J Epidemiol 2008; 18(4) 144-150
doi:10.2188/jea.JE2007438

Original Article

Incidence of Total Stroke, Stroke Subtypes, and Myocardial Infarction in the Japanese Population: The JMS Cohort Study

Shizukiyo Ishikawa,[1] Kazunori Kayaba,[2] Tadao Gotoh,[1] Naoki Nago,[3] Yosikazu Nakamura,[4] Akizumi Tsutsumi,[5] Eiji Kajii,[1] and the Jichi Medical School (JMS) Cohort Study Group.

[1]Division of Community and Family Medicine, Center for Community Medicine, Jichi Medical University
[2]School of Health and Social Services, Saitama Prefectural University
[3]Community Medicine Education Center, Japan Association for Development of Community Medicine
[4]Department of Public Health, Jichi Medical University
[5]University of Occupational and Environmental Health, Japan

Received November 7, 2007; accepted February 4, 2008; released online July 7, 2008

ABSTRACT

Background: Previous reports indicated that the incidence rate of stroke was higher in Japan than in Western countries, but the converse was true in the case of myocardial infarction (MI). However, few population-based studies on the incidence rates of stroke and MI have been conducted in Japan.

Methods: The Jichi Medical School (JMS) Cohort Study is a multicenter population-based cohort study that was conducted in 12 districts in Japan. Baseline data were collected between April 1992 and July 1995. We examined samples from 4,869 men and 7,519 women, whose mean ages were 55.2 and 55.3 years, respectively. The incidence of stroke, stroke subtypes, and MI were monitored.

Results: The mean follow-up duration was 10.7 years. A total of 229 strokes and 64 MIs occurred in men, and 221 strokes and 28 MIs occurred in women. The age-adjusted incidence rates (per 100,000 person-years) of stroke were 332 and 221 and those of MI were 84 and 31 in men and women, respectively. In the case of both sexes, the incidence rates of stroke and MI were the highest in the group of subjects aged > 70 years.

Conclusion: We reported current data on the incidence rates of stroke and MI in Japan. The incidence rate of stroke remains high, considerably higher than that of MI, in both men and women. The incidence rates of both stroke and MI were higher in men than in women.

Key words: Incidence, Stroke, Myocardial Infarction, Cohort Studies, Asian Continental Ancestry Group

INTRODUCTION

In Japan, the stroke mortality rate has declined significantly during the period from 1965 through 2000. Even after malignancy became the leading cause of death in 1981, stroke and heart disease remained the second and the third leading causes of mortality, respectively in Japan over this period.[1] Both remain major health problems in Japan as in other developed countries.

It has been reported in previous international comparative studies that the incidence and mortality of stroke were higher, and those of myocardial infarction (MI) were lower in Japan than in Western countries.[2,3] Many population-based cohort studies have been conducted in Western countries,[4-13] but few cohort studies have been carried out in Japan recently.[14,15] However, some studies examining the incidence of stroke and MI commenced several decades ago.[16-19]

It remains unclear why the mortality and incidence rates of coronary heart disease (CHD) have been lower and those of stroke higher in Japan than in Western countries. In the Ni-Hon-San study, which compared individuals of Japanese ancestry living in Japan, Hawaii, and California 35 years ago, it was confirmed that significant differences existed in the incidence rates of stroke and MI among the 3 populations.[2] While these differences may be attributable to environmental factors, they were only partially explained by known risk factors such as blood pressure, smoking, and serum cholesterol.

The objective of the present study is to examine the incidence of stroke and MI in a multicenter population-based

Address for correspondence: Shizukiyo Ishikawa, Division of Community and Family Medicine, Center for Community Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan. (E-mail: i-shizu@jichi.ac.jp)

cohort study, which commenced in the 1990s. We recently finished the follow-up for the incidence of stroke and MI.

METHODS

Subjects

The Jichi Medical School (JMS) Cohort Study was conducted to investigate the risk of cardiovascular diseases, stroke, and MI. There were 12,490 Japanese men and women from 12 communities across Japan in the present study. Baseline data were obtained between April 1992 and July 1995. A detailed description about standardized collection of baseline data has been published previously.[20] In each community, at least 1 alumnus of JMS worked as a physician and played an important role in data collection and collaboration with the local government. Mass screening examinations for cardiovascular diseases have been conducted at these sites since 1983, in accordance with the Health and Medical Service Law for the Aged. This system was used to collect the baseline data for the present study. A municipal government office in each community site sent personal invitations to all the potential participants for the examination by letter or public information. The target subjects were residents aged 40-69 years in 8 communities, those aged 20-69 years in 1 community, and those aged 35 years and older (no upper limit) in 1 community. All adults (no age limit) were examined in the other communities. The participation rate varied in each community (26%-90%), and the overall participation rate of those invited to the mass screening examination program was 65.4%.

Ethical Issues

The study design and procedure were approved by each community government and by the Ethical Committee of Epidemiologic Research at Jichi Medical University. Written informed consent for the study was obtained individually from those who responded to the mass screening examination health check-up. At visits, participants were informed that data would be obtained from questionnaires and blood samples, and that their health status would be followed up through a review of their hospital medical records if a stroke or MI was suspected to have occurred.

Follow-up System

The mass screening examination system was used to obtain the baseline data for the cohort study. This system was also used to follow-up the subjects each year. After enrollment in the study, subjects were asked whether they had a history of stroke or cardiovascular diseases. Those with such a history were asked when and which hospital they visited. Subjects who did not attend the screening examination were contacted by mail or phone. Medical records were checked if the subjects were hospitalized for any reason. Public health nurses also visited the homes of the subjects to obtain further information.

If an incident case of stroke or MI was suspected, duplicate computer tomography scans or magnetic resonance images in the case of stroke and electrocardiograms in the case of MI were requested. Death certificates were collected until the end of 2005 from public health centers with the official permission of the Agency of General Affairs and the Ministry of Health, Labour and Welfare. Data on subjects who moved out of the study area were obtained annually from the relevant municipal government.

Diagnostic Criteria

The diagnoses were carried out independently by a Diagnosis Committee, composed of 1 radiologist, 1 neurologist, and 2 cardiologists. The criteria for stroke were sudden onset of a focal and nonconvulsive neurological deficit that lasted for more than 24 h, and the stroke subtype was determined according to the criteria of the National Institute of Neurological Disorders and Stroke.[21] MI was diagnosed based on the criteria of the World Health Organization Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) Project-a multinational collaborative project that was conducted from the mid-1980s through the mid-1990s for the monitoring of coronary events.[22]

Statistical Analysis

Data regarding the variables were expressed as mean ± standard deviation (SD), except in the case of triglycerides (TGs). The distribution of TGs was very skewed and was hence expressed as geometric mean ± SD. Data regarding proportions were expressed as percentages. Incidence rates were calculated and expressed in terms of per 100,000 person-years. Direct standardization was conducted to adjust the rates to the age structure of the Japanese population in 1985. While analyzing outcomes, we excluded subjects who reported a positive history at the time of data collection, i.e., subjects with a history of stroke and MI were excluded while calculating the stroke and MI incidence rates, respectively. These analyses were performed using SAS® software version 8.2.

RESULTS

Among the 12,490 participants, 95 declined follow-up, and 7 subjects could not be contacted after baseline data collection. Thus, a total of 4,869 men and 7,519 women were followed up. The follow-up rate was 99.2%. The mean age at the time of baseline data collection was 55.2 and 55.3 years in the case of men and women, respectively. The mean duration of follow-up was 10.7 years.

The baseline characteristics of the subjects are shown in

Table 1. Baseline characteristics of the participants of the JMS Cohort Study in Japan.

	Men				Women			
	n	Mean	SD	Proportion (%)	n	Mean	SD	Proportion (%)
Age (y)	4,869	55.2	12.0		7,519	55.3	11.4	
Systolic blood pressure (mmHg)	4,665	131.4	20.5		7,283	128.2	21.0	
Diastolic blood pressure (mmHg)	4,665	79.2	12.3		7,283	76.3	12.1	
Total cholesterol (mg/dL)	4,799	184.9	34.1		7,437	196.7	34.8	
Triglycerides (mg/dL)*	4,799	108.8	(63.0-187.9)		7,436	95.6	(57.6-158.5)	
HDL cholesterol (mg/dL)	4,800	48.8	13.4		7,437	52.6	12.5	
Body-mass index (kg/m^2)	4,649	23.0	2.9		7,243	23.2	3.2	
Smoking status								
Current smoker	2,281			50.4	382			5.5
Ex-smoker	1,282			28.3	195			2.8
Non-smoker	961			21.3	6,363			91.7
Drinking status								
Current drinker	3,307			75.1	1,691			24.9
Ex-drinker	160			3.6	102			1.5
Non-drinker	937			21.3	4,987			73.6
Past history								
Stroke	62			1.3	50			0.7
Myocardial infarction	40			0.8	25			0.3
Medication								
Hypertension	452			9.3	864			11.5
Diabetes mellitus	108			2.2	119			1.6
Hyperlipidemia	57			1.2	146			1.9

SD: Standard deviation
HDL: High-density cholesterol
*: Geometric mean ±SD

Table 1. Few subjects had histories of stroke or MI. A total of 450 cases of stroke and 92 cases of MI were confirmed during the follow-up period. Of these, 229 strokes and 64 MIs occurred in men, and 221 strokes and 28 MIs occurred in women.

The crude annual incidence rates of stroke were 450.8 and 273.1 per 100,000 person-years in men and women, respectively. These rates increased with age in both sexes. The crude incidence rates of cerebral hemorrhage, cerebral infarction, and subarachnoid hemorrhage were 100.4, 324.8, and 25.6 per 100,000 person-years in men, and 63.0, 154.5, and 54.4 per 100,000 person-years in women, respectively. After adjustment for age by using the direct method, the rates of stroke were 311.5 and 221.0 per 100,000 person-years in men and women, respectively (Table 2).

The incidence rate of MI was 2.7 times higher in men than in women after the adjustment for age (Table 3).

DISCUSSION

The JMS Cohort Study is a multicenter population-based cohort study on the incidence of newly diagnosed stroke and MI. In the past few decades, rapid westernization of lifestyle has occurred in Japan. Cholesterol intake in Japan has risen dramatically since the 1960s, and the levels of serum cholesterol are now comparable to those in the United States.[17,23,24] The prevalence of hypertension has declined and is now slightly lower in Japan than in the US.[17,25,26] While the proportion of obesity has increased in some segments of the Japanese population, it remains far lower than that seen in the US.[27,28] On the other hand, the prevalence of smoking remains high among Japanese men. Data from the current study indicate the incidence rates of stroke, which appear to be lower than those reported previously, while the rates of MI appear to be stable.

In the present study, age-adjusted annual incidence rates of stroke for the total study period were 312 and 221 per 100,000 person-years in men and women, respectively. Some studies reported that in the past few decades, the incidence rate of stroke declined in Japanese cohorts starting between the 1960s and 70s.[16-19,29]

In the Hisayama study, a rapid decline in the stroke incidence rate was observed over 2 decades. In the 1960s, the age-adjusted annual incidence rates of all strokes were approximately 1200 and 600 per 100,000 person-years in men and women, respectively. The incidence rate of all strokes in the third cohort of the Hisayama study, starting in 1988, was 529 in men and 388 in women. These values are higher than those in the present study.[19] Similar trends of stroke incidence were shown in some other Japanese cohort studies.[17,18,30] The decline in stroke incidence was mainly due to a decline in the occurrence of cerebral hemorrhage. Blood pressure control and nutritional improvement contributed to this phenomenon.[17,19]

In Shiga Prefecture, around 1990, the age-adjusted

Table 2. Number of cases and incidence of stroke subtypes in men and women.

	Age (y)					Total	
	≤39	40-49	50-59	60-69	≥70	Crude	Age-adjusted*
				Men			
n	493	1,060	1,128	1,827	299	4,807	
Mean follow-up (y)	10.7	11.1	11.0	10.2	9.0	10.6	
Follow-up (person-years)	5,255	11,782	12,380	18,689	2,690	50,796	
No. of cases							
All strokes	1	15	33	143	37	229	
Cerebral hemorrhage	0	4	8	33	6	51	
Cerebral infarction	1	9	22	105	28	165	
Subarachnoid hemorrhage	0	2	3	5	3	13	
Not confirmed	0	0	0	0	0	0	
Incidence rate (/100,000 person-years)							
All strokes	19.0	127.3	266.6	765.2	1375.5	450.8	311.5
Cerebral hemorrhage	0	34.0	64.6	176.6	223.0	100.4	65.6
Cerebral infarction	19.0	76.4	177.7	561.8	1040.9	324.8	225.8
Subarachnoid hemorrhage	0	17.0	24.2	26.8	111.5	25.6	18.7
Not confirmed	0	0	0	0	0	0	
				Women			
n	677	1,536	2,108	2,777	371	7,469	
Mean follow-up (y)	10.4	11.1	11.1	10.8	9.6	10.8	
Follow-up (person-years)	7,021	16,985	23,375	29,965	3,576	80,922	
No. of cases							
All strokes	1	7	43	126	44	221	
Cerebral hemorrhage	1	1	10	33	6	51	
Cerebral infarction	0	3	13	76	33	125	
Subarachnoid hemorrhage	0	3	20	17	4	44	
Not confirmed	0	0	0	0	1	1	
Incidence rate (/100,000 person-years)							
All strokes	14.2	41.2	184.0	420.5	1230.4	273.1	221.0
Cerebral hemorrhage	14.2	5.9	42.8	110.1	167.8	63.0	39.4
Cerebral infarction	0	17.7	55.6	253.6	922.8	154.5	136.1
Subarachnoid hemorrhage	0	17.7	85.6	56.7	111.9	54.4	38.8
Not confirmed	0	0	0	0	28.0	1.2	

*: Adjusted for age by using the direct method

Table 3. Number of cases and incidence of myocardial infarction in men and women.

