TABLE 1. BACTERIAL CULTURE IN BLOOD

	Time after Infection (*h*)	Experiment 1 Culture Positive (*Positive Ratio*)	Experiment 2 Culture-Positive (*Positive Ratio*)	Experiment 2 Quantitative Culture (*cfu/10 μl Blood*)
Control mice				
	24	2/5	3/5	$8.8 \pm 5.3 \times 10^3$
	72	3/5	5/5	$2.6 \pm 1.9 \times 10^3$
Mice with emphysema				
	24	5/5	5/5	$8.9 \pm 4.1 \times 10^3$
	72	5/5	5/5	$309.3 \pm 266.5 \times 10^3$

Bacterial cultures in blood from two independent experiments are shown. In Experiment 1, two of the five control mice were positive in blood culture 24 h after infection, and three of the five mice were positive at 72 h. A small degree of *S. pneumoniae* could be observed in Experiment 2. However, all mice with emphysema were positive in both experiments 24 h after infection, and the numbers of bacteria cultured were markedly increased 72 h after infection. A total of 1.76×10^5 cfu/mouse of *S. pneumoniae* were challenged in Experiment 1, and 1.0×10^7 cfu/mouse were challenged in Experiment 2.

only a short time (30 minutes or so) before they are internalized within a macrophage (43, 45). Once the bacterium is within the macrophage, the macrophage's bactericidal mechanisms destroy the microbe. The lower BALF cytokine levels in the mice with emphysema may be due to the loss of contact with alveolar macrophages and/or to altered function of alveolar macrophages. It is also possible that chemokine production by other airway cells was blunted in the mice with emphysema (46–49).

Third, systemic responses such as serum cytokine concentrations in mice with emphysema were significantly higher than those in the control mice. The present findings suggest that severe systemic inflammatory response in mice with emphysema occurred because of severe bacteremia and that the major site of the inflammatory response moved from the alveolar space to the systemic circulation. These reversed cytokine gradients could impair neutrophil influx into the alveolar spaces after infection in the mice with emphysema. It is also possible that the loss of the capillary bed and damage to the capillary basement membrane by neutrophils elastase may have contributed to the impaired influx of neutrophils. The higher mortality of the mice with emphysema might be due to decreased microbial killing, perhaps because of the modest decrement in neutrophil recruitment, or it may reflect failed containment in the lungs and greater systemic toxicity.

Finally, Mauderly and coworkers studied the influence of preexisting pulmonary emphysema on the susceptibility of rats to inhaled diesel exhaust and showed that less soot accumulated in the lungs of emphysematous rats than in those of nonemphysematous rats. It has been suggested that emphysematous lung had an unknown function, which accelerated the removal of inhaled dust from alveolar lumens to bronchiole or blood flow, in the emphysematous rats (50). In addition, they demonstrated impaired inflammatory responses to the diesel exhaust in emphysematous rats. The numbers of neutrophils in BALF from emphysematous rats exposed to diesel exhaust were significantly less than those from nonemphysematous control rats. Emphysema prevented the expression of an exhaust-induced increase in lung collagen and reduced the exhaust-induced delay in particle clearance and the exhaust-induced increase in lavage fluid indicators of lung damage. In addition, Gross and deTreville also reported the reduced susceptibility of emphysematous rats to inhaled quartz, which was due to the reduced accumulation of particles in emphysematous lungs (51). Although there are several differences between the present study and the studies by Mauderly and coworkers and Gross and deTreville, this acceleration of removal might be one of the causes in the susceptibility of lethal streptococcal infection in mice with emphysema.

S. pneumoniae is one of the most frequent respiratory pathogens in patients with COPD and patients with community-acquired pneumonia (4, 7, 8, 10, 52, 53). Many experimental studies have been reported about respiratory tract infections using various strains of mice (19, 20, 54–57). Although some strains of mice are susceptible to *S. pneumoniae* Serotype 19, a penicillin-resistant strain, ICR mice were reported to be resistant to them (19, 56, 57). We demonstrated that control ICR mice were resistant to *S. pneumoniae* Serotype 3, a penicillin-sensitive strain. Importantly, emphysematous ICR mice died after intratracheal challenge with *S. pneumoniae* Serotype 3. This is the first study to demonstrate the differences in inflammatory response to, and the lethality of, the streptococcal infection between normal mice and mice with experimental emphysema.

Many animal models of pulmonary emphysema have been reported (24, 29). In the present experiments, we used elastase-induced emphysema in mice because we could produce experimental emphysema reproducibly and clearly. Emphysematous lesions were produced within 3 weeks after elastase instillation, and the severity of emphysematous changes was elastase dose-dependent. This experimental model has been used in other studies to produce emphysema in laboratory rodents (29). In the absence of bacterial infection, there were no significant differences in cells and cytokines in BALF and serum between the control and mice with emphysema. The present mouse model is different from the findings in the lungs of patients with COPD, who generally have chronic inflammation in the alveolar wall and infiltration of neutrophils in the airway (58, 59).

In conclusion, we have shown that mice with pulmonary emphysema are susceptible to *S. pneumoniae* infection. The mechanism of susceptibility is related to impaired host defense in the lungs during the acute phase of infection. Future studies of the susceptibility of mice with emphysema to pneumococcal pneumonia will be needed to clarify the basic mechanisms.

Acknowledgment: The authors gratefully acknowledge Eiji Tsuchida, Tsunetaka Ito, Choichiro Takahashi, and Reiko Ota of Yamagata University School of Medicine, Yamagata, Japan, and Kazunori Omori, Osamu Mukeda, and Kinue Oguro of Otsuka Pharmaceutical Co. Ltd., for technical assistance during this study. The authors would also like to thank Arjuna Celaya and Daniel Mrozek for assistance with the English.

References

1. Murray CJ, Lopez AD. Alternative projections of mortality and disability by cause 1990–2020: global burden of disease study. *Lancet* 1997;349: 1498–1504.
2. Lopez AD, Murray CC. The global burden of disease, 1990–2020. *Nat Med* 1998;4:1241–1243.
3. Gravil JH, Al-Rawas OA, Cotton MM, Flanigan U, Irwin A, Stevenson

