myogenic cells derived from an aged human are fragile and likely to lose proliferation potential under inappropriate culture conditions, these different results under different culture conditions are plausible. Actually, we have found that the proliferation capacity of human and mouse primary myogenic cells maintained in a medium containing DMEM is higher than that of the cells maintained in a medium containing Ham's F10, even though an F10-based medium was used to isolate and culture primary myogenic cells in many studies.[6,29]

Muscle-degenerative diseases such as muscular dystrophies provoke extensive replication of human muscle satellite cells.[30] Satellite cells in regenerating muscles also suffer from cellular stresses including those induced by inflammatory cytokines. Therefore, precocious growth arrest, as well as the replicative senescence of satellite cells, is likely to cause the loss of muscle-regenerative capacity in muscle-degenerative diseases. Results obtained by previous and present studies indicate a possibility of a new therapeutic strategy for sarcopenia and muscular dystrophy that overcomes the precocious growth arrest triggered by the Rb pathway. Human myogenic cells are vulnerable to cellular stresses and more likely to undergo premature growth arrest than human foreskin fibroblasts because primary cultured human fibroblasts undergo precocious growth arrest/STASIS/SIPS exclusively when exposed to stress inducers such as H_2O_2 and ultraviolet light.[21] From this point of view, the Rb pathway in human myogenic cells will be an attractive target of therapeutic intervention in muscle-degenerative diseases. The present study also shows that the total amount of pRb declined during growth arrest in primary human myogenic cells at later passages, immortalized human myogenic cells undergoing myogenesis and TKD1 cells stimulated with doxycycline. Therefore, we should consider both quantitative and qualitative control of pRb during precocious growth arrest.

The present results suggest that suppression of the Rb signaling pathway is required for immortalization of human myogenic cells in addition to telomere restoration (Figures 9Ba–f). Either Bmi-1 (ref. 9) or wild-type CDK4 (ref. 13) was coexpressed with hTERT in primary cultured human myogenic cells to block the $p16^{INK4a}$ signaling pathway, but the cells did not undergo immortalization. The results indicate that neither Bmi-1 nor the wild-type CDK4 alone allows hTERT to immortalize human myogenic cells, and that immortalization of human myogenic cells still requires secondary changes under these conditions. In fact, the combined expression of wild-type CDK4 and hTERT or Bmi-1 and hTERT results in immortalization of human myogenic cells exclusively under the optimized culture conditions supplemented with dexamethasone and growth factors,[13,14] although the role of those supplements has been unknown. It is conceivable that CDK4 kinase activity released from the inhibition by $p16^{INK4a}$ is not high enough to hyperphosphorylate Rb (Figures 9Bc and d). In contrast, CDK4R24C allows hTERT to promote slow, but continuous, proliferation in primary cultured human myogenic cells (Figure 9Be). CDK4R24C contributes to hyperphosphorylation of Rb, whereas the contribution of forced expression of wild-type CDK4 is quite limited because $p16^{INK4a}$ inhibits the kinase activity of wild-type CDK4. Our previous study indicated that E7 prevents Rb independently of $p16^{INK4a}$ and leads to immortalization of hTERT-expressing human myogenic cells[9] (Figure 9Bb). Given that the suppression of Rb, but not $p16^{INK4a}$, is quite effective in immortalization of human myogenic cells, we concluded that complete inhibition of both Rb activation and telomere shortening is necessary and sufficient for immortalization of human myogenic cells.

Combined expression of CDK4R24C, cyclin D1 and hTERT successfully and reproducibly immortalized human myogenic cells derived from normal and disease muscles, resulting in rapid proliferation without compromising differentiation potential. Cyclin D1 has a crucial role as a limiting factor of CDK4 kinase activity. Forced expression of cyclin D1 increases CDK4R24C kinase activity to an extent that is relevant for hyperphosphorylation of Rb, which then results in rapid proliferation, possibly due to the potent inhibition of Rb function (Figure 9Bf). The slower cycling of human myogenic cells immortalized by either E7 or CDK4R24C and hTERT also implies that higher CDK4 activity is required for rapid proliferation (Figures 9Bb and e). However, we cannot exclude a possibility that extraordinarily high activity of the CDK4R24C/cyclin D1 complex results in the phosphorylation of putative off-target substrates that have an essential role in the cell cycle progression and are usually phosphorylated by another member of the CDK family (Figure 9Bf).

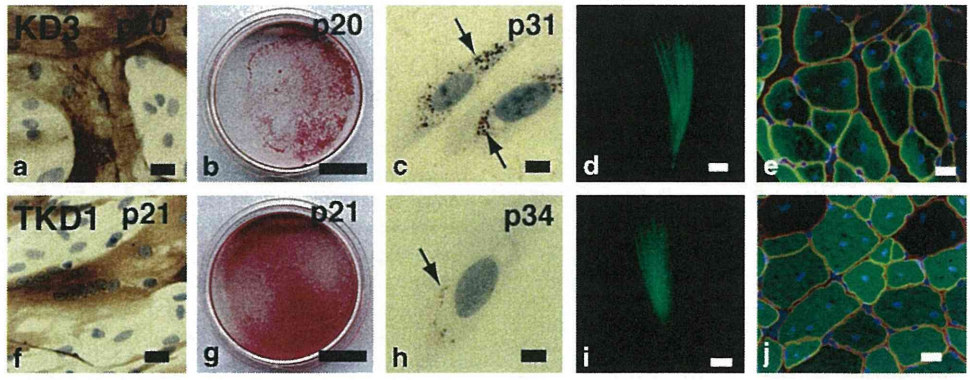


Figure 6 Multipotentiality of immortalized human myogenic cell clones KD3 and TKD1. KD3 (**a–e**) and TKD1 (**f–j**) were induced to undergo myogenic, osteogenic and adipogenic differentiation. (**a**, **f**) Cells were cultured for 5 days in primary cultured myocyte differentiation medium. Myosin heavy chain was detected by immunostaining with a horseradish peroxidase reaction. Nuclei were detected with staining with hematoxylin (blue). Scale bar, 50 μm. (**b**, **g**) The cells were cultured for 9 days in serum-containing medium supplemented with β-GP (10 mM). The cells were then stained with Alizarin Red S. Whole 35-mm dishes are shown. Scale bar, 10 mm. (**c**, **h**) The cells were cultured for 5 days in serum-containing medium supplemented with γ-linolenic acid (100 μM). Numerous lipid droplets (arrows) were stained with Oil Red O. Nuclei were detected by staining with hematoxylin (blue). Scale bar, 10 μm. (**d**, **e**, **i**, **j**) KD3 (**d**, **e**) and TKD1 (**i**, **j**) cells were labeled with modified green fluorescent protein and then 1×10^6 cells were transplanted into the TA muscle of NOD/Scid mice. (**d**, **i**) Whole TA muscles were recovered at 4 weeks after transplantation. Scale bars, 1 mm. (**e**, **j**) Pathological views (**d**, **i**). Modified green fluorescent protein (green) and laminin α2 (red) were detected by immunofluorescence. Nuclei were stained with 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate. Passage numbers of cells are shown in (**a–c** and **f–h**). Scale bar, 20 μm.