	Age (y)					Total	
	≤39	40-49	50-59	60-69	≥70	Crude	Age-adjusted*
				Men			
n	493	1,062	1,130	1,842	302	4,829	
Mean follow-up (y)	10.6	11.2	11.0	10.5	9.4	10.7	
Follow-up (person-years)	5,249	11,875	12,466	19,300	2,828	51,719	
Myocardial infarction	1	2	10	39	12	64	
Incidence rate (/100,000 person-years)	19.1	16.8	80.2	202.1	424.3	123.7	83.2
				Women			
n	677	1,539	2,110	2,796	372	7,494	
Mean follow-up (y)	10.4	11.1	11.2	10.9	9.9	10.9	
Follow-up (person-years)	7,021	17,051	23,531	30,544	3,685	81,832	
Myocardial infarction	0	0	6	14	8	28	
Incidence rate (/100,000 person-years)	0.0	0.0	25.5	45.8	217.1	34.2	30.9

*: Adjusted for age by using the direct method

incidence rates of stroke (per 100,000 person-years) were determined to be 269 in men and 168 in women after a 4-year follow-up.[31] In a cohort of urban workers, the incidence rate of stroke was considerably lower than that observed in other studies, perhaps due to a healthy worker effect.[32] The incidence of stroke was higher in men than in women in all cohort studies mentioned above, and the results of the present study were consistent with those of the previous studies.

In the US, the trend of stroke incidence was reported in the Framingham study; the incidence of stroke in the 1960 and 1970 cohorts was about half of that in the 1950 cohort. The crude annual incidence rate was approximately 200 per 100,000 person-years in the 1970 cohort.[8] The incidence rate of stroke was lower in Western countries than that reported in the present study. In the US, the annual incidence rates of stroke were between 93 and 178 per 100,000 person-years in white men.[11,13] In Europe, the MONICA Project showed that the annual incidence rates (per 100,000 person-years) of stroke were between 100 (Friuli, Italy) and 290 (Kuopio, Finland) in the case of men and between 60 (Friuli, Italy) and 190 (Novosibirsk, Russia) in the case of women. In half of the populations, the stroke incidence was twice as high in men as in women.[33] However, another collaborative study showed that annual stroke incidence rates were between approximately 300 and 500 per 100,000 person-years, which was similar to our results. Sudlow et al[10] revealed the following limitations of the MONICA Project: many parts of the world were not represented, many centers had participants aged 65 years or younger, and many centers used semi-retrospective methods.

In the Ni-Hon-San study conducted in the 1970s, Japanese men living in Japan were found to have a considerably higher stroke incidence rate than that of Japanese men living in Hawaii.[34] Interestingly, the incidence rate of stroke in the Japanese-Hawaiians 35 years ago was similar to those seen in the data reported here. These results showed that environmental factors like lifestyle, including food, were likely to be strong contributors to the difference.

Previous studies in Japan have reported the incidence rates of MI, which were lower than those for stroke. In the present study, the age-adjusted annual incidence rate (per 100,000 person-years) of MI was 80 in men and 30 in women. Despite the apparent increase in the risk for MI with westernization over the previous decades, the rates of MI do not appear to have increased to the degree they have in other Asian countries.[35,36] In the Hisayama study in Japan, the age-adjusted incidence rate of CHD did not change significantly over more than 3 decades.[19] This fact was observed among both sexes. Although the rates seen in the present study were relatively lower than those seen in other studies, the criteria used for diagnosis varied among studies, and the MONICA criteria used here include only definite cases of MI. In addition, differences were noted in the criteria for the study samples.

Annual CHD incidence rates (per 100,000 person-years) were between 200 and 500 in men and between 60 and 150 in women in Western countries.[8,12] The highest incidence rate (per 100,000 person-years) of CHD in the case of men was in North Karelia, Finland (835) and that in the case of women was in Glasgow, United Kingdom (265). Robertson et al reported a significantly greater incidence of MI and death from CHD in male Japanese-Hawaiian residents as compared with that in Japanese men living in Japan.[2] Furthermore, a substantially greater incidence of MI was seen in Japanese people living in California than in those living in Hawaii. The rate of MI incidence in Japanese people living in Japan was 1.4 (per 1,000 person-years), half that in Japanese people living in Hawaii and 3-fold lower than that in Japanese people living in California.[2] Moreover, reports of the Honolulu Heart Program and the Hiroshima/Nagasaki study, a part of the Ni-Hon-San Study, showed that the incidence of MI had not changed for about 20 years in Hawaii or in Japan.[35,37]

In the present study, the incidence rate of stroke was approximately 4 times higher than that of MI among men and 7 times higher among women. In most Western countries, the incidence rate of MI was higher than that of stroke.[8,36] The incidence rates of stroke and MI in the present study were lower than those seen in the third Hisayama cohort, which began in 1988.[19] However, the participation rate might have contributed to this difference. Serum total cholesterol levels have been increasing in recent years in Japan. Thus, the incidence rate of MI may increase in the future, and it is important to monitor the future trends of cardiovascular diseases in Japan.

There were some limitations of the current study. Although the study subjects were selected from a population-based health check-up system, they were not selected at random. Thus, the subjects were rather healthier than the general population, and the proportion of subjects treated for hypertension, diabetes mellitus, or hyperlipidemia was low. We had reported the standardized mortality ratio (SMR) of the study areas previously, and the SMRs were approximately 0.7 in both men and women.[20] The present study was carried out in primarily rural areas, and the data may not be generalizable to urban populations. However, the response and follow-up rates were quite high. In the Akabane study, the incidence of stroke in the non-responders was considerably higher than that in those who were examined in the following health check-up.[16] Not all of the study subjects could be followed up. Although we used the annual health check-up examination system, only about 60% of the participants returned for check-ups every year. Mail and telephonic follow-up was attempted in non-responders, and medical records were also checked; thus, only 7 persons were lost to follow-up. The present study was carried out in a standardized fashion in 12 different areas all over Japan and included more than 12,000 men and women. Most cohort studies conducted in Japan include only 1 area.

In conclusion, the incidence rate of stroke remains high and is remarkably higher than that of MI in both men and women. The incidence rates of both stroke and MI were higher in men than in women. Stroke remains a greater burden to healthcare in Japan than MI, whose incidence has remained paradoxically low despite apparently worsening trends of serum cholesterol and persistently high rates of smoking in men. These patterns are unlike those seen in other developed countries. Further research into the underlying causes of these differences is indicated.

ACKNOWLEDGMENT

This study was supported by Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and grants from the Foundation for the Development of the Community, Tochigi, Japan.
The authors thank J. David Curb, MD, MPH and Katsuhiko Yano, MD for their comments on the manuscript.

REFERENCES

1. Health and Welfare Statistics Association. Vital Statistics of Japan, 2000, Vol. 3. Tokyo (Japan): Health and Welfare Statistics Association; 2000.
2. Robertson TL, Kato H, Gordon T, Kagan A, Rhoads GG, Land CE, et al. Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California. Coronary heart disease risk factors in Japan and Hawaii. Am J Cardiol 1977;39:244-9.
3. Menotti A, Keys A, Kromhout D, Blackburn H, Aravanis C, Bloemberg B, et al. Inter-cohort differences in coronary heart disease mortality in the 25-year follow-up of the seven countries study. Eur J Epidemiol 1993;9:527-36.
4. Boysen G, Nyboe J, Appleyard M, Sorensen PS, Boas J, Somnier F, et al. Stroke incidence and risk factors for stroke in Copenhagen, Denmark. Stroke 1988;19:1345-53.
5. Haheim LL, Holme I, Hjermann I, Leren P. Risk factors of stroke incidence and mortality. A 12-year follow-up of the Oslo Study. Stroke 1993;24:1484-9.
6. Monique Verschuren WM, Kromhout D. Total cholesterol concentration and mortality at a relatively young age: do men and women differ? BMJ 1995;311:779-83.
7. Cholesterol, diastolic blood pressure, and stroke: 13,000 strokes in 450,000 people in 45 prospective cohorts. Prospective studies collaboration. Lancet 1995;346:1647-53.
8. 6yWRwVL3A, D'AJRVWR 5 B, Belanger A, Kannel WB. Sex and time trends in cardiovascular disease incidence and mortality: the Framingham Heart Study, 1950-1989. Am J Epidemiol 1996;143:338-50.
9. BrRvn 5 D,: KlvnDW3, 6LNV D, 2 ') DRn: 0 ,: LEHVD2. Stroke incidence, prevalence, and survival: secular trends in Rochester, Minnesota, through 1989. Stroke 1996;27:373-80.
10. Sudlow CL, Warlow CP. Comparable studies of the incidence of stroke and its pathological types: results from an international collaboration. International Stroke Incidence Collaboration. Stroke 1997;28:491-9.
11. Sacco RL, Boden-Albala B, Gan R, Chen X, Kargman DE, Shea S, et al. Stroke incidence among white, black, and Hispanic residents of an urban community: the Northern Manhattan Stroke Study. Am J Epidemiol 1998;147:259-68.
12. Tunstall-Pedoe H, Kuulasmaa K, Mahonen M, Tolonen H, Ruokokoski E, Amouyel P. Contribution of trends in survival and coronary-event rates to changes in coronary heart disease mortality: 10-year results from 37 WHO MONICA project populations. Monitoring trends and determinants in cardiovascular disease. Lancet 1999;353:1547-57.
13. Rosamond WD, Folsom AR, Chambless LE, Wang CH, McGovern PG, Howard G, et al. Stroke incidence and survival among middle-aged adults: 9-year follow-up of the Atherosclerosis Risk in Communities (ARIC) cohort. Stroke 1999;30:736-43.
14. Ohno Y, Tamakoshi A. Japan collaborative cohort study for evaluation of cancer risk sponsored by monbusho (JACC study). J Epidemiol 2001;11:144-50.
15. Watanabe S, Tsugane S, Sobue T, Konishi M, Baba S. Study design and organization of the JPHC study. Japan Public Health Center-based Prospective Study on Cancer and Cardiovascular Diseases. J Epidemiol 2001;11:S3-7.
16. Okada H, Horibe H, Yoshiyuki O, Hayakawa N, Aoki N. A prospective study of cerebrovascular disease in Japanese rural communities, Akabane and Asahi. Part 1: evaluation of risk factors in the occurrence of cerebral hemorrhage and thrombosis. Stroke 1976;7:599-607.
17. Shimamoto T, Komachi Y, Inada H, Doi M, Iso H, Sato S, et al. Trends for coronary heart disease and stroke and their risk factors in Japan. Circulation 1989;79:503-15.
18. Nakayama T, Date C, Yokoyama T, Yoshiike N, Yamaguchi M, Tanaka H. A 15.5-year follow-up study of stroke in a Japanese provincial city. The Shibata Study. Stroke 1997;28:45-52.
19. Kubo M, Kiyohara Y, Kato I, Tanizaki Y, Arima H, Tanaka K, et al. Trends in the incidence, mortality, and survival rate of cardiovascular disease in a Japanese community: the Hisayama study. Stroke 2003;34:2349-54.
20. Ishikawa S, Gotoh T, Nago N, Kayaba K; Jichi Medical School (JMS) Cohort Study Group. The Jichi Medical School (JMS) Cohort Study: design, baseline data and standardized mortality ratios. J Epidemiol 2002;12:408-17.
21. Adams HP, Jr., Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, et al. Classification of subtype of acute ischemic stroke: Definitions for use in a multicenter clinical trial: TOAST: Trial of ORG 10172 in Acute Stroke Treatment. Stroke 1993;24:35-41.
22. WHO MONICA Project Principal Investigators. The World Health Organization MONICA Project (monitoring trends and determinants in cardiovascular disease): a major international collaboration.. J Clin Epidemiol 1988;41:105-14.
23. Arai H, Yamamoto A, Matsuzawa Y, Saito Y, Yamada N, Oikawa S, et al. Serum lipid survey and its recent trend in the general Japanese population in 2000. J Atheroscler Thromb 2005;12:98-106.
24. Carroll MD, Lacher DA, Sorlie PD, Cleeman JI, Gordon DJ, Wolz M, et al. Trends in serum lipids and lipoproteins of adults, 1960-2002. JAMA 2005;294:1773-81.