RD. Home treatment of exacerbations of chronic obstructive pulmonary disease by an acute respiratory assessment service. *Lancet* 1998; 351:1853–1855.
4. Connors AF Jr, Dawson NV, Thomas C, Harrell FE Jr, Desbiens N, Fulkerson WJ, Kussin P, Bellamy P, Goldman L, Knaus WA. Outcomes following acute exacerbation of severe chronic obstructive lung disease. *Am J Respir Crit Care Med* 1996;154:959–967.
5. Saint S, Bent S, Vittinghoff E, Grady D. Antibiotics in chronic obstructive pulmonary disease exacerbations: a meta-analysis. *JAMA* 1995:273: 957–960.
6. Hurd SS. International efforts directed at attacking the problem of COPD. *Chest* 2000:117(5 Suppl 2):336S–338S.
7. Soler N, Torres A, Ewig S, Gonzalez J, Celis R, El-Ebiary M, Hernandez C, Rodriguez-Roisin R. Bronchial microbial patterns in severe exacerbations of chronic obstructive pulmonary disease (COPD) requiring mechanical ventilation. *Am J Respir Crit Care Med* 1998:157:1498–1505.
8. Miravitlles M, Espinosa C, Fernandez-Laso E, Martos JA, Maldonado JA, Gallego M. Relationship between bacterial flora in sputum and functional impairment in patients with acute exacerbations of COPD: Study Group of Bacterial Infection in COPD. *Chest* 1999:116:40–46.
9. Kessler R, Faller M, Fourgaut G, Mennecier B, Weitzenblum E. Predictive factors of hospitalization for acute exacerbation in a series of 64 patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1999;159:158–164.
10. Wilson R. The role of infection in COPD. *Chest* 1998;113:242S–248S.
11. Dewan NA, Rafique S, Kanwar B, Satpathy H, Ryschon K, Tillotson GS, Niederman MS. Acute exacerbation of COPD: factors associated with poor treatment outcome. *Chest* 2000;117:662–671.
12. Grossman RF. The value of antibiotics and the outcomes of antibiotic therapy in exacerbations of COPD. *Chest* 1998;113:249S–255S.
13. Fagon JY, Chastre J. Severe exacerbations of COPD patients: the role of pulmonary infections. *Semin Respir Infect* 1996;11:109–118.
14. Arunabh, Fein AM. Management of acute exacerbations in chronic obstructive pulmonary disease. *Curr Opin Pulm Med* 2000;6:122–126.
15. Griffith DE, Mazurek GH. Pneumonia in chronic obstructive lung disease. *Infect Dis Clin North Am* 1991;5:467–484.
16. Ewig S, Torres A. Severe community-acquired pneumonia. *Clin Chest Med* 1999;20:575–587.
17. Zalacain R, Sobradillo V, Amilibia J, Barron J, Achotegui V, Pijoan JI, Llorente JL. Predisposing factors to bacterial colonization in chronic obstructive pulmonary disease. *Eur Respir J* 1999;13:343–348.
18. Vegelin AL, Bissumbhar P, Joore JC, Lammers JW, Hoepelman IM. Guidelines for severe community-acquired pneumonia in the western world. *Neth J Med* 1999;55:110–117.
19. Takashima K, Tateda K, Matsumoto T, Ito T, Iizawa Y, Nakao M, Yamaguchi K. Establishment of a model of penicillin-resistant *Streptococcus pneumoniae* pneumonia in healthy CBA/J mice. *J Med Microbiol* 1996:45:319–322.
20. Tateda K, Takashima K, Miyazaki H, Matsumoto T, Hatori T, Yamaguchi K. Noncompromised penicillin-resistant pneumococcal pneumonia CBA/J mouse model and comparative efficacies of antibiotics in this model. *Antimicrob Agents Chemother* 1996;40:1520–1525.
21. Bedos JP, Azoulay-Dupuis E, Vallee E, Veber B, Pocidalo JJ. Individual efficacy of clarithromycin (A-56268) and its major human metabolite 14-hydroxy clarithromycin (A-62671) in experimental pneumococcal pneumonia in the mouse. *J Antimicrob Chemother* 1992;29:677–685.
22. LeVine AM, Reed JA, Kurak KE, Cianciolo E, Whitsett JA. GM-CSF-deficient mice are susceptible to pulmonary group B streptococcal infection. *J Clin Invest* 1999;103:563–569.
23. Barry B, Muffat-Joly M, Gehanno P, Pocidalo JJ. Effect of increased dosages of amoxicillin in treatment of experimental middle ear otitis due to penicillin-resistant *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1993;37:1599–1603.
24. Karlinsky JB, Snider GL. Animal models of emphysema. *Am Rev Respir Dis* 1978;117:1109–1133.
25. Otto-Verberne CJ, Ten Have-Opbroek AA, Franken C, Hermans J, Dijkman JH. Protective effect of pulmonary surfactant on elastase-induced emphysema in mice. *Eur Respir J* 1992;5:1223–1230.
26. Janoff A, Dearing R. Prevention of elastase-induced experimental emphysema by oral administration of a synthetic elastase inhibitor. *Am Rev Respir Dis* 1980;121:1025–1029.
27. Christensen TG, Korthy AL, Snider GL, Hayes JA. Irreversible bronchial goblet cell metaplasia in hamsters with elastase-induced panacinar emphysema. *J Clin Invest* 1977;59:397–404.
28. Lucey EC, Goldstein RH, Stone PJ, Snider GL. Remodeling of alveolar walls after elastase treatment of hamsters: results of elastin and collagen mRNA *in situ* hybridization. *Am J Respir Crit Care Med* 1998; 158:555–564.
29. Snider GL, Lucey EC, Stone PJ. Animal models of emphysema. *Am Rev Respir Dis* 1986;133:149–169.
30. Moore TA, Newstead MW, Strieter RM, Mehrad B, Beaman BL, Standiford TJ. Bacterial clearance and survival are dependent on CXC chemokine receptor-2 ligands in a murine model of pulmonary *Nocardia asteroides* infection. *J Immunol* 2000;164:908–915.
31. Takashima K, Tateda K, Matsumoto T, Iizawa Y, Nakao M, Yamaguchi K. Role of tumor necrosis factor alpha in pathogenesis of pneumococcal pneumonia in mice. *Infect Immun* 1997:65:257–260.
32. O'Brien DP, Briles DE, Szalai AJ, Tu AH, Sanz I, Nahm MH. Tumor necrosis factor alpha receptor I is important for survival from *Streptococcus pneumoniae* infections. *Infect Immun* 1999:67:595–601.
33. Benton KA, VanCott JL, Briles DE. Role of tumor necrosis factor alpha in the host response of mice to bacteremia caused by pneumolysin-deficient *Streptococcus pneumoniae*. *Infect Immun* 1998;66:839–842.
34. Janoff EN, Fasching C, Orenstein JM, Rubins JB, Opstad NL, Dalmasso AP. Killing of *Streptococcus pneumoniae* by capsular polysaccharide-specific polymeric IgA, complement, and phagocytes. *J Clin Invest* 1999;104:1139–1147.
35. Standiford TJ, Wilkowski JM, Sisson TH, Hattori N, Mehrad B, Bucknell KA, Moore TA. Intrapulmonary tumor necrosis factor gene therapy increases bacterial clearance and survival in murine gram-negative pneumonia. *Hum Gene Ther* 1999;10:899–909.
36. Rezaiguia S, Garat C, Delclaux C, Meignan M, Fleury J, Legrand P, Matthay MA, Jayr C. Acute bacterial pneumonia in rats increases alveolar epithelial fluid clearance by a tumor necrosis factor-alpha-dependent mechanism. *J Clin Invest* 1997;99:325–335.
37. Takabatake N, Nakamura H, Abe S, Inoue S, Hino T, Saito H, Yuki H, Kato S, Tomoike H. The relationship between chronic hypoxemia and activation of the tumor necrosis factor-alpha system in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2000;161:1179–1184.
38. Saito H, Nakamura H, Kato S, Inoue S, Inage M, Ito M, Tomoike H. Percutaneous *in vivo* gene transfer to the peripheral lungs using plasmid-liposome complexes. *Am J Physiol Lung Cell Mol Physiol* 2000; 279:L651–L657.
39. Heemskerk-Gerritsen BA, Dijkman JH, Ten Have-Opbroek AA. Stereological methods: a new approach in the assessment of pulmonary emphysema. *Microsc Res Tech* 1996;34:556–562.
40. Saetta M, Shiner RJ, Angus GE, Kim WD, Wang NS, King M, Ghezzo H, Cosio MG. Destructive index: a measurement of lung parenchymal destruction in smokers. *Am Rev Respir Dis* 1985;131:764–769.
41. Fukuoka T, Kawada H, Kitayama A, Koga T, Kubota M, Harasaki T, Kamai Y, Ohya S, Yasuda H, Iwata M, *et al.* Efficacy of CS-834 against experimental pneumonia caused by penicillin-susceptible and -resistant *Streptococcus pneumoniae* in mice. *Antimicrob Agents Chemother* 1998;42:23–27.
42. Doerschuk CM, Mizgerd JP, Kubo H, Qin L, Kumasaka T. Adhesion molecules and cellular biomechanical changes in acute lung injury: Giles F. Filley Lecture. *Chest* 1999:116(Suppl 1):37S–43S.
43. Raynolds HY. Integrated host defense against infections. In: Crystal RG, West JB, Barnes PJ, Weibel ER, editors. The lung: scientific foundations. 2nd ed. Philadelphia, PA: Lippincott-Raven Publishers; 1997. p. 2353–2365.
44. Raynolds HY. Lung inflammation: normal host defence or a complication of some diseases. *Ann Rev Med* 1987;38:295–323.
45. Green GM, Kass EH. The role of alveolar macrophages in the clearance of bacteria from the lung. *J Exp Med* 1964;119:167–175.
46. Abe S, Nakamura H, Inoue S, Takeda H, Saito H, Kato S, Mukaida N, Matsushima K, Tomoike H. Interleukin-8 gene repression by clarithromycin is mediated by the activator protein-1 binding site in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2000;22:51–60.
47. Prieto A, Reyes E, Bernstein ED, Martinez B, Monserrat J, Izquierdo JL, Callol L, de LUCAS P, Alvarez-Sala R, Alvarez-Sala JL, *et al.* Defective natural killer and phagocytic activities in chronic obstructive pulmonary disease are restored by glycophosphopeptical (inmunoferon). *Am J Respir Crit Care Med* 2001;163:1578–1583.
48. Pilette C, Godding V, Kiss R, Delos M, Verbeken E, Decaestecker C, De Paepe K, Vaerman JP, Decramer M, Sibille Y. Reduced epithelial expression of secretory component in small airways correlates with airflow obstruction in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;163:185–194.
49. Aaron SD, Angel JB, Lunau M, Wright K, Fex C, Le Saux N, Dales RE. Granulocyte inflammatory markers and airway infection during acute

exacerbation of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2001;163:349–355.

50. Mauderly JL, Bice DE, Cheng YS, Gillett NA, Griffith WC, Henderson RF, Pickrell JA, Wolff RK. Influence of preexisting pulmonary emphysema on susceptibility of rats to inhaled diesel exhaust. *Am Rev Respir Dis* 1990;141:1333–1341.
51. Gross P, deTreville RT. Emphysema and pneumoconiosis: an experimental study on their interrelationship. *Arch Environ Health* 1969;18:340–349.
52. Ruiz M, Ewig S, Marcos MA, Martinez JA, Arancibia F, Mensa J, Torres A. Etiology of community-acquired pneumonia: impact of age, comorbidity, and severity. *Am J Respir Crit Care Med* 1999;160:397–405.
53. Torres A, Serra-Batlles J, Ferrer A, Jimenez P, Celis R, Cobo E, Rodriguez-Roisin R. Severe community-acquired pneumonia: epidemiology and prognostic factors. *Am Rev Respir Dis* 1991;144:312–318.
54. Azoulay-Dupuis E, Vallee E, Veber B, Bedos JP, Bauchet J, Pocidalo JJ. *In vivo* efficacy of a new fluoroquinolone, sparfloxacin, against penicillin-susceptible and -resistant and multiresistant strains of *Streptococcus pneumoniae* in a mouse model of pneumonia. *Antimicrob Agents Chemother* 1992;36:2698–2703.
55. Bedos JP, Rieux V, Bauchet J, Muffat-Joly M, Carbon C, Azoulay-Dupuis E. Efficacy of trovafloxacin against penicillin-susceptible and multiresistant strains of *Streptococcus pneumoniae* in a mouse pneumonia model. *Antimicrob Agents Chemother* 1998;42:862–867.
56. Moine P, Vallee E, Azoulay-Dupuis E, Bourget P, Bedos JP, Bauchet J, Pocidalo JJ. *In vivo* efficacy of a broad-spectrum cephalosporin, ceftriaxone, against penicillin-susceptible and -resistant strains of *Streptococcus pneumoniae* in a mouse pneumonia model. *Antimicrob Agents Chemother* 1994;38:1953–1958.
57. Briles DE, Crain MJ, Gray BM, Forman C, Yother J. Strong association between capsular type and virulence for mice among human isolates of *Streptococcus pneumoniae*. *Infect Immun* 1992;60:111–116.
58. Crooks SW, Bayley DL, Hill SL, Stockley RA. Bronchial inflammation in acute bacterial exacerbations of chronic bronchitis: the role of leukotriene B4. *Eur Respir J* 2000:15:274–280.
59. Hill AT, Bayley D, Stockley RA. The interrelationship of sputum inflammatory markers in patients with chronic bronchitis. *Am J Respir Crit Care Med* 1999;160:893–898.

Dual response to Fas ligation in human endothelial cells: apoptosis and induction of chemokines, interleukin-8 and monocyte chemoattractant protein-1

Minako Yamaoka-Tojo, Seiji Yamaguchi, Joji Nitobe, Shuichi Abe, Sumito Inoue, Naoki Nozaki, Masaki Okuyama, Makoto Sata, Isao Kubota, Hidenori Nakamura and Hitonobu Tomoike

Background **To maintain the integrity of tissues, endothelial cells play critical roles. Fas ligand (FasL) is well known to deliver a death signal through its receptor, Fas. The Fas/FasL system may concomitantly induce expressions of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) besides triggering apoptosis in endothelial cells. We also investigated whether an inhibitor of caspase-8 (Z-IETD-FMK) does modulate IL-8 and MCP-1 secretion.**

Methods and results **After treatment with interferon-gamma (IFN-γ), human recombinant FasL (hr FasL) or Fas agonistic antibody (CH-11) was added to cultured human endothelial cells. IFN-γ up-regulated Fas mRNA levels. Fas ligation promoted apoptosis assessed by fluorescent-activated cell sorter (FACS) analysis in a dose-dependent manner and induced prominent DNA fragmentation. Simultaneously, IL-8 and MCP-1 were secreted from the endothelial cells in response to hr FasL or CH-11 in a dose-dependent manner ($P < 0.01$). Fas-neutralizing agent (Fas-Fc) suppressed the Fas-mediated secretions of IL-8 and MCP-1 ($P < 0.01$) both as well as the Fas-mediated apoptosis. On the other hand, whereas Z-IETD-FMK suppressed apoptosis, the inhibitor enhanced the Fas-mediated secretions of both IL-8 and MCP-1 beyond the value of the Fas stimulation alone ($P < 0.01$), suggesting an enhanced signalling for the chemokine expression.**

Conclusion **In human endothelial cells, the Fas/FasL system induces both IL-8 and MCP-1 secretions probably via a caspase-8 independent pathway. The Fas/FasL system may amplify the inflammatory cascade in the vascular injury and atherogenesis by recruiting leukocytes at the region of apoptotic endothelial damage.** ***Coron Artery Dis*** **14:89–94 © 2003 Lippincott Williams & Wilkins.**

Coronary Artery Disease 2003, 14:89–94

Keywords: Fas ligand, interleukin-8, monocyte chemoattractant protein-1, interferon-γ

The First Department of Internal Medicine, Yamagata University School of Medicine, Yamagata, Japan.