25. van den Hoogen PCW, Feskens EJM, Nagelkerke NJD, Menotti A, Nissinen A, Kromhout D, et al. The relation between blood pressure and mortality due to coronary heart disease among men in different parts of the world. N Engl J Med 2000;342:1-8.
26. Stamler J, Elliott P, Dennis B, Dyer AR, Kesteloot H, Liu K, et al. INTERMAP: background, aims, design, methods, and descriptive statistics (nondietary). J Hum Hypertens 2003;17:591-608.
27. Yoshiike N, Seino F, Tajima S, Arai Y, Kawano M, Furuhata T, et al. Twenty-year changes in the prevalence of overweight in Japanese adults: the National Nutrition Survey 1976-95. Obes Rev 2002;3:183-90.
28. Flegal KM, Carroll MD, Ogden CL, Johnson CL. Prevalence and trends in obesity among ᵀS adults, 1999-2000. JAMA 2002;288:1723-7.
29. Kodama K. Stroke trends in Japan. Ann Epidemiol 1993;3:524-8.
30. Komachi Y, Tanaka H, Shimamoto T, Handa K, Iida M, Isomura K, et al. A collaborative study of stroke incidence in Japan: 1975-1979. Stroke 1984;15:28-36.
31. Kita Y, Okayama A, ᵀeshima H, Wada M, Nozaki A, Choudhury SR, et al. Stroke incidence and case fatality in Shiga, Japan 1989-1993. Int J Epidemiol 1999;28:1059-65.
32. Kitamura A, Iso H, Iida M, Naito Y, Sato S, Jacobs DR, et al. Trends in the incidence of coronary heart disease and stroke and the prevalence of cardiovascular risk factors among Japanese men from 1963 to 1994. Am J Med 2002;112:104-9.
33. Stegmayr B, Asplund K, Kuulasmaa K, Rajakangas AM, Thorvaldsen P, Tuomilehto J. Stroke incidence and mortality correlated to stroke risk factors in the WHO MONICA Project. An ecological study of 18 populations. Stroke 1997;28:1367-74.
34. Takeya Y, Popper JS, Shimizu Y, Kato H, Rhoads GG, Kagan A. Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California: incidence of stroke in Japan and Hawaii. Stroke 1984;15:15-23.
35. Kodama K, Sasaki H, Shimizu Y. Trend of coronary heart disease and its relationship to risk factors in a Japanese population: a 26-year follow-up, Hiroshima/Nagasaki study. Jpn Circ J 1990;54:414-21.
36. Truelsen T, Mahonen M, Tolonen H, Asplund K, Bonita R, Vanuzzo D; WHO MONICA Project. Trends in stroke and coronary heart disease in the WHO MONICA Project. Stroke 2003;34:1346-52.
37. Reed D, Maclean C. The nineteen-year trends in CHD in the Honolulu Heart Program. Int J Epidemiol 1989;18:S82-7.

© Med Sci Monit, 2008; 14(3): CR107-111
PMID: 18301352

www.MEDSCIMONIT.COM
Clinical Research

CR

Received: 2007.08.22
Accepted: 2007.12.03
Published: 2008.03.01

The UCP2–866 A/A genotype is associated with low density lipoprotein particle sizes in the general population

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Taku Hamada[1 BCDEF], Kazuhiko Kotani[1,2 ABCD], Shinji Fujiwara[3,4 AB], Yoshiko Sano[1 AB], Masayuki Domichi[1 AB], Kokoro Tsuzaki[1 BC], Kaoru Takahashi[5 G], Naoki Sakane[1 ABCDE]

[1] Division of Preventive Medicine and Diabetes Education, Clinical Research Institute for Endocrinology and Metabolic Disease, National Hospital Organization Kyoto Medical Center, Kyoto, Japan
[2] Division of Health Administration and Promotion, Faculty of Medicine, Tottori University, Tottori, Japan
[3] Division of Community and Family Medicine, Center for Community Medicine, Jichi Medical University, Tochigi Japan
[4] Mima City National Health Insurance Koyadaira Clinic, Tokushima, Japan
[5] Department of Public Health and Genetic Epidemiology, Kobe University Graduate School of Medicine, Kobe, Japan

Source of support: This study was supported in part by a grant-in-aid from the Japan Foundation for Community Development

Summary

Background: It has been reported that a common G→A single nucleotide polymorphism (SNP) at the position -866 of the uncoupling protein-2 promoter (UCP2–866 G/A SNP) modulates UCP2 expression in adipose tissue and pancreatic β-cell function, and lipid profiles. Reduced low density lipoprotein (LDL) particle size is a significant predictor of the development for coronary artery disease. The purpose of this study was to investigate whether the UCP2–866 G/A SNP was associated with serum LDL particle characteristics in a general Japanese population.

Material/Methods: In 279 subjects (age 65±13 years), body mass index (BMI), percent body fat, blood pressure, and blood biochemical profiles were measured. The UCP2–866 G/A SNP was determined with a fluorescence-based allele-specific DNA primer assay system. LDL particle characteristics were analyzed by high-resolution polyacrylamide gel electrophoresis.

Results: The frequency of the -866 A allele was 47.8%. There was no difference in triglyceride, total cholesterol, LDL-cholesterol, HDL-cholesterol, and small dense LDL levels between genotypes. However, subjects with the -866 A/A genotype had significantly lower mean LDL particle size levels (263.5±4.9Å) than those with the -866 G/G genotype (264.6±4.9Å, P=0.034). Multiple regression analysis revealed that the -866 A/A genotype was a significant variable contributing to the variance in the reduced LDL particle size levels (P=0.012).

Conclusions: The -866 A/A genotype may contribute to reduced LDL particle size levels, a significant risk factor for the development of coronary artery disease.

key words: UCP2 • polymorphism • lipid • LDL particle size • small dense LDL

Full-text PDF: http://www.medscimonit.com/fulltxt.php?ICID=836574
Word count: 2241
Tables: 2
Figures: –
References: 37
Author's address: Naoki Sakane, M.D,Ph.D., Division of Preventive Medicine and Diabetes Education, Clinical Research Institute for Endocrinology and Metabolic Disease, National Hospital Organization Kyoto Medical Center, e-mail: nsakane@kyotolan.hosp.go.jp

BACKGROUND

The uncoupling proteins (UCPs) are one of the families of inner mitochondria membrane transporters that dissipate proton gradients, releasing stored energy as heat [1]. UCP1 is expressed mainly in brown adipose tissue and is responsible for nonshivering thermogenesis in infants and small mammals, although its role in adult humans is unclear [1,2]. UCP3 is predominantly expressed in skeletal muscle [3]. In contrast, UCP2 is widely expressed, including in white adipose tissue, skeletal muscle, pancreatic islets, and the central nervous system [1,2,4]. Among studies on the physiological function of UCP2, some found its mRNA levels to be related with plasma free fatty acid concentrations [5,6]. Furthermore, disruption of the UCP2 gene enhances production of reactive oxygen species (ROS) generation in transgenic mice [7], and increases the size of atherosclerotic lesions and markers of oxidative stress in low-density lipoprotein (LDL) receptor-deficient mice [8]. Thus, UCP2 can be speculated to play some role in lipid metabolism [1].

The clinical importance of a common functional G→A single nucleotide polymorphism (SNP) at position -866 in the promoter region of the UCP2 gene (UCP2-866 G/A SNP) has been studied [9–15]. This polymorphism is associated with enhanced UCP2 mRNA expression in human adipose tissue *in vivo* [9,11] and an increased trans-activating effect in a human adipocyte cell line *in vitro* [9]. Also, it has been reported that the UCP2–866 G/A SNP is associated with a reduced risk of obesity in the Caucasian population [9], β-cell dysfunction the in middle-aged [10], and hypertension in type 2 diabetic subjects [15]. More importantly, a genetic association of the UCP2–866 G/A SNP with asymptomatic carotid atherosclerosis has been found in the middle-aged humans, suggesting a protective role for UCP2 in atherogenesis as proposed from studies with animal and cell culture models [16].

It has been shown that reduced LDL particle size is a significant predictor for the development for coronary artery disease [17–19]. In fact, subjects with a predominance of small, dense LDL particles have a two to three-fold increased risk of coronary artery disease [20]. To the best of our knowledge, no previous study has investigated the genetic associations between the UCP2–866 G/A SNP and LDL particle sizes. Therefore, the purpose of this study was to investigate whether the UCP2–866 G/A SNP contributes to LDL particle sizes in the general Japanese population.

MATERIAL AND METHODS

Subjects

We studied 279 subjects, aged 65±13 years (mean ±SD), which included 123 men and 156 women, recruited from community-dwelling volunteers during an annual health check up of residents of Mima city, Tokushima in Japan. Eligible subjects were subjectively healthy without any known history and clinical features of cardiovascular, kidney, or liver disease, and were not taking medication known to influence body weight, blood pressure, and glucose levels. All subjects provided written informed consent before being enrolled in the study. The study protocol was approved by the Ethics committee of the National Hospital Organization Kyoto Medical Center. To assess lifestyle habits, each of the participants filled out a questionnaire that included questions regarding the drinking of alcohol, smoking, and other lifestyle-related factors. Drinking was assessed from the frequency of drinking and the amount of alcohol consumed on a weekly basis. With respect to smoking, individuals were classified as either a non-smoker, a past-smoker, or a current smoker. After an overnight fast, body weight was measured using a body fat analyzer (HBS-354-W OMRON, Tokyo, Japan), and venous blood samples were then collected for serum analyses. Body mass index (BMI) was calculated as weight (kilograms) divided by height squared (kg/m^2).

Serum lipids, lipoprotein cholesterol, and biochemical analyses

The serum total cholesterol (Wako Pure Chemical Industries, Tokyo, Japan), high-density lipoprotein (HDL)-cholesterol, LDL-cholesterol, and triglyceride levels were determined by enzymatic methods (DAIICHI PURE CHEMICALS, Tokyo, Japan). Blood glucose was measured by the hexokinase method (SHINO-TEST, Tokyo, Japan). HbA1c was measured by the hexokinase method. Serum leptin was measured with an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, USA). Serum adiponectin levels were measured with an enzyme-linked immunosorbent assay kit (Otsuka Pharmaceutical Co, Tokyo, Japan).

Determination of serum lipoprotein particle size

The mean LDL particle size and subclasses were evaluated by using high-resolution polyacrylamide gel electrophoresis (LipoPrint system, Quantimetrix Inc., Redondo Beach, California, USA) [21]. The profiles consist of one fraction of very low density lipoprotein (VLDL), three of midband (MID), seven of LDL, and one of HDL in the LipoPrint system. Briefly, 25 µl of serum sample and 200 µl of liquid loading gel containing a lipophilic dye to stain cholesterol in each lipoprotein were applied to a 3% polyacrylamide gel tube and then mixed several times by vigorously inverting. Next, these samples were photopolymerized at room temperature for 30 min and then electrophoresed for 65 min (3 mA/gel tube). After the electrophoresis, scanning was done with a ScanMaker i900 (MICROTEK Co., USA) and the lipoprotein subfractions, VLDL, MID, LDL and HDL, were simultaneously analyzed by using an accessory software program kit (Quantimetrix Inc, Redondo Beach, California, USA). The stained bands of lipoprotein fractions were identified by their mobility (Rf) using VLDL as the starting reference point (VLDL=0) and HDL as the leading reference point (HDL=1).

Genotyping of the -866 G/A SNP in the UCP2 gene

A noninvasive genotyping sampling method has been implemented for collecting buccal mucosa cells using cytobrushes. After the phenol-extraction procedure, 0.2 to 2 µg of DNA per subject was obtained. Genotypes were determined with a fluorescence-based allele-specific DNA primer assay system (Toyobo Gene Analysis, Tsuruga, Japan) [22]. The polymorphic region of UCP2 was amplified using the polymerase chain reaction with allele-specific sense primers labeled at the 5' end with either fluorescein isothiocyanate

Table 1. Clinical and lipid characteristics of Japanese subjects according to the genotype of the UCP2–866 G/A polymorphism.

UCP2 genotype	Wild-type G/G (n=80)	Heterozygous G/A (n=131)	Homozygous A/A (n=68)	P value		
				G/G vs. G/A	G/A vs. A/A	G/G vs. A/A
Gender (male/female)	40/40	47/84	36/32	ns	ns	ns
Smoking (never, past/current)	69/11	114/17	55/13	ns	ns	ns
Age (yr)	65±14	65±12	65±14	ns	ns	ns
Body mass index (kg/m^2)	24.0±3.1	24.0±3.2	24.2±2.9	ns	ns	ns
Systolic blood pressure (mmHg)	141±19	137±19	137±22	ns	ns	ns
Diastolic blood pressure (mmHg)	78±11	77±12	77±12	ns	ns	ns
HbA1c (%)	5.6±0.8	5.6±1.2	5.7±1.1	ns	ns	ns
Fasting blood glucose (mM)	5.5±1.2	5.5±1.7	5.8±2.1	ns	ns	ns
Total cholesterol (mM)	4.70±0.87	4.97±0.94	4.68±0.89	ns	ns	ns
LDL-cholesterol (mM)	3.00±0.72	3.15±0.84	2.96±0.74	ns	ns	ns
HDL-cholesterol (mM)	1.33±0.36	1.46±0.37	1.34±0.39	0.038	ns	ns
Triglyceride (mM)	2.66±1.53	2.49±1.03	2.79±0.89	ns	ns	ns
Small dence LDL (%)	7.76±6.64	6.71±6.18	8.65±6.21	ns	ns	ns
LDL particle size (Å)	264.6±4.9	265.3±4.44	263.5±4.9	ns	ns	0.034
Leptin (ng/mL)	4.7±4.8	4.7±5.5	3.8±3.9	ns	ns	ns
Adiponectin (µg/mL)	8.2±5.1	9.1±6.3	7.3±5.2	ns	ns	ns

Values are the means ±SD. Gender difference, and smoking among genotypes were analyzed by χ^2 test or the Turkey's test after one-way ANOVA.