This study was supported by grants 10307016 and 12670645 from the Ministry of Education, Science, and Culture, Japan.

This work was presented in part at the Scientific Sessions of the American Heart Association (AHA), Dallas on November 8–11, 1998, and published in abstract form (Circulation. 1998. 98: I-397). Further parts of this work were presented at the Scientific Sessions of AHA, Anaheim on November 11–14, 2001, and published in abstract form (*Circulation* 2001; 104:II-171).

Correspondence and requests for reprints to: Minako Yamaoka-Tojo, Department of Internal Medicine and Cardiology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa, 228-8555, Japan.
Tel: + 81 42 778 8111; fax: +81 42 778 8441;
e-mail: myamaoka@med.kitasato-u.ac.jp

Received 25 September 2002 **Revised** 02 December 2002
Accepted 06 December 2002

Introduction

Infiltrating leukocytes through damaged endothelial cells may be responsible for tissue injury and a variety of disease processes. One of the important features in the endothelial cell damage is apoptotic cell death. Fas ligand (FasL) is a cytokine that induces apoptosis by binding to its surface receptor, Fas [1]. When Fas is activated in the tissues, the cells may secrete chemotactic factors in the process of the cell death, resulting in amplified recruitment of leukocytes and tissue injury. Indeed, it has been suggested that Fas may transduce cell activation signals independently or as an alternative to cell death [2,3].

Chemokines are polypeptide molecules that cause direct migration of leukocytes [4]. Interleukin-8 (IL-8) has a potent chemotactic propensity for neutrophils and thus serves to amplify the pro-inflammatory cascade [5–8]. Monocyte chemoattractant protein-1 (MCP-1) is one of the critical chemoattractant proteins for monocytes, and plays a pivotal role in atherogenesis [6, 9–11]. It is not fully understood whether Fas stimulation can induce chemokine in endothelial cells besides triggering apoptosis.

Here, we demonstrated that cultured human endothelial cells, ECV304 cells [12–15], secrete IL-8 and MCP-1 in response to Fas stimulation besides triggering apoptosis. We also tested whether Fas stimulation enhances expression of IL-8 and MCP-1 in ECV304 cells by shutting off downstream cascades of caspase-8.

0954-6928 © 2003 Lippincott Williams & Wilkins

DOI: 10.1097/01.mca.0000057869.37565.62

Materials and methods

Reagents

The monoclonal antibody to the Fas antigen (IgM; clone CH-11) was purchased from Medical and Biological Laboratories Co., Ltd. (MBL, Nagoya, Japan). Human soluble recombinant FasL (hr FasL) [16,17] and human Fas chimeric protein (Fas-Fc) [18] were provided from Bioscience Research Laboratory, Mochida Pharmaceutical Co. (Tokyo, Japan). The hr FasL in *Pichia* pastoris harbouring the FasL expression plasmid was produced as previously reported [16]. Caspase-8 inhibitor (Z-IETD-FMK; FLICE inhibitor or Z-Ile-Glu-Thr-Asp-CH2F) was purchased from MBL. Human recombinant interferon-γ [IFN-γ was purchased from Life Technologies, Inc. (Gaithersburg, Maryland, USA)]. Foetal bovine serum was from COSMO BIO Co., Ltd. (Tokyo, Japan). Proteinase K and DNase-free RNase were from Boehringer Mannheim Biochemicals (Boehringer, Mannheim, Germany).

Cell culture

The human endothelial cell line, ECV340, represents a spontaneously transformed human endothelial cell line established from human umbilical vein [12–15] (Health Science Research Resources Bank, Osaka, Japan). ECV340 cells were maintained and propagated in Medium 199 (Gibco BRL, Grand Island, New York, USA) containing 10% heat inactivated foetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were kept in a humidified incubation at 37°C with 5% CO_2. After treatment with IFN-γ (100 U/ml) for 12 h, the cells were incubated with hr FasL or Fas agonistic antibody (CH-11) in serum-free medium for 20 h. Caspase-8 inhibitor (Z-IETD-FMK; 10 μmol/l) or Fas neutralizing agent (Fas-Fc; 10 μg/ml) was added 30 min before Fas stimulation. The culture supernatants were collected and centrifuged at 1500 rpm for 5 min at 4°C and stored at −20°C until the time of assay.

Analysis of DNA fragmentation

For DNA fragmentation analysis, ECV340 cells were detached by exposure to 0.5%. trypsin for 2 min, rinsed twice with 10% fetal calf serum in phosphate-buffered saline (PBS), and then centrifuged. The pellet was incubated in a lysis buffer containing 1 mol/l Tris-HCl (pH 7.4), 0.5 mol/l ethylenediamine tetra-acetic acid (EDTA) (pH 8.0), and in 10% Triton X-100 at 4°C for 10 min. Only fragmented DNA was extracted and followed by incubation with proteinase K and DNase-free RNase A [19]. The DNA was extracted by isopropanol precipitation and finally re-suspended in TE buffer [10 mol/l Tris-HCl [pH 8.0] and 1 mol/l EDTA]. The DNA was electrophoretically fractionated on 2% agarose gel and stained with ethidium bromide.

Reverse-transcription and PCR analysis

Total cellular RNA from ECV304 cells was by the acid guanidinium thiocynate–phenol–chloroform methods using the RNAzol™ B (TEL-TEST, Inc., Friendwood, Texas, USA) [20]. Human Fas mRNA expression was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) (RNA PCR kit; TaKaRa, Otsu, Japan) using synthesized oligonucleotide primers encoding human Fas antigen, 5′–GACCCAGAATACCAAGTGCA–3′ (positions 557 through 576) and 5′–TCTGTTCTGCTGC-TGTGTTCTTGG–3′ (positions 994 through 1013) [21]. The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers were purchased from Clonetech (Palo Alto, California, USA) [22] and define an amplicon of 450 base pairs.

Quantification of apoptosis by flow cytometry

The percentage of cells undergoing apoptosis was quantitatively determined by virtue of their ability to bind annexin V and exclude propidium iodide (PI) using flow cytometry (Apoptosis Detection Kit; R & D Systems, Minneapolis, Minnesota, USA). In brief, ECV304 cells were washed in cold PBS twice and then re-suspended in a small volume of binding buffer at a concentration 1×10^6 cells/ml. Fluorescein-labelled annexin V and PI were added to the cells. Cells expressing phosphatidylserine on the outer leaflet of cell membranes will bind annexin V. Cells with a compromised cell membrane will allow PI to bind to the cellular DNA. The cells analyzed by flow cytometry could be classified into three potential populations: live cells are stained with neither FITC-annexin V nor PI, necrotic cells stained with both fluorochromes while cells undergoing apoptosis stained only with the FITC-annexin V reagent [23]. Analysis was performed using a FACSCalibur™ flow cytometer (Becton Dickinson, Mountain View, California, USA) equipped with Cell Quest software (Becton Dickinson & Co., San Jose, California, USA).

Enzyme-linked immunosorbent assay (ELISA)

Interleukin-8 and MCP-1 levels in cell culture supernatants from different treatments were determined by using ELISA kits (Quantikine; R&D Systems) according to the manufacture's instructions. Briefly, samples were incubated on microtiter plate wells pre-coated with a monoclonal antibody specific for IL-8 (or MCP-1). After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-8 (or MCP-1) was added to the wells. Colour developed in proportion to the amount of IL-8 (or MCP-1) bound in the initial step. The minimum detectable dose of IL-8 was 10 pg/ml and that of MCP-1 was 5 pg/ml. Samples were assayed in triplicate.

Statistical analysis

All values are expressed as the mean ± SEM. Statistical comparisons were made by one-way analysis of variance (ANOVA), and were followed by the Scheffe's F test. Differences were considered statistically significant at $P < 0.05$.

Results

Fas-mediated cell death in human endothelial cells

Fas agonistic antibody (CH-11; 100 ng/ml) induced DNA fragmentation in ECV304 cells (Fig. 1, lane 3). Fas-mediated cell death was more prominent in the presence of IFN-γ, suggesting that ECV304 cells become susceptible to Fas stimulation in the pre-treatment of IFN-γ (Fig. 1, lane 4). Fas neutralizing agent (Fas-Fc) attenuated Fas-mediated DNA fragmentation (Fig. 1, lane 5).

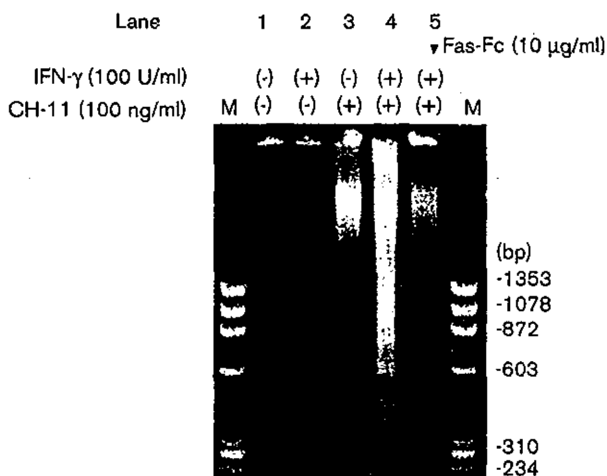
Fas mRNA in human endothelial cells with IFN-γ treatment

Fas mRNA transcript was analyzed by RT-PCR to examine the synergic effect of IFN-γ on apoptosis of ECV304 cells (Fig. 2). Amplification products corresponding to Fas were detected in human endothelial cells after pre-treatment with IFN-γ (100 U/ml) for 12–48 h, which increased Fas-mediated cell death in human endothelial cells. In our study, Fas mRNA could be detected after treatment with IFN-γ.

Dual response to Fas ligation in endothelial cells

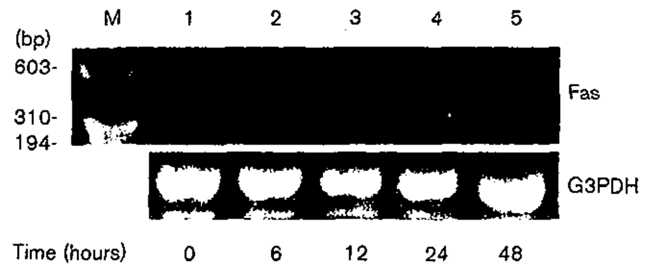
Figure 3a shows the effects of Fas-ligation with agonistic anti-Fas antibody (CH-11) in the presence or absence of IFN-γ (100 U/ml). Cell apoptosis was analyzed with log fluorescence dot plot of Annexin V-fluorescein and PI stained using flow cytometry. Figure 3b represents the effects of CH-11 on the production of IL-8 and MCP-1 in addition to the induction of endothelial cell apoptosis. Treatments of CH-11 increased dose-dependently the levels of IL-8 in the culture medium ($P < 0.01$). MCP-1 release in the culture was increased with 500 ng/ml of CH-11 ($P < 0.01$). The stimulation of CH-11 also increased apoptotic cells ($P < 0.01$). Dual response of endothelial cells to CH-11, cell apoptosis and chemokine release, was observed in a dose-dependent manner.

Fig. 1



DNA fragmentation in human endothelial cells, ECV304 cells. The cells were incubated in Medium-199 containing 10% FBS with or without interferon (IFN)-γ (100 U/ml) for 12 h and then incubated in serum free medium with or without Fas agonistic antibody (CH-11, 100 ng/ml) for 20 h. Fas neutralizing agent (Fas-Fc) was added 30 min before CH-11 stimulation. Molecular weight markers from *Hae*III-digested phiX 174 are indicated (M).

Fig. 2



Reverse transcriptase-polymerase chain reaction analysis of human Fas and G3PDH transcripts in human endothelial cells, ECV304 cells. The cells were treated with 100 U/ml of interferon (IFN)-γ up to 48 h in Medium-199 with 10% Foetal bovine serum (FBS). Fas mRNA expressions was detected for 12–48 hours treatment with IFN-γ.

Dual response to human recombinant Fas ligand (FasL) binding in endothelial cells

Endothelial cell apoptosis and chemokine secretion in response to FasL binding were analyzed (Fig. 3c). Interleukin-8 secretion was increased in the culture medium with treatment of FasL ($P < 0.01$). MCP-1 release was also augmented with FasL ($P < 0.01$). The stimulation of FasL (50 ng/ml) increased apoptotic cells ($P < 0.01$). Dual response to FasL revealed in a dose-dependent manner. The lower dose of FasL inducing the cell apoptosis can also stimulate chemokine secretion in the endothelial cells.

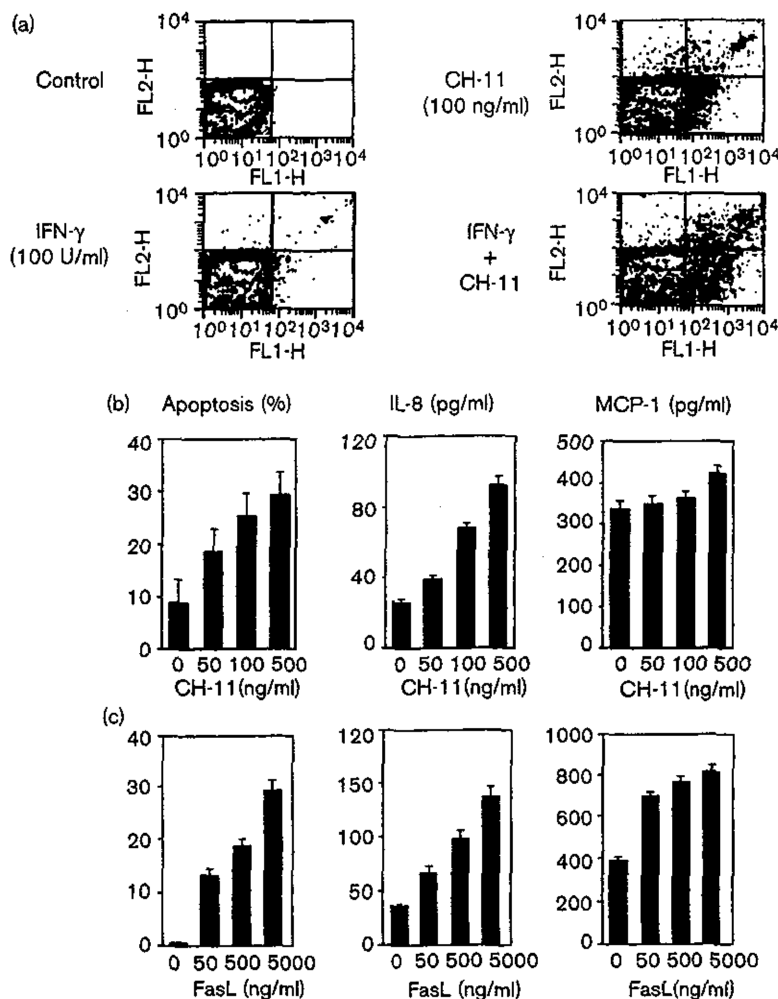
Enhancement of Fas-mediated chemokine secretion by caspase-8 inhibitor

Apoptosis of the endothelial cells induced by CH-11 (100 ng/ml) was completely inhibited by caspase-8 inhibitor (Z-IETD-FMK) and Fas-Fc (Fig. 4). The Z-IETD-FMK enhanced the levels of IL-8 and MCP-1 in the cell culture supernatant with CH-11. The level of IL-8 with stimulation of CH-11 was increased from 67.7 ± 1.4 to 99.7 ± 2.8 pg/ml by the treatment of Z-IETD-FMK ($P < 0.01$). The level of MCP-1 with stimulation of CH-11 was also increased from 360.4 ± 6.9 to 444.3 ± 9.4 pg/ml by Z-IETD-FMK ($P < 0.01$). Therefore, the Fas-mediated signal seems to diverge to two pathways, one of which leads to the caspase cascade-mediated apoptosis and the other leads to the production of IL-8 and MCP-1.

Discussion

Recent research points to a prominent role of immunity and inflammation in the pathogenesis of atherosclerosis

Fig. 3



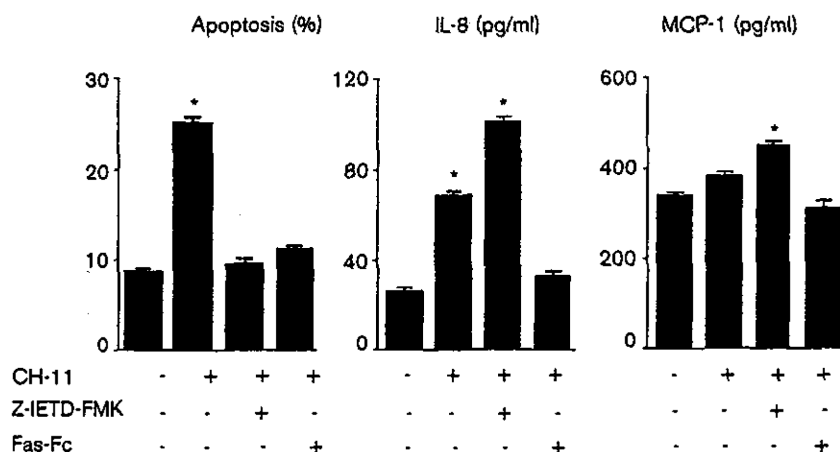
Apoptosis and chemokine secretion with Fas-stimulation in human endothelial cells, ECV304 cells. Log fluorescence dot plot of Annexin V-fluorescein and propidium iodide (PI) stained human endothelial cells (a). Analysis was performed on a FACSCalibur™. Effects of Fas ligation with agonistic anti-Fas antibody (CH-11) and human recombinant Fas ligand (hr FasL) on the production of IL-8 and MCP-1 (b and c). The CH-11 and hr FasL was applied for 20 h after treatment of IFN-γ (100 U/ml, 12 h). Results are shown as means ± SEM of three experiments (*$P < 0.01$).

[24,25]. The arterial endothelium in the atherosclerotic regions is activated and expresses chemokines and leukocyte adhesion molecules [24,25]. Fas is a well-characterized apoptosis inducer. In this study, Fas ligation induced IL-8 and MCP-1 expression besides triggering apoptosis in human endothelial cells.

Both Fas and FasL are expressed in arterial tissue, including human atherosclerotic plaque [26,27]. FasL expression in vascular walls seems to play two distinct roles in response to the circumstance exposed to the vessel; the induction of rapid growth in vascular intimal lesions [28] and conversely, the protection of the vessel by inhibiting invasion of leukocytes within the vessel wall [29]. The present study highlights apoptosis and chemokine expression by the Fas/FasL system in human endothelial cells. We demonstrated that both recombinant FasL and agonistic anti-Fas antibody induced IL-8 and MCP-1 secretion besides triggering apoptosis in human endothelial cells. This finding may suggest that FasL can recruit leukocytes at the region of FasL-induced apoptotic loss of endothelial cells.

A growing body of experimental evidence supports the pivotal role of chemokines in the pathogenesis of atherosclerosis [30]. Interleukin-8 is a cytokine with potent chemotactic properties for neutrophils and T lymphocytes, and thus serves to amplify the inflammatory cascade [5]. Interleukin-8 appears late in the inflammatory response and stimulates neutrophilic respiratory burst, degranulation, and adherence to the endothelial surface. Furthermore, trans-endothelial neutrophil migration triggered by tumour necrosis factor-alpha (TNF-α) is thought to be IL-8 dependent [31]. Monocyte

Fig. 4



Effects of caspase-8 inhibitor (Z-IETD-FMK) or Fas neutralizing agent (Fas-Fc) on percentage of apoptotic cells and chemokine production. Z-IETD-FMK or Fas-Fc was added 30 min before CH-11 stimulation for 20 h with pre-treatment of IFN-γ for 12 h. Note that caspase-8 inhibitor attenuated apoptosis but increased secretion of IL-8 and MCP-1. Results are shown as means ± SEM of four experiments (*$P < 0.01$).

chemoattractant protein-1 is a member of the C–C chemokine family and a potent chemotactic factor for monocytes [32]. Monocyte chemoattractant protein-1 mRNA has been shown to be expressed in human atherosclerotic plaque *in vivo* [33]. The endothelial expression of MCP-1 is apparently essential for the earliest cellular responses of atherogenesis. The Fas/FasL system may contribute to progression of atherogenesis by secreting IL-8 and MCP-1 in human endothelial cells. Interestingly, the secretion of IL-8 and MCP-1 induced by Fas stimulation may occur independently of apoptotic pathway since the Z-IETD-FMK efficiently inhibited Fas-mediated apoptosis but enhanced Fas-induced chemokine secretion. Therefore, the Fas-mediated signal appears to diverge to two pathways, one of which leads to the caspase cascade-mediated apoptosis and the other leading to the IL-8 and MCP-1 secretions independent of apoptosis. Furthermore, Z-IETD-FMK enhanced the Fas-mediated secretions of both IL-8 and MCP-1 beyond the value of the Fas stimulation alone, suggesting an enhanced signalling for the chemokine expression by shutting off the caspase cascade. Therefore, there may be two mechanisms where the Fas-mediated production of chemokines is up regulated by Z-IETD-FMK. One of the mechanisms is simply because the number of the living endothelial cells is increased by Z-IETD-FMK. The other is that the signal for the chemokine expression is enhanced when the apoptotic signal is shut down by Z-IETD-FMK. To clarify the mechanism, further detailed experiments are required.