(5'-CAT TGT TGG CTG TTC ACG xGT-3') or Texas red (5'-AATT GTT GGC TGT TCA CGx AT-3') and with an antisense primer labeled at the 5' end with biotin (5'-CCT GGT TCG CCT TTA ATT G-3'). The reaction mixture (25 µL) contained 20 ng of DNA, 5 pmol of each primer, 0.2 mmol/L of each deoxynucleoside triphosphate, 3.5 mmol/L $MgCl_2$, and 1 U of rTaq DNA polymerase (Toyobo, Osaka, Japan) in polymerase buffer. The amplification protocol was an initial denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 68°C for 30 sec; and a final extension at 68°C for 2 min. The amplified DNA was incubated in a solution containing streptavidin conjugated magnetic beads in the wells of a 96-well plate at room temperature. The plate was then placed on a magnetic stand, and the supernatants from each well were transferred to the wells of a 96-well plate containing 0.01 mol/L NaOH and measured for fluorescence with a microplate reader (Fluoroscan Ascent; Dainippon Pharmaceutical, Osaka, Japan) at excitation and emission wavelengths of 485 nm and 538 nm, respectively, for fluorescein isothiocyanate and of 584 nm and 612 nm, respectively, for Texas red.

Statistical analysis

All of the statistical analyses were performed with the Statistical Package of Social Science (SPSS for Windows, version 11.0, SPSS Inc, Chicago, IL). Data are expressed as means ±SD. The allele frequency was determined by gene counting. Deviations in genotype distribution from Hardy-Weinberg equilibrium were analyzed with the χ^2 test. A one-way ANOVA was performed to compare the continuous variables among genotype groups. The Turkey's test was performed after the ANOVA as a post-hoc comparison test. Differences in gender and smoking between the genotypes were assessed with the χ^2 test. A multiple regression analysis was used to evaluate the impact of the -866 A/A genotype, age, gender, lipids and lifestyle factors such as BMI, drinking, and smoking on mean LDL particle size. $P<0.05$ was accepted as a statistically significant level.

Moreover, to observe the difference in mean LDL particle size between the -866G/G and A/A genotypes, based on information from the prior reports (approximately 60% G/G and 40% A/A) in respective genotype proportions [12], 262 Å and 267 Å [SD: 10 Å] in controls and coronary artery diseased patients in respective LDL size levels [23], the sample size was estimated at $n=2\times SD^2\times(\alpha+Z\beta)^2/\Delta^2$, assuming $\alpha=0.05$, $\beta=0.20$ (power=0.80), $Z\alpha=1.96$, $Z\beta=0.842$, $\Delta=5$, and SD=10; therefore the sample size for the G/G and A/A genotypes was calculated at 79 and 53 subjects, respectively.

Results

As shown in Table 1, the -866 G/G genotype of the UCP2 polymorphism had an overall frequency of 28.7%, whereas the G/A genotype and the A/A genotype frequencies were 47.0% and 24.3%, respectively. There was no notable difference in the genotype distribution between genders. The genotype frequency did not differ from that expected for Hardy-Weinberg equilibrium (p=0.75). There was no significant difference in age, BMI, blood pressure,

Table 2. Multiple regression analysis with LDL particel size as the response variable.

Explanatory variable	β-coefficient	P value
Age	0.006	0.899
Gender	0.050	0.398
BMI	0.177	0.001
Smoking	0.164	0.002
Alcohol drinking	0.118	0.040
Total cholesterol	−0.321	0.001
HDL-cholesterol	0.385	0.001
Triglyceride	−0.394	0.001
The -866 A/A genotype	−0.113	0.012

r^2=0.5. β, standard regression coefficient. Alcohol drinking; every day =0, 4–5 times a week = 1, 2–3 times a week = 2, once a week = 3, none = 4.
Smoking; never/past = 0, current = 1. Genotypes; the -866 G/G and G/A genotype = 0, the -866 A/A genotype = 1.

HbA1c, fasting blood glucose, total cholesterol, LDL-cholesterol and HDL-cholesterol, triglyceride, small dense LDL, leptin, and adiponectin levels, and the frequency of statin treatment among the genotypes, respectively. However, subjects with the -866 G/A genotype had significantly higher HDL-cholesterol levels than those with the -866 G/G genotype. Furthermore, subjects with the A/A genotype had significantly smaller serum LDL particles than those with the G/G genotype. A multiple regression analysis showed that the -866 A/A genotype was independently associated with a reduced LDL particle size (P=0.012), when adjusted for age, gender, BMI, total cholesterol, HDL-cholesterol, triglyceride, alcohol intake, and smoking (Table 2).

Discussion

In the present study, the frequency of the -866 A allele (47.8%) was similar to that reported in other studies including Japanese subjects [12,15], and the most important findings were that the -866 A/A genotype in the promoter region of the UCP2 gene may itself reduce LDL particle size in the general Japanese population, with being independent of potential confounding factors such as age, gender, BMI, alcohol drinking, and smoking. It has been reported that LDL particle size is highly predictive of increased risk for coronary artery disease [17–19]. LDL particles of a reduced size level are easily oxidized [24,25], and are associated with increased atherosclerotic burden [26,27]. In support of this, there are findings that subjects with smaller LDL particles have a two to three-fold greater risk of developing coronary artery disease [17–19], and our results seems useful in the early identification of subjects susceptible to reduction in LDL particle size.

The underlying mechanism responsible for the reduction in LDL particle size accompanying the -866 A/A genotype remains unclear. The LDL subclass distribution is influenced by several factors including age, sex, diet, obesity and genetic factors [28,29]. UCP2 belongs to the mitochondria anion family and has been shown to modulate the production of ROS by decreasing the mitochondrial membrane potential [30,31]. ROS can affect lipid metabolism, and the UCP2 gene may influence the involvement of ROS on lipid regulation [30,31]. *In vitro*, overexpression of UCP2 in macrophages limits steady-state levels of intracellular ROS [32], whereas UCP2 antisense oligonucleotides increased levels of intracellular ROS in murine endothelial cells [33]. Indeed, disruption of the UCP2 gene promoted the generation of ROS in transgenic mice [7]. These results together with other findings in middle-aged subjects suggest a protective role for UCP2 in atherosclerosis by reducing the amount of ROS generated [7,8,16]. Dhamrait et al. [34] reported a doubling of the risk of coronary heart disease for subjects with the -866 A/A genotype in a prospective study of healthy men. Furthermore, there is evidence that individuals with the -866 A/A genotype had significantly decreased adipose UCP2mRNA levels relative to those with the -866 G/G genotype [11]. It may be difficult to diminish the amount of ROS generated through decreased UCP2mRNA levels with the -866 A/A genotype, and as a result, it is conceivable that the -866 A/A genotype reduces LDL particle size. Further examinations are needed to clarify these points.

The present results indicating no genotype-related differences in triglyceride, total cholesterol, or LDL-cholesterol levels are consistent with previous findings in cohorts of non-diabetic Austrian [9] and Intalian [35] subjects, but not all findings [36]. Also, the findings that subjects with the -866 G/A genotype had increased HDL-cholesterol levels are in contrast to other previous studies [9,35,36]. Although the underlying mechanisms have yet to be elucidated, we have known about the influence of the UCP3 gene on increased HDL-cholesterol levels, in relation to the -866G/A promoter SNP in UCP2 [37], thus these analyses are thought to be of future benefit. Reis et al. [36] found that the -866 G/G genotype of UCP2 had a protective effect against high triglyceride, total cholesterol, and LDL-cholesterol levels, and conversely, the presence of the -866 A allele in either the homozygous or heterozygous form was associated with an unfavorable effect on these parameters in French Caucasian subjects with type 2 diabetes [36]. Although the reason for these contrasting results between studies is unclear, the differences in subjects examined (degree of BMI) or in the experimental design may be responsible. Anyway, our results that the -866 G/A genotype was independently and significantly associated with increased HDL-cholesterol levels, even considering the variable LDL particle size, also seem to be noteworthy.

Conclusions

The -866 A/A genotype in the promoter region of the UCP2 gene was associated with a reduced LDL particle size in the general Japanese population. The present results suggest the homozygous form to play a role in the genetic predisposition to a reduction of LDL particle size which that is a significant risk factor of the development for coronary artery disease. Further studies are necessary to clarify the contribution of the UCP2 -866 G/A SNP to lipid metabolism including LDL particle size and HDL-cholesterol levels, in addition to the occurrence of coronary artery disease.

REFERENCES:

1. Dalgaard LT, Hansen T, Urhammer SA et al: The uncoupling protein 3–55 C>T variant is not associated with Type II diabetes mellitus in Danish subjects. Diabetologia, 2001; 44: 1065–67
2. Saleh MC, Wheeler MB, Chan CB: Uncoupling protein-2: evidence for its function as a metabolic regulator. Diabetologia, 2002; 45: 174–87
3. Hesselink MK, Mensink M, Schrauwen P: Human uncoupling protein-3 and obesity: an update. Obes Res, 2003; 11: 1429–43
4. Fleury C, Neverova M, Collins S et al: Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. Nat Genet, 1997; 15: 269–72
5. Argyropoulos G, Brown AM, Peterson R et al: Structure and organization of the human uncoupling protein 2 gene and identification of a common biallelic variant in Caucasian and African-American subjects. Diabetes, 1998; 47: 685–87
6. Argyropoulos G, Harper ME: Uncoupling proteins and thermoregulation. J Appl Physiol, 2002; 92: 2187–98
7. Arsenijevic D, Onuma H, Pecqueur C et al: Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. Nat Genet, 2000; 26: 435–39
8. Blanc J, Alves-Guerra MC, Esposito B et al: Protective role of uncoupling protein 2 in atherosclerosis. Circulation, 2003; 107: 388–90
9. Esterbauer H, Schneitler C, Oberkofler H et al: A common polymorphism in the promoter of UCP2 is associated with decreased risk of obesity in middle-aged humans. Nat Genet, 2001; 28: 178-83
10. Krempler F, Esterbauer H, Weitgasser R et al: A functional polymorphism in the promoter of UCP2 enhances obesity risk but reduces type 2 diabetes risk in obese middle-aged humans. Diabetes, 2002; 51: 3331–35
11. Wang H, Chu WS, Lu T et al: Uncoupling protein-2 polymorphisms in type 2 diabetes, obesity, and insulin secretion. Am J Physiol Endocrinol Metab, 2004; 286: E1–7
12. Sasahara M, Nishi M, Kawashima H et al: Uncoupling protein 2 promoter polymorphism -866G/A affects its expression in beta-cells and modulates clinical profiles of Japanese type 2 diabetic patients. Diabetes, 2004; 53: 482–85
13. Bulotta A, Ludovico O, Coco A et al: The common -866G/A polymorphism in the promoter region of the UCP-2 gene is associated with reduced risk of type 2 diabetes in Caucasians from Italy. J Clin Endocrinol Metab, 2005; 90: 1176–80
14. Yamasaki H, Sasaki H, Ogawa K et al: Uncoupling protein 2 promoter polymorphism -866G/A affects peripheral nerve dysfunction in Japanese type 2 diabetic patients. Diabetes Care, 2006; 29: 888–94
15. Ji Q, Ikegami H, Fujisawa T et al: A common polymorphism of uncoupling protein 2 gene is associated with hypertension. J Hypertens, 2004; 22: 97–102
16. Oberkofler H, Iglseder B, Klein K et al: Associations of the UCP2 gene locus with asymptomatic carotid atherosclerosis in middle-aged women. Arterioscler Thromb Vasc Biol, 2005; 25: 604–10
17. Cho HK, Shin G, Ryu SK et al: Regulation of small dense LDL concentration in Korean and Scottish men and women. Atherosclerosis, 2002; 164: 187–93
18. Gardner CD, Fortmann SP, Krauss RM: Association of small low-density lipoprotein particles with the incidence of coronary artery disease in men and women. JAMA, 1996; 276: 875–81
19. Lamarche B, Tchernof A, Moorjani S et al: Small, dense low-density lipoprotein particles as a predictor of the risk of ischemic heart disease in men. Prospective results from the Quebec Cardiovascular Study. Circulation, 1997; 95: 69–75
20. Austin MA, Mykkanen L, Kuusisto J et al: Prospective study of small LDLs as a risk factor for non-insulin dependent diabetes mellitus in elderly men and women. Circulation, 1995; 92: 1770–78
21. Hoefner DM, Hodel SD, O'Brien JF et al: Development of a rapid, quantitative method for LDL subfractionation with use of the Quantimetrix Lipoprint LDL System. Clin Chem, 2001; 47: 266–74
22. Yamada Y, Izawa H, Ichihara S et al: Prediction of the risk of myocardial infarction from polymorphisms in candidate genes. N Engl J Med, 2002; 347: 1916–23
23. Gardner CD, Fortmann SP, Krauss RM: Association of small low-density lipoprotein particles with the incidence of coronary artery disease in men and women. JAMA, 1996; 276: 875–81
24. Arad Y, Rumsey SC, Galeano NF et al: LDL has optimal size for maximal binding to the LDL receptor. Circulation, 1992; 86: I-551
25. Tribble DL, Holl LG, Wood PD et al: Variations in oxidative susceptibility among six low density lipoprotein subfractions of differing density and particle size. Atherosclerosis, 1992; 93: 189–99
26. Toshima S, Hasegawa A, Kurabayashi M et al: Circulating oxidized low density lipoprotein levels. A biochemical risk marker for coronary heart disease. Arterioscler Thromb Vasc Biol, 2000; 20: 2243–47
27. Ehara S, Ueda M, Naruko T et al.: Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. Circulation, 2001, 17(103): 1955–60
28. Mauger JF, Lichtenstein AH, Ausman LM et al: Effect of different forms of dietary hydrogenated fats on LDL particle size. Am J Clin Nutr, 2003; 78: 370–75
29. Bosse Y, Chagnon YC, Despres JP et al: Compendium of genome-wide scans of lipid-related phenotypes: adding a new genome-wide search of apolipoprotein levels. J Lipid Res, 2004; 45: 2174–84
30. Negre-Salvayre A, Hirtz C, Carrera G et al: A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. FASEB J, 1997; 11: 809–15
31. Cannon B, Shabalina IG, Kramarova TV et al: Uncoupling proteins: a role in protection against reactive oxygen species – or not? Biochim Biophys Acta, 2006; 1757: 449–58
32. Ryu JW, Hong KH, Maeng JH et al: Overexpression of uncoupling protein 2 in THP1 monocytes inhibits beta2 integrin-mediated firm adhesion and transendothelial migration. Arterioscler Thromb Vasc Biol, 2004; 24: 864–70
33. Duval C, Negre-Salvayre A, Dogilo A et al: Increased reactive oxygen species production with antisense oligonucleotides directed against uncoupling protein 2 in murine endothelial cells. Biochem Cell Biol, 2002; 80: 757–64
34. Dhamrait SS, Stephens JW, Cooper JA et al: Cardiovascular risk in healthy men and markers of oxidative stress in diabetic men are associated with common variation in the gene for uncoupling protein 2. Eur Heart J, 2004; 25: 468–75
35. Sesti G, Cardellini M, Marini MA et al: A common polymorphism in the promoter of UCP2 contributes to the variation in insulin secretion in glucose-tolerant subjects. Diabetes, 2003; 52: 1280–83
36. Reis AF, Dubois-Laforgue D, Bellanne-Chantelot C et al: A polymorphism in the promoter of UCP2 gene modulates lipid levels in patients with type 2 diabetes. Mol Genet Metab, 2004; 82: 339–44
37. Cha MH, Kim IC, Kim KS et al: Association of UCP2 and UCP3 gene polymorphisms with serum high-density lipoprotein cholesterol among Korean women. Metabolism, 2007; 56: 806–13