Atherosclerotic lesions are characterized by the injury of endothelial cells and the infiltration of immune competent cells. The dual pathway initiated by Fas ligation may be necessary for the mechanism of atherosclerosis, involving not only apoptotic processes but also the provocation of angiogenic and pro-inflammatory responses, and including macrophage and/or lipid accumulation, and plaque growth. If we can regulate these two pathways, mediating endothelial cell apoptosis and activating both IL-8 and MCP-1, the Fas/FasL system may serve a beneficial function in recruiting professional scavengers, especially macrophages, that help clear the cell and matrix debris [34]. Our findings suggest an important role for the Fas/FasL system in the pathophysiology of atherosclerosis, and provide a direction for more specific immuno-modulating therapy.

In vitro and *in vivo* studies demonstrated that IFN-γ could promote atherosclerosis [35–38] within the complex cytokine network. We cultured human endothelial cells under the treatment of IFN-γ. Under the conditions of our assays, pre-treatment of IFN-γ increased endothelial cell apoptosis and secretions of IL-8 and MCP-1 induced by CH-11 or recombinant FasL. CH-11 can trigger apoptosis in the presence of IFN-γ in human vascular smooth muscle cells (hVSMCs) [26]. Exogenous IFN-γ also induced arteriosclerotic changes in the absence of leukocytes in hVSMCs [12]. In the present study, the effect of IFN-γ on the susceptibility of human endothelial cells to Fas-mediated cell death may result at least in part from alterations in Fas mRNA levels, which was also documented [39].

In conclusion, the Fas/FasL system stimulates the secretion of IL-8 and MCP-1 in human endothelial cells in addition to triggering apoptosis via a pathway independent of caspase cascade. When Fas-inducing apoptosis occurs in endothelial cells, both leukocyte-mediated

tissue injury and tissue leukocytic invasion may concomitantly ensue.

Acknowledgements

This study was supported by grants 10307016 and 12670645 from the Ministry of Education, Science, and Culture, Japan. The authors thank Bioscience Research Laboratory, Mochida Pharmaceutical Co. (Tokyo, Japan) for providing hr FasL and Fas-Fc.

References

1 Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression of the Fas ligand, a novel member of the tumour necrosis factor family. *Cell* 1993; **75**:1169–1178.
2 Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, Schooley KA, *et al.* Fas ligand mediates activation-induced cell death in human T lymphocytes. *J Exp Med* 1995; **181**:71–77.
3 Miwa K, Asano M, Horai R, Iwakura Y, Nagata S, Suda T. Caspase 1-independent IL-1beta release, and inflammation induced by apoptosis inducer Fas ligand. *Nat Med* 1998; **11**:1287–1292.
4 Baggiolini M, Dewald B, Moser B. Interleukin-8, and related chemotactic cytokines-CXC and CC chemokines. *Adv Immunol* 1994; **55**:97–179.
5 Strieter RM, Kunkel SL, Schowell HJ, Remick DG, Phan SH, Ward PA, *et al.* Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-alpha, LPS, and IL-1beta. *Science* 1989; **243**:1467–1471.
6 Koch AE, Kunkel SL, Pearce WH, Shah MR, Parikh D, Evanoff HL, *et al.* Enhanced production of the chemotactic cytokines interleukin-8 and monocyte chemo-attractant protein-1 in human abdominal aortic aneurysms. *Am J Pathol* 1993; **142**:1423–1431.
7 Keane MP, Arenberg DA, Lynch III JP, White RI, Iannettoni MD, Burdick MD, *et al.* The CXC chemokines, IL-8 and IP-10, regulate angiogenic activity in idiopathic pulmonary fibrosis. *J Immunol* 1997; **159**:1437–1443.
8 Simonini A, Moscucci M, Muller DW, Bates ER, Pagani FD, Burdick MD, *et al.* IL-8 is an angiogenic factor in human coronary atherectomy tissue. *Circulation* 2000; **101**:1519–1526.
9 Rollins BJ, Wong GG. The human homologue of the JE gene encodes a monocyte secretory protein. *Mol Cell Biol* 1989; **9**:4687–4695.
10 Libby P, Hansson GK. Involvement of the immune system in human atherogenesis: current knowledge and unanswered questions. *Lab Invest* 1991; **64**:5–15.
11 Fruebis J, Gonzalez V, Silvestre M, Palinski W. Effect of probucol treatment on gene expression of VCAM-1, MCP-1, and M-CSF in the aortic wall of LDL receptor-deficient rabbits during early atherogenesis. *Arterioscler Thromb Vasc Biol* 1997; **17**:1289–1302.
12 Takahashi K, Sawasaki Y, Hata J, Mukai K, Goto T. Spontaneous transformation, and immortalisation of human endothelial cells. *In Vitro Cell Dev Biol* 1990; **8**:265–274.
13 Marier JAM, Statuo M, Ragnotti G. Endogenous interleukin 1 alpha be transported to the nuclear to exert its activity in human endothelial cells. *Mol Cell Biol* 1994; **14**:1845–1851.
14 Hughes SE. Functional characterization of the spontaneously transformed human umbilical vein endothelial cell line ECV304: use in an *in vitro* model of angiogenesis. *Exp Cell Res* 1996; **225**:171–185.
15 Okuyama M, Yamaguchi S, Yamaoka M, Nitobe J, Fujii S, Yoshimura T, *et al.* Nitric oxide enhances expression and shedding of TNF receptor (p55) in endothelial cells. *Arterioscler Thromb Vasc Biol* 2000; **20**:1506–1511.
16 Tanaka M, Suda T, Yatomi T, Nakamura N, Nagata S. Lethal effect of recombinant human Fas ligand in mice pre-treated with *Propionibacterium acnes*. *J Immunol* 1997; **158**:2303–2309.
17 Yamaoka M, Yamaguchi S, Okuyama M, Nitobe J, Nakamura N, Suzuki T, *et al.* Fas ligand induced cardiomyocytes apoptosis. *J Mol Cell Cardiol* 2000; **32**:881–889.
18 Suda T, Nagata S. Purification, and characterization of the Fas-ligand that induces apoptosis. *J Exp Med* 1994; **179**:873–879.
19 Selins KS, Cohen JJ. Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *J Immunol* 1987; **139**:3199–3206.
20 Puissant C, Houdebine LM. An improvement of the single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Biotechniques* 1990; **8**:148–149.
21 Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasajima T, *et al.* Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. *Circ Res* 1994; **75**:426–433.
22 Autieri MV, Yue TL, Ferstein GZ, Ohlstein E. Antisense oligonucleotides to the p65 subunit of NF-kB inhibit human vascular smooth muscle cell adherence and proliferation and prevent neointima formation in rat carotid arteries. *Biochem Biophys Res Commun* 1995; **213**:827–836.
23 Homburg CH, de Haas M, von dem Borne AE, Verhoeven AJ, Reutelingsperger CP, Roos D. Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis *in vitro*. *Blood* 1995; **85**:532–540.
24 Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999; **340**:115–126.
25 Frostegard J, Ulfgren AK, Nyberg P, Hedin U, Swedenborg J, Andersson U, *et al.* Cytokine expression in advanced human atherosclerotic plaques: dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis* 1999; **145**:33–43.
26 Geng YJ, Henderson LE, Levesque EB, Muszynzki M, Libby P. Fas is expressed in human atherosclerotic intima and promotes apoptosis of cytokine-primed human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 1997; **17**:2200–2208.
27 Cai W, Devaux B, Schaper J. The role of Fas/APO 1 and apoptosis in the development of human atherosclerotic lesions. *Atherosclerosis* 1997; **131**:177–186.
28 Schneider DB, Vassalli G, Wen S, Driscoll RM, Sassani AB, De Young MB, *et al.* Expression of Fas ligand in arteries of hypercholesterolemic rabbits accelerates atherosclerotic lesion formation. *Arterioscler Thromb Vasc Biol* 2000; **20**:298–308.
29 Sata M, Walsh K. TNF alpha regulation of Fas ligand expression on endothelium modulates leukocyte infiltration of the blood vessel wall. *Nat Med* 1998; **4**:415–420.
30 Shin WS, Szuba A, Rockson SG. The role of chemokines in human cardiovascular pathology: enhanced biological insights. *Atherosclerosis* 2002; **160**:91–102.
31 Smart SJ, Casale TB. TNF-alpha-induced trans-endothelial neutrophil migration is IL-8 dependent. *Am J Physiol* 1994; **266**: L238–L245.
32 Rollins BJ. Chemokines. *Blood* 1997; **90**:909–928.
33 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993; **362**:801–809.
34 Kockx MM, Herman AG. Apoptosis in atherosclerosis: beneficial or detrimental? *Cardiovasc Res* 2000;**45**:736–746.
35 Gupta S, Pablo AM, Jiang X, Wang N, Tall AR, Schindler C. IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest* 1997; **99**:2752–2761.
36 Nagano H, Mitchell RN, Taylor MK, Hasegawa S, Tilney NL, Libby P. Interferon-gamma deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts. *J Clin Invest* 1997; **100**:550–557.
37 Tellides G, Tereb DA, Kirkiles-Smith NC, Kim RW, Wilson JH, Schechner JS, *et al.* Interferon-gamma elicits arteriosclerosis in the absence of leukocytes. *Nature* 2000; **403**:207–211.
38 Whitman SC, Ravisankar P, Elam H, Daugherty A. Exogenous interferon-gamma enhances atherosclerosis in apolipoprotein E−/− mice. *Am J Pathol* 2000; **157**:1819–1824.
39 Richardson BC, Lalwani ND, Johnson KJ, Marks RM. Fas ligation triggers apoptosis in macrophages but not endothelial cells. *Eur J Immunol* 1994; **24**:2640–2645.