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 30, pp. 21220–21229, July 25, 2008

Cyclin-dependent Kinase Inhibitor, p21$^{WAF1/CIP1}$, Is Involved in Adipocyte Differentiation and Hypertrophy, Linking to Obesity, and Insulin Resistance*[S]

Received for publication, March 6, 2008 Published, JBC Papers in Press, April 29, 2008, DOI 10.1074/jbc.M801824200

Noriyuki Inoue[‡], Naoya Yahagi[§], Takashi Yamamoto[‡], Mayumi Ishikawa[‡], Kazuhisa Watanabe[‡], Takashi Matsuzaka[§], Yoshimi Nakagawa[‡], Yoshinori Takeuchi[‡], Kazuto Kobayashi[‡], Akimitsu Takahashi[‡], Hiroaki Suzuki[‡], Alyssa H. Hasty[¶], Hideo Toyoshima[‡], Nobuhiro Yamada[‡], and Hitoshi Shimano[‡§1]

From the [‡]Department of Internal Medicine (Metabolism and Endocrinology) and [§]Graduate School of Comprehensive Human Sciences and Center for Tsukuba Advanced Research Alliance, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki, 305-8575, Japan and [¶]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee 37232

Both adipocyte hyperplasia and hypertrophy are determinant factors for adipocyte differentiation during the development of obesity. p21$^{WAF1/CIP1}$, a cyclin-dependent kinase inhibitor, is induced during adipocyte differentiation; however, its precise contribution to this process is unknown. Using both *in vitro* and *in vivo* systems, we show that p21 is crucial for maintaining adipocyte hypertrophy and obesity-induced insulin resistance. The absence of p21 in 3T3-L1 fibroblasts by RNA-mediated interference knockdown or in embryonic fibroblasts from p21$^{-/-}$ mice impaired adipocyte differentiation, resulting in smaller adipocytes. Despite normal adipose tissue mass on a normal diet, p21$^{-/-}$ mice fed high energy diets had reduced adipose tissue mass and adipocyte size accompanied by a marked improvement in insulin sensitivity. Knockdown of p21 in enlarged epididymal fat of diet-induced obese mice and also in fully differentiated 3T3-L1 adipocytes caused vigorous apoptosis by activating p53. Thus, p21 is involved in both adipocyte differentiation and in protecting hypertrophied adipocytes against apoptosis. Via both of these mechanisms, p21 promotes adipose tissue expansion during high fat diet feeding, leading to increased downstream pathophysiological consequences such as insulin resistance.

The process of adipocyte differentiation has been extensively characterized in cultures of preadipocyte clonal cell lines such as mouse 3T3-L1 and 3T3-F442A (1, 2). Adipogenesis requires a sequence of events including growth arrest of proliferating preadipocytes, coordinated reentry into the cell cycle with limited clonal expansion, and growth arrest before terminal differentiation during which lipid accumulation occurs. Thus, it is reasonable to assume that factors involved in cell cycle regulation may have important roles in the adipocyte differentiation process. Cell cycle progression in mammals is governed by various complexes of cyclins and cyclin-dependent kinases (CDKs)[2] as well as their inhibitors, of which p21 and p27 are the most widely studied. It was previously suggested that expression of p21 and p27 is changed during the adipogenesis (3). p21 has been reported to be induced by Foxo1 and has been implicated in entry of adipocytes into the clonal expansion phase of adipogenesis; meanwhile, p27 is critical for adipocyte hyperplasia (3–5). The expression of both p21 and p27 is altered during adipocyte differentiation; however, their precise role in adipogenesis, especially at the time of terminal differentiation, remains unclear.

Obesity is caused by over-nutrition and decreased physical activity and is characterized by excess storage of lipids in adipose tissue, which can be accounted for by both adipocyte hyperplasia and hypertrophy. Obesity often precedes insulin resistance and precipitates type 2 diabetes and cardiovascular diseases (6). Impaired adipocyte differentiation is also related to insulin resistance. Based upon the potential roles of CDK inhibitors in cell cycle progression and apoptosis, it is reasonable to speculate that CDK inhibitors could be involved in obesity and insulin resistance through regulation of adipocyte differentiation and adipose tissue growth.

p21$^{WAF1/CIP1}$ is the major CDK inhibitor and halts the cell cycle at G_1 (Refs. 7–10; for review, see Refs. 11 and 12). p21 is a well known target of p53, a stress response. In addition to causing G_1 arrest, p21 protects cells from apoptosis (12). We recently found that p21 is a direct gene target of SREBPs, crucial transcription factors involved in lipogenesis and adipogenesis (13, 14). We also reported that p21 expression was highly upregulated in hypertrophic adipose tissue and in the liver in models of obesity (15, 16). These data prompted us to explore the role of p21 in adipocyte differentiation and hypertrophy and to determine the involvement of p21 in obesity and insulin resistance.

* This research was supported in part by Grant-in-Aid for Scientific Research and Scientific Research on Priority Areas, 2006, 1805200 from the Ministry of Education, Science, Sports, and Culture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. A1–A5 and Tables A1–A3.

[1] To whom correspondence should be addressed. Tel.: 81-29-853-3053; Fax: 81-29-863-3074, E-mail: shimano-tky@umin.ac.jp.

[2] The abbreviations used are: CDK, cyclin-dependent kinase; RNAi, RNA-mediated interference; MEF, mouse embryonic fibroblast; HFHS, high fat high sucrose; PPAR, peroxisome proliferator-activated receptor; DEXA, dual energy x-ray absorptiometry; TUNEL, terminal dUTP nick-end labeling; C/EBP, CCAAT/enhancer-binding protein.

EXPERIMENTAL PROCEDURES

Cell Culture and Adipocyte Differentiation—3T3-L1 cells (ATCC) were maintained in high glucose Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal calf serum in 5% CO_2. Cells were seeded at 2.5×10^5 cell/6-cm collagen-coated dishes (BD Biosciences) (day 4) and cultured to confluence (day 2). After 2 days, cells were replaced with differentiation induction medium A (5 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine) (day 0). After 48 h cells were changed to differentiation induction medium B (5 mg/ml insulin) (day 2). Medium was renewed every other day.

Preparation of Recombinant Adenovirus—cDNAs encoding the full-length fragment of mouse $p21^{Waf1/Cip1}$ were integrated into adenoviral vectors. RNAi adenovirus vectors, RNAi-LacZ (LacZi), p21 RNAi-137 (137i), and p21 RNAi-275 (275i) were constructed with BLOCK-iT™ U6 RNAi Entry Vector kit and BLOCK-iT™ Adenoviral RNAi Expression system (Invitrogen). Each adenoviral vector was propagated in 293A cells and purified by cesium chloride density centrifugation (17, 18).

Oil Red O Stain—Cells were fixed with 10% formalin in phosphate-buffered saline for 10 min at 37 °C and stained with Oil Red O for 60 min at room temperature. Cells were washed with distilled water, and the retained dye was eluted by isopropanol (19).

Mice—This project was approved by The Animal Care Committee of University of Tsukuba and performed under its guidelines. The $p21^{Waf1/Cip1}$ knock-out mice (B6;129S2-*Cdkn1a*^{tm1Tyj}/J) were originally purchased from CLEA Japan Inc. and The Jackson Laboratory, provided by Dr. Tyler Jacks (20) and were backcrossed onto the C57BL/6J background within our colony for five or more generations. Mice were genotyped by PCR according to methods provided by The Jackson Laboratory. Mice were maintained on a normal chow diet for a 14-h light/10-h dark cycle. For diet-induced obesity, the mice were fed on a high fat high sucrose (HFHS) diet as previously described (21) at 10 weeks of age for 6 (or indicated) weeks. We performed weekly collections of blood for metabolic analysis (measurement of blood glucose, insulin, triglyceride, total cholesterol, and free fatty acid) and measured body weight. They were subjected to intravenous glucose tolerance tests 4 weeks after the HFHS diet started, insulin tolerance tests 5 weeks after the diet started, dual energy x-ray absorptiometry (DEXA) analysis, and sacrifice at 6 weeks. Tissue samples were fixed in 10% formalin for microscopy analysis and preserved in liquid N_2 for the Northern blot analysis.

DEXA Analysis—PIXImus2 DEXA (GE Medical Systems LUNAR) was used to measure lean body tissue and percent fat mass (22).

Determination of Adipocyte Number and Size—Epididymal fat pads from normal mice (~50 mg) and HFHS-fed mice (~150 mg) were fixed in osmium tetroxide-collidine buffer for 3 days. Adipocytes of diameter from 25 to 125 μm in normal mice or from 25 to 250 μm in HFHS mice were collected by filtration through 25-, 125-, and 250-μm nylon screens. Size and number of adipocytes were measured with a Z-2 Coulter counter for normal mice and a Multisizer 3 Coulter counter for HFHS mice (Beckman Coulter) as previously described (22–24).

Metabolic Studies—Glucose tolerance tests were performed on male mice fasted overnight (9–12 h). Glucose solutions were injected at 2 g/kg of body weight via tail vein. Blood glucose and insulin were measured 0, 5, 15, 30, and 60 min after injection. Insulin tolerance tests were performed on male mice fasted for 3 h. Mice were intraperitoneally injected with human regular insulin at 0.5 units/kg or 0.75 units/kg of body weight (Humulin R 40 units/ml, Eli Lilly), and blood glucose was measured 0–120 min after injection with ANTSENSE II (Horiba Ltd.). Plasma glucose, triglyceride, cholesterol, and non-esterfied fatty acids were determined with Glu ICII, TG IE, T-Cho IE, and non-esterfied fatty acid IC kits from WAKO. Plasma insulin and leptin were determined with the mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (TMB) (AKRIN-011T, Shibayagi) and the mouse leptin ELISA kit (Morinaga Institute of Biological Science, Inc.), respectively.

Northern Blot Analysis—Total RNA was prepared from livers, white adipose tissue, brown adipose tissue, muscle, and cultured cells using TRIzol reagent (Invitrogen). For Northern blot analysis, equal aliquots of total RNA from 5–6 mice and culture cells were pooled (5–10 μg of total RNA), denatured with formaldehyde and formamide, subjected to electrophoresis in a 1% agarose gel, and transferred to Hybond N membranes (Amersham Biosciences) for hybridization. cDNA probes were labeled with [α-^{32}P]dCTP (3,000–10,000 cpm) using Rediprime™ Random Prime Labeling System (Amersham Biosciences). The filters were hybridized with the radiolabeled probe in Rapid-hyb buffer (Amersham Biosciences) at 65 °C and washed in 0.1 × SSC, 0.1% SDS at 65 °C. Blots were exposed to Kodak BIOMAX MS1 (Eastman Kodak Co.) and BAS 2500 with BAStation software (Fuji Photo Film).