An Acyl-CoA Synthetase Gene Family in Chromosome 16p12 May Contribute to Multiple Risk Factors

Naoharu Iwai, Toshifumi Mannami, Hitonobu Tomoike, Koh Ono, Yoshitaka Iwanaga

Abstract—We recently reported that genetic polymorphisms of SAH, an acyl-CoA synthetase for fatty acids, might contribute to multiple risk factors, especially hypertriglyceridemia. There are at least 4 members in this SAH gene family, *SAH*, *MACS1*, *MACS2*, and *MACS3*, and these 4 members are clustered in human Ch16p12. It is possible either that the previously observed associations were due to linkage disequilibrium with truly important polymorphisms in other members of the SAH gene family or that other polymorphisms in this gene family may also influence multiple risk factors. Thus, we performed association studies between genetic polymorphisms in this *SAH* region and multiple risk factors, using a large cohort representing the general population in Japan. The L513S polymorphism in *MACS2* was shown to significantly influence the triglyceride level and the waist-to-hip ratio. The previously observed associations between an *SAH* polymorphism and the waist-to-hip ratio appear to be due to linkage disequilibrium with the L513S polymorphism. Haplotype analysis indicated that a haplotype defined by the I/D polymorphism of *SAH* and the L513S polymorphism in *MACS2* was highly significantly associated with the triglyceride level. This study confirmed the importance of this chromosomal region in the pathogenesis of hypertriglyceridemia and visceral obesity. (***Hypertension*. 2003;41:1041-1046.**)

Key Words: epidemiology ■ fatty acids ■ genetics ■ hyperlipidemia ■ obesity

Differential screening was used to isolate *SAH* (*S*pontaneously hypertensive rat–Clone *A*–*H*ypertension-associated) from a genetically hypertensive rat strain, spontaneously hypertensive rat (SHR).[1] The expression of *SAH* in the kidneys of SHR is markedly higher than that in the kidneys of a normotensive control stain, Wistar-Kyoto rat. The rat *SAH* is localized on chromosome 1 near the most prominent QTL for blood pressure and had been expected to contribute to hypertension in SHR.[2,3] However, subsequent congenic analysis excluded rat *SAH* from the genes that contribute to hypertension in SHR.[4,5]

Recently, SAH protein has been reported to be significantly homologous to bovine xenobiotic-metabolizing medium-chain fatty acids: CoA ligase.[6] We revealed that human SAH had acyl-CoA synthetase activity toward medium chain fatty acids and that a genetic polymorphism of *SAH* might contribute to multiple risk factors, including hypertriglyceridemia, obesity, and hypertension.[7] It is likely that a genetic polymorphism of *SAH* might influence triglyceride metabolism, energy expenditure, and fat metabolism by influencing fatty acid metabolism.

A homology search of *SAH* in the human genome indicates that there are at least 4 members in this *SAH* gene family, *SAH*, *MACS1*, *MACS2*, and *MACS3* (Figure). Moreover, these 4 appear to be clustered in chromosome 16p12 (see Results). It is possible that the associations seen between the *SAH* polymorphism and multiple risk factors in the preceding study[7] might be due to linkage disequilibrium with genetic polymorphisms in other members of this gene family and that genetic polymorphisms in other members of this gene family might also contribute to multiple risk factors. Thus, to extend our previous work, we searched for genetic variations in this chromosomal region and performed association studies between polymorphisms in this region and multiple risk factors using a large cohort representing the general population in Japan.

Methods

DNA Studies

Genomic DNA from 36 subjects was used for sequence screening for polymorphisms. The promoter region and all of the exons of the *MACS1*[8] (Medium Chain Acyl-CoA Synthetase 1; MACS1) gene were sequenced according to the human draft sequence. The genome structures of *MACS2* (GenBank accession: AX451437) and *MACS3* (GenBank accession: AK000588) had not been determined at the beginning of the present study. We determined exon-intron boundaries on the basis of homology to *SAH* and *MACS1* and amplified intronic sequences by primers residing on the neighboring exons to determine the flanking sequences of exons. Based on the flanking sequences, all of the coding exons of *MACS2* and *MACS3* were amplified and sequenced. Primer sequences can be provided on request. The polymorphisms were determined by use of the TaqMan system (PE Applied Biosystems). The sequences of the primers and probes used in the TaqMan method can be provided on request.

Received December 10, 2002; first decision January 10, 2003; revision accepted February 21, 2003.

From the National Cardiovascular Center, Suita, Osaka, Japan.

Correspondence to Naoharu Iwai, MD, Research Institute, National Cardiovascular Center, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan. E-mail niwai@res.ncvc.go.jp

© 2003 American Heart Association, Inc.

Hypertension is available at http://www.hypertensionaha.org

DOI: 10.1161/01.HYP.0000064944.60569.87

```
        1                                                             60
SAH     MLRHAKCFQRLAIFGSVRALHKDNRTATPQNFSNYESMKQDFKLGIPEYFNFAKDVLDQW
MACS2         MHWLRKVQGLCTLWGTQMSSRTLYINSRQLVSLQWGHQEVPAKFNFASDVLDHW
MACS3 MRPWLRHLVLQALRNSRAFCGSHGKPAPLPVPQKIVATWEAISLGRQLVPEYFNFAHDVLDVW
MACS1    MQWLMRFRTLWGIHKSFHNIHPAPSQLRCRSLSEFGAPRWNDYEVPEEFNFASYVLDYW

        61                                                           120
        TDKEKAGKKPSNPAFWWINRNGEEMRWSFEELGSLSRKFANILSEACSLQRGDRVILILP
        ADMEKAGKRLPSPALWWVNGKGKELMWNFRELSENSQQAANVLSGACGLQRGDRVAVVLP
        SRLEEAGHRPPNPAFWWVNGTGAEIKWSFEELGKQSRKAANVLGGACGLQPGDRMMLVLP
        AQKEKEGKRGPNPAFWWVNGQGDEVKWSFREMGDLTRRVANVFTQTCGLQQGDHLALMLP

        121                                                          180
        RVPEWWLANVACLRTGTVLIPGTTQLTQKDILYRLQSSKANCIITNDVLAPAVDAVASKC
        RVPEWWLVILGCIRAGLIFMPGTIQMKSTDILYRLQMSKAKAIVAGDEVIQEVDTVASEC
        RLPEWWLVSVACMRTGTVMIPGVTQLTEKDLKYRLQASRAKSIITSDSLAPRVDAISAEC
        RVPEWWLVAVGCMRTGIIFIPATILLKAKDILYRLQLSKAKGIVTIDALASEVDSIASQC

        181                                                          240
        ENLHSKLIVSENSREGWGNLKELMKHASDSHTCVKTKHNEIMAIFFT-SGTSGYPKMTAHT
        PSLRIKLLVSEKSCDGWLNFKKLLNQASTTHHCVETGSQEASAIYFT-SGTSGLPKMAEHS
        PSLQTKLLVSDSSRPGWLNFRELLREASTEHNCMRTKSRDPLAIYFTKREPPGAPKMVEHS
        PSLKTKLLVSDHSREGWLDFRSLVKSASPEHTCVKSKTLDPMVIFFT-SGTTGFPKMAKHS

        241                                                          300
        HSSFGLGLSVNGRFWLDLTPSDVMWNTSDTGWAKSAWSSVFSPWIQGACVFTHHLPRFEP
        YSSLGLKAKMDAG-WTGLQASDIMWTISDTGWILNILCSLMEPWALGACTFVHLLPKFDP
        QSSYGLGFVASGRRWVALTESDIFWNTTDTGWVKAAW-TLFSAWPNGSCIFVHELPRVDA
        HGLALQPSFPGSRKLRSLKTSDVSWCLSDSGWIVATIWTLVEPWTAGCTVFIHHLPQFDT

        301                                                          360
        TSILQTLSKYPITVFCSAPTVYRMLVQNDITSYKFKSLKHCVSAGEPITPDVTEKWRNKT
        LVILKTLSSYPIKSMMGAPIVYRMLLQQDLSSYKFPHLQNCVTVGESLLPETLENWRAQT
        KVILNTLSKFPITTLCCVPTIFRLLVQEDLTRYQFQSLRHCLTGGEALNPDVREKWKHQT
        KVIIQTLLKYPINHFWGVSSIYRMILQQDFTSIRFPALEHCYTGGEVVLPKDQEEWKRRT

        361                                                          420
        GLDIYEGYGQTETVLICGNFKGMKIKPGSMGKPSPAFDVKIVDVNGNVLPPGQEGDIGIQ
        GLDIRESYGQTETGLTCMVSKTMKIKPGYMGTAASCYDVQIIDDKGNVLPPGTEGDIGIR
        GVELYEGYGQSETVVICANPKGMKIKSGSMGKASPPYDVQIVDDEGNVLPPGEEGNVAVR
        GLLLYENYGQSETGLICATYWGMKIKPGFMGKATPPYDVQVIDDKGSILPPNTEGNIGIR

        421                                                          480
        VLPNRPFGLFTHYVDNPSKTASTLRGNFYITGDRGYMDKDGYFWFVARADDVILSSGYRI
        VKPIRPIGIFSGYVDNPDKTAANIRGDFWLLGDRGIKDEDGYFQFMGRADDIINSSGYRI
        IRPTRPFCFFNCYLDNPEKTAASEQGDFYITGDRARMDKDGYFWFMGRNDDVINSSSYRI
        IKPVRPVSLFMCYEGDPEKTAKVECGDFYNTGDRGKMDEEGYICFLGRSDDIINASGYRI

        481                                                          540
        GPFEVENALNEHPSVAESAVVSSPDPIRGEVVKAFVVLNPDYKSHDQEQLIKEIQEHVKK
        GPSEVENALMEHPAVVETAVISSPDPVRGEVVKAFVVLALQFLSHDPEQLTKELQQHVKS
        GPVEVESALAEHPAVLESAVVSSPDPIRGEVVKAFIVLTPAYSSHDPEALTRELQEHVKR
        GPAEVESALVEHPAVAESAVVGSPDPIRGEVVKAFIVLTPQFLSHDKDQLTKELQQHVKS

        541
        TTAPYKYPRKVEFIQELPKTISGKTKRN
        VTAPYKYPRKIEFVLNLPKTVTGKIQRAKLRDKEWKMSGKARAQ
        VTAPYKYPRKVAFVSELAKDGFWKDPKE
        VTAPYKYPRNVEFVSELPKTITGKIERKELRKKETGQM
```

The *SAH* gene family. Amino acid sequences of *SAH* and *MACS1*, *MACS2*, and *MACS3* are shown. Identical amino acid residues among members are indicated. L513S polymorphism of *MACS2* is indicated by bold letter "**L**".

The expression levels of *MACS1*, *MACS2*, *MACS3*, and *SAH* mRNA were assessed by PCR, with the use of a human cDNA panel (Clontech) with 2 independent sets of primers.

Subjects

The selection criteria and design of the Suita Study have been described previously.[7] The genotypes were determined in 1976 consecutive subjects (written informed consent was obtained), who constituted the latter half of the study population in the preceding study. The study protocol was approved by the institutional ethics committee.