Cell Proliferation Assay—For determination of cell proliferation, a 5-bromo-2′-deoxyuridine labeling and detection kit I was used according to the manufacturer's instructions (Roche Applied Science).

Apoptosis Assay—For the assessment of apoptosis, the In Situ Cell Death Detection kit (Roche Applied Science) was used. The test principle of this kit was labeling DNA strand breaks by terminal deoxynucleotidyltransferase, which catalyzes the polymerization of labeled nucleotides to free 3′-OH DNA ends in a template-independent manner (TUNEL reaction).

Immunoblot Analysis—Cells were harvested in 1% Triton X-100 buffer (25 mM Hepes (pH 7.9), 50 mM KCl, 5 mM EDTA, 5 mM $MgCl_2$, 1% Triton X-100, 1 mM dithiothreitol, and protease inhibitor (Roche Applied Science)). Protein concentrations were determined using the BCA protein assay kit (Pierce). Samples were fractionated on 8, 12, or 15% SDS-PAGE followed by transfer to Immobilon Transfer membranes (Millipore). Blots were subsequently incubated in Tris-buffered saline-Tween overnight at 4 °C with 1:2000 anti-$p21^{waf1}$, anti-$p27^{Kip1}$, anti-C/EBPα, anti-C/EBPβ, anti-p53 (Santa Cruz) Ser-15 phosphorylation of p53, Ser-20 phosphorylation of p53, Ser-46 phosphorylation of p53 (Cell Signaling), and α-tubulin (Calbiochem). After incubation with horseradish peroxidase-conjugated secondary antibod-

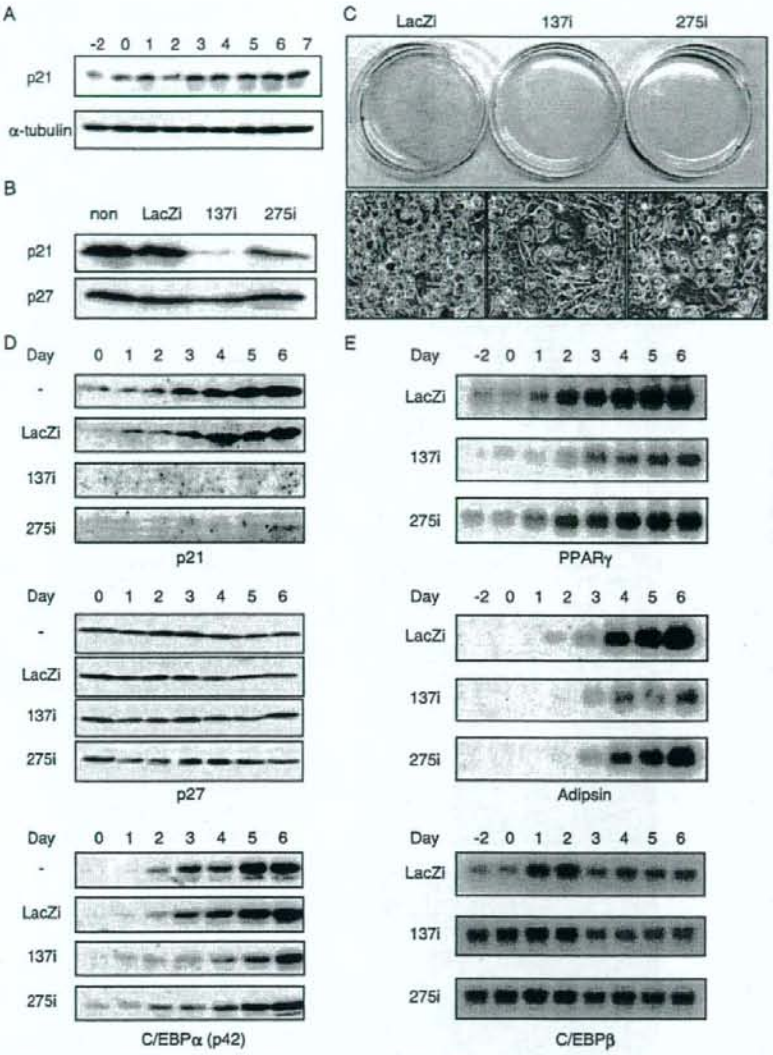

FIGURE 1. **Inhibition of adipocyte differentiation in 3T3-L1 cells by knockdown of p21.** *A*, changes of p21 protein in differentiating 3T3-L1 cells. Total cellular proteins were extracted from cells at the indicated days of differentiation and subjected to immunoblot analysis with antibodies against p21 and α-tubulin as a loading control. *B*, efficacy of p21 knockdown in 3T3-L1 preadipocytes infected with p21RNAi adenoviruses. Expression of p21 and p27 level is shown by immunoblotting 24 h after the infection of p21 RNAi adenovirus (*137i* or *275i*) or LacZi adenovirus (*LacZi*) as a negative control. Note that 137i exhibits nearly complete suppression with a higher efficiency of p21 knockdown than 275i. *C*, effect of p21 gene knockdown on adipocytes differentiation. 3T3-L1 adipocytes were infected with adenoviruses at day 3. The Oil Red O staining of cells from day 6 post-differentiation induction is shown. *D* and *E*, time course analysis of protein (*D*) and mRNA (*E*) expression in 3T3-L1 cells after infection of p21 RNAi adenoviruses at day 3. Cellular total RNA (5 μg) and protein (80 μg) were subjected to SDS-PAGE followed by immunoblot analysis for p21, p27, and C/EBPα (*D*) and to Northern blot analysis for PPARγ, adipsin, and C/EBPβ (*E*), respectively.

ies, blots were detected by ECL or ECL Advance Western blotting detection kit (Amersham Biosciences).

In Vivo Adenovirus Injection into White Adipose Tissue—After 3 weeks of HFHS feeding, mice were anesthetized by halothane before tissue dissection. The adenovirus (2.5×10^{11} optical particle unit) was injected on each side of the epididymal fat pad (25).

Statistical Analysis—Data are expressed as the mean ± S.E. Statistical significance was assessed using the Student's *t* test. Data sets involving more than two groups are assessed by Dunnett's test (SAS Institute Inc.).

RESULTS

Effects of the Acute Absence of p21 on Adipocyte Differentiation in Vitro—3T3-L1 fibroblasts were differentiated into adipocytes, and p21 expression was assessed. Consistent with previous works, there was a rapid induction of p21 protein at the time of differentiation into adipocytes (Fig. 1*A*) (3). Expression of p21 was sustained at both the mRNA and protein levels during terminal differentiation and during the subsequent hypertrophic phase of lipid accumulation. To determine the effects of acute p21 deficiency, knockdown of p21 using two different adenoviral vectors with RNAi (137i and 275i) before induction of differentiation was performed (Fig. 1*B*). p21 deficiency caused a dose-dependent suppression of adipocyte differentiation as assessed by Oil Red O staining (Fig. 1*C*). The inhibition of p21 resulted in no changes in p27 levels (Fig. 1*D*). However, reduction in p21 levels was accompanied by reduced levels of C/EBPα and PPARγ, transcription factors involved in adipocyte differentiation, along with adipsin, a marker for adipogenesis (Fig. 1*E*). However, C/EBPβ, known to function at an initial stage of adipogenesis was rather enhanced by p21 inhibition, suggesting that the absence of p21 might impair the later stage of adipocyte differentiation (Fig. 1*E*).

Effects of the Chronic Absence of p21 during Adipocyte Differentiation in Vitro—To determine the effect of chronic absence of p21, mouse embryonic fibroblasts (MEFs) from $p21^{-/-}$ mice were induced to differentiate into adipocytes (supplemental Fig. A1). Before differentiation, $p21^{-/-}$ MEF cells exhibited an increased cell number and uptake of 5-bromo-2′-deoxyuridine as compared with $p21^{+/+}$ control cells, indicating enhanced cell

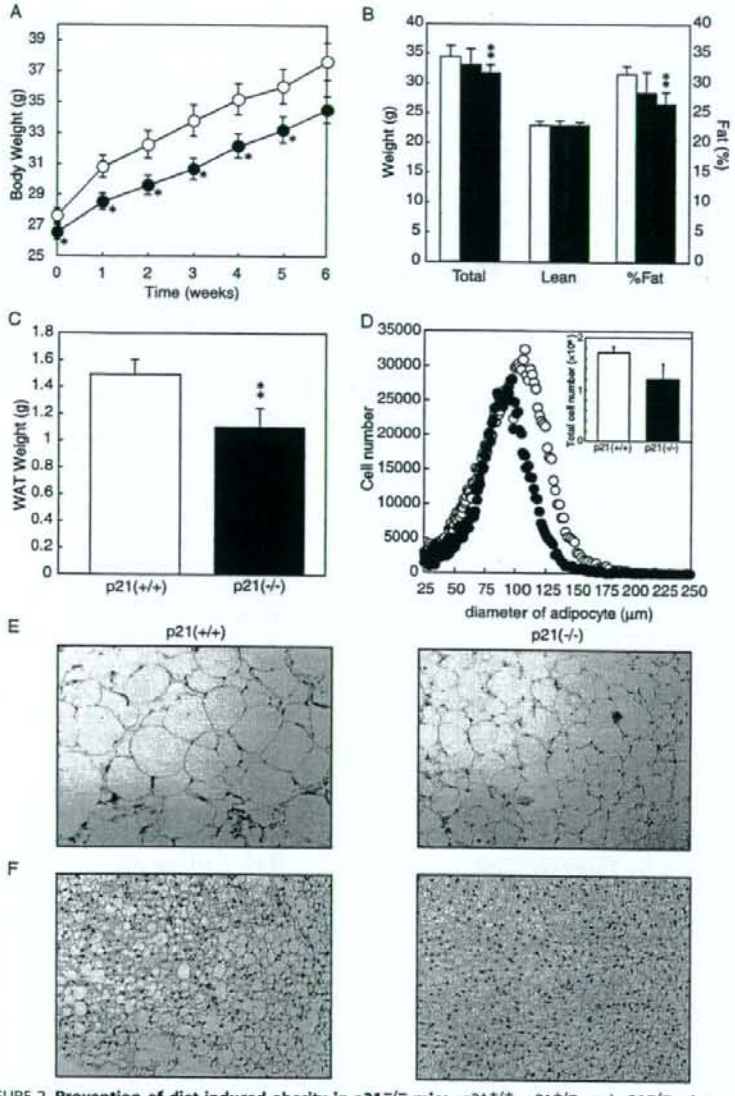

FIGURE 2. **Prevention of diet-induced obesity in p21$^{-/-}$ mice.** p21$^{+/+}$, p21$^{+/-}$, and p21$^{-/-}$ mice were placed on a high fat and high sucrose (HFHS) diet at 10 weeks of age for a total of 6 weeks. *A*, body weight curves on a HFHS diet. *B*, total and lean mass and % fat in p21$^{+/+}$ (*white bars*), p21$^{+/-}$ (*gray bars*), and p21$^{-/-}$ (*black bars*) mice as estimated by DEXA. *C*, weight of epididymal fat pads in p21$^{+/+}$ and p21$^{-/-}$ mice. *WAT*, white adipose tissue. *D*, size distribution and total cell number (*inset*) of adipocytes from white adipose tissue. *E* and *F*, microscopic examination of white (*E*) and brown (*F*) adipose tissues (hematoxylin/eosin stain). Values represent the means ± S.E. from 14 mice per group. $p < 0.05$ (*) and at $p < 0.01$ (**) for p21$^{-/-}$ compared with p21$^{+/+}$ groups.

growth in the absence of p21 (supplemental Fig. A1, *A* and *B*). Upon induction of differentiation, p21$^{-/-}$ MEF cells began to differentiate sooner; however, at terminal differentiation the size of the resultant adipocytes was smaller than differentiated wild type p21$^{+/+}$ MEFs (supplemental Fig. A1*C*). These data demonstrate that p21 contributes to, but is not indispensable, for adipocyte differentiation.

Metabolic Effects of the Chronic Absence of p21 in Vivo—Consistent with the results from cultured cells, adiposity of chow diet-fed p21$^{-/-}$ mice was normal. Growth curves were nearly identical between p21$^{-/-}$ and p21$^{+/+}$ mice (supplemental Fig. A2*A*). Body weight, total fat weight (by DEXA), and adipocyte size did not significantly changed with only a minimal trend toward a decrease in p21$^{-/-}$ mice (supplemental Fig. A2, *B* and *C*, and A3, A–C). There were no significant changes in plasma glucose, insulin, lipids, or leptin levels (supplemental Table A1). Insulin sensitivity and secretion did not change in p21$^{-/-}$ mice as assessed by glucose and insulin tolerance tests (supplemental Fig. A2, *D* and *E*, and Table A1).