The characteristics of the subjects analyzed in the present study are summarized in Table 1, according to L513S polymorphism of *MACS2*. Hypertension was defined as systolic blood pressure >140 mm Hg, diastolic blood pressure >90 mm Hg, or the current use of antihypertensive medication. Total cholesterol and triglyceride levels were determined by enzymatic methods and kits (L-TC WAKO, Wako Pure Chemical, and Clinimate TG-2, Daiichi Chemicals). Homeostasis model assessment of insulin resistance (HOMA) was calculated as follows[9]: HOMA=[fasting insulin (μU/mL)× fasting glucose (mmol/L)]/22.5. Total immunoreactive insulin was measured by a kit (TOSOH), with the use of a 2-site immunoenzymometric assay.

Statistical Analysis

Values are expressed as mean±SEM. All statistical analyses were performed with the JMP statistical package (SAS Institute Inc). Multiple linear regression and multiple logistic analyses were performed with other covariates. Residuals of the waist-to-hip ratio and triglycerides were calculated by adjusting for age, gender, alcohol consumption (ethanol mL/d), and smoking (cigarettes/d). In some settings, the probability value was corrected (*Pc*) by the Bonferroni method. Principal component analysis was performed on the basis of correlations.

Linkage disequilibrium[10] and haplotype analyses were performed using the SNPAlyze statistical package (Dynacom Inc, http://www.dynacom.co.jp/; accessed March 5, 2003). Haplotype estimation was performed by the expectation-maximization algorithm.[11] To measure linkage disequilibrium between SNPs, Lewontin's D′ was calculated.[12]

Results

Confirmation of the *SAH* Gene Family

A BLAST search revealed the existence of 3 transcripts homologous to *SAH*, namely *MACS1* to *MACS3*. The complete genome structure of *MACS1* has been described previously.[8] The genome structure of *MACS3* and part of the genome structure of *MACS2* have not been reported, and we determined flanking sequences of coding exons of *MACS2* and *MACS3* for sequence screening of polymorphisms.

The polymorphisms found in the present study are summarized in Table 2. Polymorphisms in introns were not studied in detail and are not included in Table 2.

TABLE 1. Characteristics of the Study Population

Phenotype	SS (n=125)	LS (n=731)	LL (n=1120)	P
Men, %	54.4	47.2	47.5	NS
Age, y	60.1 (1.1)	59.6 (0.4)	60.1 (0.4)	NS
Alcohol consumption, mL/d	15.6 (2.0)	15.6 (0.8)	14.6 (0.8)	NS
Smoking, cigarettes/d	5.4 (0.9)	4.4 (0.4)	4.3 (0.3)	NS
HTN, %	42.4	37.4	38.7	NS
HDL, mmol/L	1.40 (0.04)	1.51 (0.02)	1.53 (0.01)	0.0025
TChol, mmol/L	5.42 (0.08)	5.42 (0.03)	5.45 (0.03)	NS
TG, mmol/L	1.74 (0.09)	1.42 (0.04)	1.40 (0.03)	0.0059
R-TG, mmol/L	+0.36 (0.01)	0.00 (0.01)	−0.04 (0.04)	0.0089
W/H	0.914 (0.006)	0.905 (0.003)	0.897 (0.002)	0.0034
R-W/H	+0.011(0.006)	+0.005 (0.002)	−0.004 (0.002)	0.0011
BMI, kg/m^2	23.4 (0.3)	22.9 (0.1)	22.6 (0.1)	0.0090
FBS, mmol/L	5.56 (0.09)	5.47 (0.04)	5.42 (0.03)	NS
HOMA	2.43 (0.17) (n=60)	1.90 (0.07) (n=422)	1.79 (0.05) (n=624)	0.0015
Insulin, μU/mL	9.5 (0.6) (n=60)	9.7 (0.2) (n=422)	7.3 (0.2) (n=624)	0.0036

Characteristics of the study population are shown according to the L513S polymorphism of the *MACS2* genotype. HTN indicates hypertensive subjects; HDL, HDL cholesterol; TChol, total cholesterol; TG, triglycerides; R-TG, residuals of TG; W/H, waist-to-hip ratio; R-W/H, residuals of W/H; BMI, body mass index; FBS, fasting blood glucose; HOMA, homeostasis model assessment of insulin resistance. R-TG and R-W/H were calculated by adjusting for age, gender, alcohol consumption, and smoking.

The expression of *MACS1* was not detected, as described below, which may downplay the importance of this gene. The AC repeat polymorphism in the promoter may not be suitable for high-throughput genotyping and was neglected in the present study. The polymorphisms in exons 8, 11, 12, and 13 were in complete linkage disequilibrium in the 36 subjects sequenced, and we selected the exon 12 polymorphism for the association study.

We found 3 polymorphisms in the coding region of *MACS2*, which were selected for the association study. The L513S polymorphism may have some functional meaning, since hydrophobic leucine is replaced by hydrophilic serine.

We found 4 polymorphisms in the coding region of *MACS3*. The Q159H (exon 3) and P353R (exon 7 to 1) polymorphisms were in complete linkage disequilibrium with the T534M (exon 12) and H361R (exon 7 to 2) poly-

TABLE 2. Polymorphisms in the Ch16p12 SAH Region

Gene	Region		Sequence	AA Change	Minor Allele Frequency
MACS1					
	Promoter		TGTTTAGAAA (CA)n TTGGAGAGGT	...	0.417
	Ex8		CTCCACCCTA[C/T]GACGTCCAGG	TAC(Y)/TAT(Y)	0.417
	Ex10		GGGACAGAGG[A/T]AAGATGGATG	GGA(G)/GGT(G)	0.070
	Ex11		AGGTTGAAAG[T/C]GCTTTGGTGG	AGT(S)/AGC(S)	0.417
	Ex12		ACCCAAGGAA[A/G]GTGAGTGAGG	AAA(K)/AAG(K)	0.417
	Ex13	3'UTR	CTGCACACCT[A/G]AGGCAAATCC	...	0.417
MACS2					
	Ex9		CACAGGGATT[G/A]ACTTGCATGG	TTG(L)/TTA(L)	0.222
	Ex11		GGGACGGGCA[G/A]ATGATATCAT	GAT(D)/AAT(N)	0.097
	Ex13		GTCCTGGCCT[T/C]GCAGTTCCTG	TTG(L)/TCG(S)	0.208
MACS3					
	Ex3		ACCGGCTGCA[G/C]GCGTCCAGGG	CAG(Q)/CAC(H)	0.167
	Ex7(1)		GCCCTCAACC[C/G]TGACGTGAGG	CCT(P)/CGT(R)	0.457
	Ex7(2)		AAGTGGAAAC[A/G]CCAGACCGGT	CAC(H)/CGC(R)	0.457
	Ex12		AGAGGCACTA[C/T]CGCGGGAACT	ACG(T)/ATG(M)	0.167

Polymorphisms in the SAH region are shown. Minor allele frequencies are obtained from the 36 subjects sequenced. Polymorphisms indicated by bold letters are used for genotyping of the study population.

TABLE 3. Linkage Disequilibrium Between Polymorphisms

Genotype	SAH12	M1/E12	M2/E9	M2/E11	M2/E13	M3/E7	M3/E12
SAH I/D	**−0.9999**	**0.5302**	−0.2939	−0.0415	0.1851	−0.0337	0.4589
	15.5322	**241.7198**	15.4449	0.1314	56.3709	0.6984	93.1298
SAH12		**−0.6890**	0.1353	**−0.7164**	0.1830	0.4778	**−0.7965**
		18.1148	4.0146	**2.1522**	4.4001	9.8528	**1.5742**
M1/E12			0.1697	0.4467	0.4434	0.2454	**0.9778**
			12.8313	46.1666	140.5322	101.3379	**181.6169**
M2/E9				**0.9732**	**−0.8225**	**0.9480**	0.3467
				983.5424	**97.3028**	**463.9159**	104.8876
M2/E11					**−0.7896**	**0.9397**	0.2992
					45.6709	**233.7887**	147.0224
M2/E13						**0.6942**	−0.2010
						409.5116	2.6518
M3/E7							0.3393
							25.5663

Linkage disequilibriums between polymorphisms are shown. D′ (upper) and χ^2 (lower) values are indicated. Bold letters indicate polymorphisms in strong linkage disequilibrium. The *SAH* I/D and intron 12 polymorphisms have been described previously.[7]

morphisms, respectively. Thus, we selected the H361R (exon 7 to 2) and T534M (exon 12) polymorphisms for the association study. We also determined 2 polymorphisms of *SAH*, I/D polymorphism in the promoter and A/G polymorphism in intron 12, which were concluded to be associated with multiple risk factors in the preceding study in 4039 subjects.[7]

Linkage disequilibrium among these polymorphisms is shown in Table 3. Although the locus for *MACS3* has not been clarified, strong linkage disequilibrium between the *MACS3* and *MACS2* polymorphisms indicates that *MACS3* may reside in this human chromosome 16p12 region near the *MACS2* locus.

Expression of the *MACS* Gene Family

RT-PCR analysis of expression levels of *MACS1*, *MACS2*, and *MACS3* and *SAH* revealed that *MACS2* and *MACS3* and *SAH* were expressed mainly in the kidney and liver. However, we could not detect PCR product from *MACS1* in any of the tissues examined including the spleen, thymus, prostate, testis, ovary, small intestine, colon, lymph node, heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas.

Association Study

Association studies between the polymorphisms in Table 2 and various phenotypes in the 1976 subjects revealed that the L513S polymorphism in *MACS2* strongly influenced triglycerides (TG), HDL cholesterol, waist-to-hip ratio (W/H), and body mass index (BMI) (Table 1). More intriguingly, an index for insulin resistance (HOMA) was influenced by the L513S polymorphism. Since members of the *SAH* gene family appear to have acyl-CoA synthetase activity toward fatty acids, it is likely that principal phenotypes influenced by this gene family may be the triglyceride level and/or visceral obesity (waist-to-hip ratio).

The effects of other polymorphisms on the triglyceride level and W/H ratio are indicated in Table 4. Residuals of the triglyceride level (R-TG) and W/H ratio (R-W/H) were calculated by adjusting for age, sex, alcohol consumption, and smoking. Residuals of the triglyceride level were also calculated after excluding subjects with hypolipidemic drugs to correctly assess the influence of polymorphisms on the triglyceride level (R-TG′). The influence of a *SAH* polymorphism on triglycerides and W/H ratio, which was evident in 4039 subjects in the preceding study, was weak in the present group of 1976 subjects, who comprised a subset (latter part) of the preceding 4039 subjects.