Next, the role of p21 in adipogenesis in diet-induced obesity was evaluated. Our preliminary data indicated that gene expression of p21 was highly induced by high fat diet, suggesting a role of this protein in the hypertrophic change of adipocytes (data not shown). When p21$^{-/-}$ mice were fed the HFHS diet, which rapidly induces obesity and insulin resistance, the degree of body weight gain was ameliorated compared with strain-matched C57BL/6 p21$^{+/+}$ controls (Fig. 2*A*). The retarded body weight gain in p21$^{-/-}$ mice was due to decreased fat mass as estimated by DEXA (Fig. 2*B*). The weight of epididymal fat pads was also significantly decreased in p21$^{-/-}$ mice (Fig. 2*C*). Total fat weight of p21$^{+/-}$ mice was between those of p21$^{+/+}$ and p21$^{-/-}$ mice, demonstrating the dose-dependent effect of p21 on adiposity (Fig. 2*B*). The size distribution of adipocytes from the epididymal adipose tissue, as estimated by a Coulter counter, also confirmed a significant decrease in the size of p21$^{-/-}$ adipocytes (Fig. 2*D*). Consistently, histological analysis of adipose tissue demonstrated that the marked enlargement of adipocytes in HFHS-fed mice was suppressed in p21$^{-/-}$ mice (Fig. 2*E*). The presence of large lipid vacuoles, which emerged in the cytosol of brown fat cells of HFHS diet-fed p21$^{+/+}$ mice, was decreased in p21$^{-/-}$ mice (Fig. 2*F*). In fact, brown adipose tissue of p21$^{-/-}$ mice fed HFHS diets appeared similar to

$p21^{+/+}$ mice on a chow diet (compare Fig. 2*F* and supplemental Fig. A3*D*).

The amelioration of obesity was accompanied by marked improvement of insulin resistance (Fig. 3). Improved insulin sensitivity in HFHS-fed $p21^{-/-}$ mice was supported by decreased plasma insulin levels despite similar or slight trends of decreasing plasma glucose levels in fasted and fed conditions and in intravenous glucose tolerance tests (supplemental Table A2, Fig. 3, *A* and *B*). Insulin tolerance tests showed a more prominent decrease in plasma glucose levels after injection of insulin in $p21^{-/-}$ mice than in control mice (Fig. 3*B*). These data indicate that gene disruption of p21 causes a partial protection from diet-induced obesity and a marked improvement in insulin resistance. This protection from diet-induced obesity was not due to differences in food intake (2.95 ± 0.10 *versus* 2.86 ± 0.05 g/day for $p21^{+/+}$ and $p21^{-/-}$ mice, respectively; $n = 6$; data were collected over a 4-week period).

Gene Expression in Metabolic Tissues of $p21^{-/-}$ Mice—Expression of various genes in white adipose tissue of HFHS-fed $p21^{+/+}$ and $p21^{-/-}$ mice were estimated by Northern blot analysis (Fig. 4*A*). Adipogenic transcription factors such as PPARγ, C/EBPα, and C/EBPβ were not different between $p21^{+/+}$ and $p21^{-/-}$ mice. Expression of leptin and ADRP, known to be up-regulated in white adipose tissue in obesity, was suppressed in $p21^{-/-}$ mice on a HFHS diet, whereas adipsin, known to be suppressed in obesity, was up-regulated in $p21^{-/-}$ adipose tissues to varying degrees. As estimated by reverse transcription-PCR (supplemental Fig.A5*A*), decreased leptin and increased adiponectin were observed, consistent with amelioration of obesity in $p21^{-/-}$ mice on a HFHS diet (supplemental Fig. A5*A*). Expression of monocyte chemoattractant protein 1 and tumor necrosis factor α was also decreased, indicating that macrophage infiltration could be suppressed by the absence of p21 (supplemental Fig. A5*B*). PGC1α in brown adipose tissue, SCD-1 in the liver, and PPARδ in skeletal muscle are all known to be involved in energy consumption. These genes were decreased in diet-induced obesity in $p21^{+/+}$ mice, and their levels were normalized in $p21^{-/-}$ mice, indicating a shift of energy balance toward to expenditure (Fig. 4*B*).

Potential Involvement of Apoptosis in Mechanisms by Which p21 Deficiency Protects against Diet-induced Obesity—To determine the mechanism by which the absence of p21 protects against diet-induced obesity and insulin resistance, p21 was suppressed in 3T3-L1 cells after they had already differentiated into mature adipocytes. In contrast to infection of adenoviral RNAi before the differentiation protocol (Fig. 1, *C–E*), acute suppression of p21 at day 6 caused inhibition of lipid accumulation, detachment of the lipid-containing cells, and a marked change in color of the medium (Fig. 5*A*). TUNEL staining confirmed that these changes significantly involve apoptosis (Fig. 5, *B* and *C*). Supporting these observations, immunoblot analysis indicated that inhibition of p21 was followed by a time-dependent induction of caspase-3, the terminal player of apoptosis, but no change in expression of Bcl-2, a gene that plays a protective role from apoptosis (Fig. 6*B*). p53 phosphorylated at Ser-46, associated with apoptosis (26), was induced at day 8, 48 h after infection with p21RNAi adenovirus (Fig. 6*B*). These data suggest that p21 inhibition during the terminal differentiation of adipocytes could induce apoptosis, suppressing the hypertrophic changes required for completion of differentiation. p53, the most potent inducer of p21, is known to be activated in

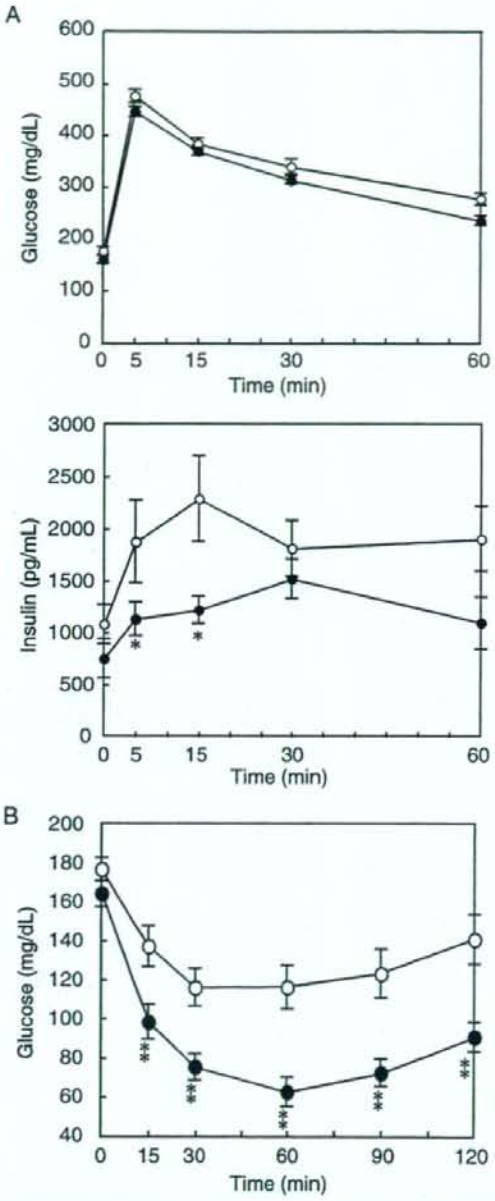

FIGURE 3. **Protection of $p21^{-/-}$ mice from insulin resistance caused by diet-induced obesity.** Glucose tolerance test (*A*) and insulin tolerance test (*B*) on $p21^{+/+}$ (*white circles*) and $p21^{-/-}$ mice (*black circles*). Mice were fed the HFHS diet for 4 weeks and injected with 2 mg/kg D-glucose (*A*) or 0.75 units/kg insulin for 5 weeks under the HFHS diet condition (*B*). The panels show serum glucose concentration (*upper panel of A* and *B*) and insulin concentration (*lower panel of A*) at the indicated time (min). Values represent the means ± S.E. from 14 mice per group. $p < 0.05$ (*) and at $p < 0.01$ (**) for $p21^{-/-}$ compared with $p21^{+/+}$ groups.

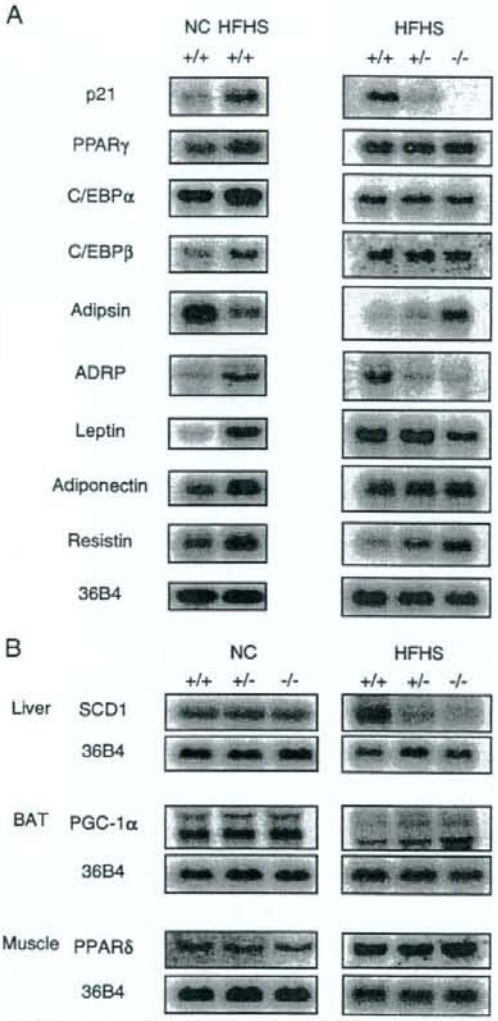

FIGURE 4. **Gene expression of $p21^{-/-}$ mice on a HFHS diet.** *A*, Northern blot analysis in epididymal fat pads in $p21^{+/+}$ and $p21^{-/-}$ mice fed a HFHS diet for 6 weeks. *Left panel*, $p21^{+/+}$ mice on a normal chow (*NC*) diet as a control and HFHS diet. *Right panel*, $p21^{+/+}$, $p21^{+/-}$, and $p21^{-/-}$ mice on a HFHS diet for 6 weeks. *B*, Northern blot analysis of livers, blown adipose tissues (*BAT*) and skeletal muscles in p21 knock-out mice was shown. *Left panel*, $p21^{+/+}$, $p21^{+/-}$, and $p21^{-/-}$ mice on a normal chow diet as a control. *Right panel*, $p21^{+/+}$, $p21^{+/-}$, and $p21^{-/-}$ mice fed a HFHS diet for 6 weeks. All mice were sacrificed at fasted state for 12 h. Total RNA was extracted from the organs of six mice. Equal aliquots of the RNA samples were pooled for each organ (10 μg) and subjected to Northern blot analysis for hybridization with the indicated cDNA probes (36B4 as a loading control).

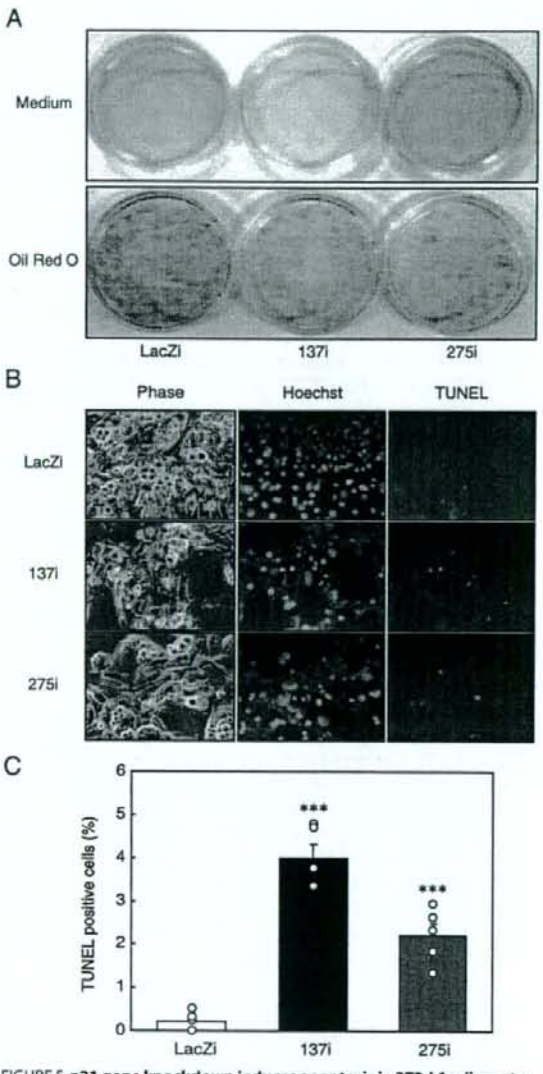

FIGURE 5. **p21 gene knockdown induces apoptosis in 3T3-L1 adipocytes.** *A*, 3T3-L1 cells were differentiated into adipocytes, and p21 RNAi adenovirus was infected at day 6. Media (*upper panel*) and Oil Red O staining of 3T3-L1 cells (*lower panel*) 4 days after infection of p21 RNAi (*137i* or *275i*), or control LacZi adenovirus (day 10). *B*, apoptosis of 3T3-L1 adipocytes after p21 knockdown at day 10. Microscopic examinations of the cells with light phase contrast for morphology (phase (*left*)), Hoechst 33342 for nuclear staining (Hoechst (*middle*)), and TUNEL staining for apoptotic cells (TUNEL (*right*)) are shown at a magnification of ×400. *C*, emergence of apoptotic cells was determined by the ratio of TUNEL positive cells to Hoechst 33342-positive cells. Values represent the mean ± S.E. from six dishes per group. ***, at $p < 0.0001$ for 137i and 275i *versus* LacZi adenovirus-infected cells.

response to cellular stresses. p53 protein has been detected in 3T3-L1 cells and remains constant during intracellular accumulation of lipids in Fig. 6*A*, consistent with the previous report (27, 28). However, phosphorylation of p53 protein at Ser-15 and Ser-20 is the key process for p21 trans-activation (29, 30). In accordance with p21 induction, the phosphorylation of these sites was elevated during the adipocyte differentiation. In contrast, phosphorylation of p53 at Ser-46 was reported to be associated with apoptosis (26). This apoptosis-related change of p53 was induced at the late stage of differentiation in the 3T3-L1 adipocytes after the Ser-15 and Ser-20 phosphorylation (Fig. 6*A*).

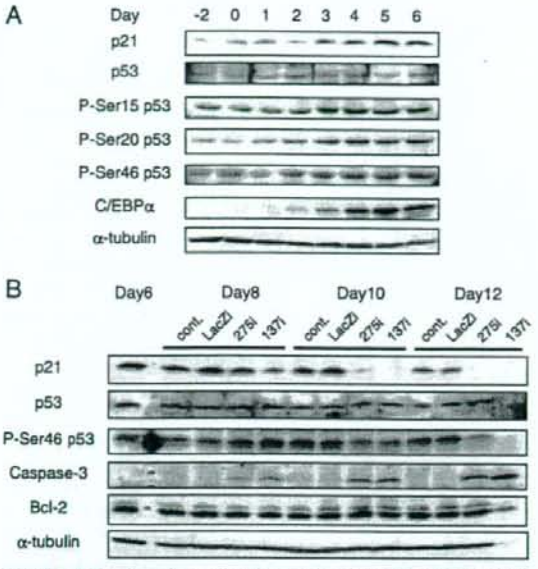

FIGURE 6. **Activation of caspase-3 by p21 knockdown in 3T3-L1 adipocytes after intrinsic activation of p21/p53.** *A*, time-course changes of p53 and p21 activation in 3T3-L1 cells during adipocyte differentiation. 3T3-L1 fibroblasts were differentiated into adipocytes according to the standard protocol (see "Experimental Procedures"). The level of p53, p21 proteins, and phosphorylation (*P*) of p53 at Ser-15, Ser-20, and Ser-46 crucial for p21 trans-activation were analyzed at the indicated days by immunoblot analysis. *B*, 3T3-L1 cells were induced to differentiate into adipocytes and infected with p21 RNAi adenovirus at day 6. Changes of p21, p53, Ser-46 phosphorylation of p53, caspase-3, Bcl-2, and α-tubulin (as a loading control) proteins in 3T3-L1 adipocytes at the indicated days after the induction for adipocyte differentiation were determined by immunoblot analysis.

To examine the involvement of apoptosis in the suppression of p21 *in vivo*, adenoviral RNAi for p21 was injected directly into epididymal adipose tissue of HFHS-fed mice. Significant inhibition of p21 expression was obtained 4 days after the infection (Fig. 7*A*), causing a trend to loss of body weight (Fig. 7*B*) accompanied by decreased glucose and insulin levels in insulin and glucose tolerance tests, respectively (Fig. 7, *C* and *D*). TUNEL staining demonstrated induction of adipocyte apoptosis by p21 knockdown (Fig. 7*E*). These data implicated that p21 could be induced by diet-induced obesity and contributes to protection from apoptosis during adipocyte hypertrophy.

DISCUSSION

Role of p21 in Adipocyte Differentiation and Apoptosis—Our current study clearly demonstrates that p21 plays a crucial role in later stages of differentiation and hypertrophy of adipocytes. p21 is induced during adipogenesis and sustained during terminal differentiation. p21 knockdown experiments demonstrated that p21 contributes to but is not absolutely required for adipocyte differentiation. Emergence of vigorous apoptosis upon acute p21 knockdown during the terminal phase of 3T3-L1 differentiation and in hypertrophic adipose tissue during diet-induced obesity in mice reveals that p21 is crucial for adipocyte hypertrophy rather than for the initial stages of differentiation. *In vivo*, p21 was highly induced in enlarged adipose tissue of diet-induced obese mice, and its absence ameliorated obesity. In contrast to acute knockdown experiments of p21, apoptosis was not detected in adipose tissue from $p21^{-/-}$ mice on a HFHS diet or in $p21^{-/-}$ MEF throughout adipocyte differentiation (data not shown). We speculate that in the absence of p21, adipocytes might not become hypertrophic enough to cause apoptosis.

In normal physiology, it is conceivable that the increased accumulation of intracellular lipids, which occurs during differentiation, is a cellular stress to large adipocytes, leading to activation of the p53/p21 pathway. In this situation p21 might be required for survival of hypertrophic adipocytes. This potential of p21 is mediated through its anti-apoptotic actions rather than its growth arrest properties. It has long been speculated that adipocyte hypertrophy involves apoptosis (31, 32), but evidence of apoptosis was not appreciably observed. Our data provide evidence that in the presence of p21, hypertrophic adipocytes are relatively protected against apoptosis, possibly explaining this discrepancy.

Role of p53 in Sustained p21 Expression in Adipocytes—Previous studies have shown that the transcription factors Foxo1, SREBP-1c/ADD1, and C/EBP family are involved in adipocyte differentiation, and all three have strong transcriptional activity for the p21 promoter (4, 13, 33, 34). Each of these factors likely contributes to the induction of p21 during the initial stages of differentiation in normal adipocytes. Although expression of p53, a dominant regulator of p21, remains stable at both mRNA and protein levels during adipocyte differentiation, we found that Ser-15 and Ser-20 of p53, crucial sites for trans-activation of p21 (29, 35) were hyperphosphorylated during the later stages of differentiation. It can be speculated that sustained p21 expression during terminal differentiation or obesity-related activation is mediated through p53 activation. We previously observed that the p53/p21 pathway is activated in hypertrophied adipocytes, such as from leptin-deficient ob/ob mice, and also in fatty livers (15, 16). This is consistent with our current observation in adipose tissue of HFHS diet-induced obese mice. p53 is the key sensor of indications of cellular stress such as DNA damage and ultimately determines whether the cell will go through cell growth arrest or apoptosis. p21 is an established cell cycle arrest inducer but has been also implicated to be involved in inhibition of apoptosis (12). In 3T3L1 adipocytes, p53 activation, as indicated by phosphorylation at Ser-15 and Ser-20, could result from lipid accumulation. Furthermore, phosphorylation of p53 at Ser-46 at the later stage of differentiation indicated the potential risk of apoptosis (26) that was masked by p21. Our current data suggest that hypertrophic adipocytes in obesity are also under considerable stress and are vulnerable to apoptosis, which under normal conditions, could be inhibited by activation of p21. Thus, we postulate that p21 plays a role in adipogenesis by halting cell cycle but, more importantly, by preventing the apoptosis of hypertrophic adipocytes.

Effect of p21 on Obesity and Insulin Resistance—Of clinical relevance, amelioration of high fat diet-induced obesity in

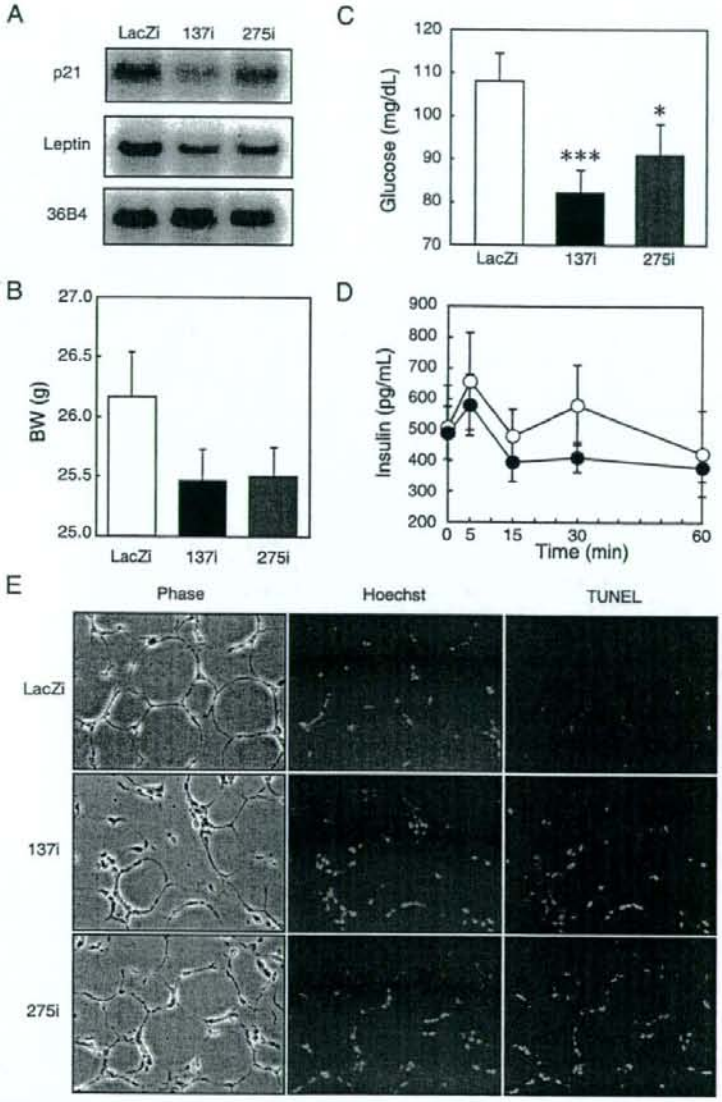

FIGURE 7. **Local p21 knockdown in white adipose tissue from diet-induced obesity mice causes apoptosis.** C57BL/6J mice (6 weeks of age) were fed a HFHS diet for 3 weeks. Adenoviral p21 RNAi (137i (*black*) or 275i (*gray*)) or LacZi (*white*) as a control was directly injected into epididymal white fat pads. After injection of adenovirus, these mice were fed a HFHS diet. Four days after the local infection, mice were sacrificed, and adipose tissues were collected. Total RNA of equally pooled aliquots (10 μg) extracted from the fat pads of six mice was subjected to Northern blot analysis for p21 and leptin (*A*). Body weights (*BW*), blood glucose concentrations 5 min after intraperitoneal injection of insulin 4 days after the local infection (*C*), and plasma insulin concentrations on an intravenous glucose tolerance test 6 days after the local infection (*D*) are shown in the diet-induced obesity mice after p21 knockdown in white adipose tissue ($n = 12$). Shown are TUNEL assays for apoptotic cells with light phase contrast microscopy and Hoechst 33342 staining for nuclear staining 4 days after the local infection (*E*). Values represent the means ± S.E. from 12 mice per group. $p < 0.05$ (*) and at $p < 0.001$ (***) for 137i and 275i *versus* LacZi adenovirus infected mice.

we speculate that it could be strain and/or sex differences in which we used male $p21^{-/-}$ mice backcrossed to C57BL6 *versus* the original work on female mice on the undescribed back ground. We presume that p21 in adipocytes plays the major role in obesity and insulin resistance since even partial knockdown of p21 in epididymal fat pads effectively reproduced the apoptosis and decreased the size of adipocytes as observed in $p21^{-/-}$ mice on HFHS diet. Because decreased size of adipose tissue was associated with reduction in monocyte chemoattractant protein 1 and tumor necrosis factor α expression (supplemental Fig. A5*B*), macrophage infiltration and subsequent proinflammatory responses in obesity were suppressed in $p21^{-/-}$ mice. The absence of p21 in macrophages could contribute to inhibition of inflammation in adipose tissues as reported in atherosclerosis (36). There were only mild changes in the expression of genes related to energy metabolism in liver and skeletal muscle of $p21^{-/-}$ mice on HFHS diet, indicating that they are not the primary organs responsible for anti-obesity in the absence of p21. It should be noted that in brown adipose tissue, PGC1α was slightly restored in $p21^{-/-}$ mice. Diet-induced obesity is usually accompanied by fat accumulation and decreased burning function in this tissue (6), which was prevented in HFHS-fed $p21^{-/-}$ mice Considering the similar food intake between $p21^{+/+}$ and $p21^{-/-}$ mice, it is possible that p21 deficiency contributed to amelioration of diet-induced obesity and insulin resistance at least partially by restoring the fat-burning function of brown fat tissue.

A New Link between Cell Growth and Metabolism—Our study provides evidence that factors responsible for the regulation of cell growth and survival are also involved in metabolism. A recent report on a direct interaction between p21 and Akt substantiates this novel link between a CDK inhibitor and metabolism (37). The p53/p21 pathway might play a determining role in the fate of hyper-

$p21^{-/-}$ mice was associated with improved insulin sensitivity. Adiposity in $p21^{-/-}$ mice was originally reported to be slightly increased as estimated by epididymal fat pads (22). The cause of this discrepancy with our data is currently unknown; however,