To avoid the problems of multiple testing, a principal component analysis was also performed. After performing a correlation analysis among TG, HDL, W/H, and BMI, the principal components were identified. The first principal component explained 50.5% of the total variance, and the influence of genotype on this component was analyzed by 1-way ANOVA (Table 4). The first principal component was defined as [0.422 (TG)+0.499 (HDL)+0.531 (W/H)+0.539 (BMI)]. Although the pathophysiological meaning of this component is difficult to discern at a glance, it was significantly affected by the L513S polymorphism (Table 4).

To clarify the possible contribution of polymorphisms other than the L513S polymorphism to triglycerides and W/H ratio, diplotypes defined by L513S and another polymorphism were determined in the study population. The effects of various haplotypes on triglycerides and W/H ratio were also evaluated.

There are 4 haplotypes defined by the L513S and I/D (SAH) polymorphisms: L513-D (haplotype1, allele frequency 0.559, 95% CI, 0.533 to 0.585), L513-I (haplotype2, allele frequency 0.200, 95% CI, 0.180 to 0.216), S513-D (haplotype3, allele frequency 0.136, 95% CI, 0.119 to 0.155), and S513-I (haplotype4, allele frequency 0.105, 95% CI, 0.086 to 0.120). The effects of the diplotypes defined by these 4 haplotypes on the triglyceride level are shown in Table 5. One-way ANOVA indi-

TABLE 4. Polymorphisms of SAH Gene Family and Triglycerides and W/H Levels

Phenotype	SAH I/D	SAH12	M1/E12	M2/E9	M2/E11	M2/E13	M3/E7	M3/E12	10D	D33
R-TG (n=1976)										
F value	2.1400	0.6510	0.9534	3.0167	1.2921	4.7360	0.3173	2.5916	**3.0537**	**18.3860**
P	0.1179	0.5216	0.3856	0.0492	0.2749	0.0089	0.7281	0.0752	**0.0012**	**<0.0001**
Pc	1.0000	1.0000	1.0000	1.0000	1.0000	0.2848	1.0000	1.0000	**0.0384**	**<0.0032**
df	2	2	2	2	2	2	2	2	**9**	**1**
R-TG′ (n=1898)										
F value	1.3562	0.6926	0.8046	4.2791	1.7569	**6.0809**	0.3752	3.0618	**3.5432**	**19.9302**
P	0.2579	0.5004	0.4474	0.0140	0.1713	**0.0023**	0.6872	0.0470	**0.0002**	**<0.0001**
Pc	1.0000	1.0000	1.0000	0.4480	1.0000	**0.0736**	1.0000	1.0000	**0.0064**	**<0.0032**
df	2	2	2	2	2	**2**	2	2	**9**	**1**
R-W/H (n=1976)										
F value	0.3093	0.8124	0.0952	1.0303	1.5800	**6.8456**	0.5537	0.8507	1.7752	2.4432
P	0.7340	0.4439	0.9092	0.3571	0.2062	**0.0011**	0.5749	0.4273	0.0681	0.1182
Pc	1.0000	1.0000	1.0000	1.0000	1.0000	**0.0352**	1.0000	1.0000	1.0000	1.0000
df	2	2	2	2	2	**2**	2	2	9	1
1st PC (n=1976)										
F value	0.4884	0.7813	2.5228	0.8158	2.0186	**9.7314**	1.1155	0.2028	**2.8083**	**9.7354**
P	0.6137	0.4580	0.0805	0.4424	0.1331	**<0.0001**	0.3279	0.8165	**0.0028**	**0.0018**
Pc	1.0000	1.0000	1.0000	1.0000	1.0000	**<0.0032**	1.0000	1.0000	**0.0448**	**0.0288**
df	2	2	2	2	2	**2**	2	2	**9**	**1**

The influence of polymorphisms on R-TG, R-TG′, R-W/H, and the first principal component (1st PC) were analyzed by 1-way ANOVA. R-TG and R-W/H were calculated by adjusting for gender, age, alcohol, and smoking (n=1976). R-TG′ was calculated after excluding subjects who were receiving hypolipidemic drugs (n=1898). The 1st PC was calculated as described in the text. The effects of the 10 diplotypes (10D) and the Diplotype 33 (D33) are also indicated. In D33, the 10 diplotypes (see TABLE 5) are recategorized into 2 groups, i.e., diplotype 33 and others. The haplotypes are defined in the text.

P values are corrected (*Pc*) by multiplying 32 [(8 genotypes+7 haplotypes+1 recategorization)×2 (possibly independent 2 phenotypes: triglyceride and waist-to-hip ratio)] (Bonferroni).

cated that the diplotype had significant effects on R-TG (P=0.0012) and R-TG′ (P=0.0002). As shown in Table 5, the diplotype 33 had significantly higher R-TG and R-TG′ levels. Thus, we recategorized the 10 diplotypes into 2 groups, that is, diplotype 33 and others. The influence of this diplotype 33 on the triglyceride level was highly significant even after correction by the Bonferroni method (P<0.0001 and Pc<0.0032, Table 4).

TABLE 5. Influence of Diplotype on Triglycerides Levels

Diplotype	n=1976 (n′=1898)	R-TG	*P*	R-TG′	*P*
11	632 (606)	0.01 (0.04)	<0.0001	−0.01 (0.04)	<0.0001
12	411 (393)	−0.12 (0.05)	<0.0001	−0.12 (0.05)	<0.0001
13	320 (306)	0.02 (0.06)	0.0001	0.01 (0.06)	<0.0001
14	332 (323)	0.02 (0.06)	0.0001	0.05 (0.06)	0.0001
22	75 (71)	0.13 (0.13)	0.0046	0.15 (0.13)	0.0074
23	4 (4)	0.41 (0.55)	NS	0.42 (0.54)	NS
24	81 (78)	−0.21 (0.12)	<0.0001	−0.23 (0.12)	<0.0001
33	46 (46)	0.69 (0.16)		0.70 (0.26)	
34	52 (49)	0.01 (0.15)	0.0023	0.03 (0.15)	0.0023
44	23 (22)	0.12 (0.23)	0.0444	0.19 (0.23)	0.0678

The influence of the diplotype on the triglyceride level was assessed by 1-way ANOVA. Haplotypes are defined in the text. The diplotype XY indicates the genotype with X and Y haplotypes. Thus, diplotype 23 indicates the genotype with one haplotype 2 and one haplotype 3. One-way ANOVA indicated that the diplotype had a significant influence on R-TG (P=0.0012) and R-TG′ (P=0.0002) (see TABLE 4).

P values indicate significant differences from the diplotype 33 group (by Fisher protected least significant difference test).

Discussion

We recently reported that genetic polymorphisms in *SAH* influenced multiple risk factors, including TG, HDL cholesterol, BMI, W/H ratio, and blood pressure status.[7] Since then, 3 other genes with high homology to *SAH* have been identified to cluster in the *SAH* region, chromosome16p12. Thus, it is possible either that the previously observed associations were due to linkage disequilibrium with truly important polymorphisms in other members of the SA gene family or that other polymorphisms in this gene family may also influence multiple risk factors.

In the present study, to evaluate the above-mentioned hypotheses, we performed extensive association studies between genetic polymorphisms in this region and multiple risk factors using a large cohort representing the general population in Japan. The L513S polymorphism in *MACS2* was shown to significantly influence TG, HDL, W/H, BMI, and HOMA index.

Because the L513S genotype appeared to influence various phenotypes including TG, HDL, W/H, and BMI, a principal component analysis was performed to avoid the problems of multiple testing. The L513S polymorphism had a highly significant influence on the first principal component. However, the pathophysiological meaning of this component is difficult to discern.

The members of the *SAH* gene family seem to have acyl-CoA synthetase activity toward medium chain fatty acids.[6–8] Thus, it is logically highly likely from the biological viewpoint that principal phenotypes influenced by this gene family may be the TG level and/or visceral obesity. Therefore, we studied the influence of polymorphisms on the TG level and W/H ratio (an excellent index of visceral obesity) (Table 4). Diplotype 33 had a highly significant influence on the TG level and the L513S polymorphism of *MACS2* had a weak but significant influence on the W/H ratio. Therefore, most of the previously observed associations between a *SAH* polymorphism and multiple risk factors appear to be due to linkage disequilibrium with the L513S polymorphism and haplotype 3.

In conclusion, the present study confirmed the importance of this chromosomal region, especially *MACS2* and *SAH*, in the pathogenesis of hypertriglyceridemia and visceral obesity. Intriguingly, this locus has been reported to be one of the suggestive loci for body mass index in the Framingham Heart Study.[13]

Perspectives

Human MACS1, human SAH, and bovine counterparts have been reported to act as acyl-CoA synthetases for various fatty acids, especially medium-chain fatty acids (MCFA).[6–8,14] MCFA are abundant in milk, coconut oil, and various synthetic oils. The activation of MCFA takes place mostly in the mitochondrial matrix by acyl-CoA synthetase for MCFA. Most of the MCFA incorporated into hepatocytes is subject to β-oxidation. Some of the acyl-CoA produced during MCFA oxidation is directed toward ketone body production, and the rest is directed to de novo synthesis of long-chain fatty acids, which are then incorporated into triglycerides or other complex lipids.[15,16] Recently, it has been proposed that medium-chain triglycerides may help to prevent obesity.[17] Therefore, it is highly likely that members of the *SAH* gene family (possible acyl-CoA synthetases for MCFA) may play some important roles in triglyceride metabolism, energy expenditure, fat metabolism, and, therefore, insulin resistance. However, the precise in vivo functions of the members of this gene family and the functional properties of the L513S polymorphism remain to be clarified and await further investigation.

Acknowledgments

This study was supported by the Program for Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan. We express our gratitude to Prof Soichiro Kitamura for his support of this study. We also thank Ms Akemi Fukumoto for technical assistance.

References

1. Iwai N, Inagami T. Isolation of preferentially expressed genes in the kidneys of hypertensive rats. *Hypertension*. 1991;17:161–169.
2. Iwai N, Tsujita Y, Kinoshita M. Isolation of a chromosome 1 region that contributes to high blood pressure and salt sensitivity. *Hypertension*. 1998;32:636–638.
3. Frantz SA, Kaiser M, Gardiner SM, Gauguier D, Vincent M, Thompson JR, Bennett T, Samani NJ. Successful isolation of a rat chromosome 1 blood pressure quantitative trait locus in reciprocal congenic strains. *Hypertension*. 1998;32:639–646.
4. Hubner N, Lee Y-A, Lindpaintner K, Ganten D, Kreutz R. Congenic substitution mapping excludes Sa as a candidate locus for a blood pressure quantitative trait locus on rat chromosome 1. *Hypertension*. 1999;34:643–648.
5. St Lezin E, Liu W, Wang J-M, Yang Y, Qi N, Kren V, Zidek V, Kurtz TW, Pravenec M. Genetic analysis of rat chromosome 1 and Sa gene in spontaneous hypertension. *Hypertension*. 2000;35:225–230.
6. Vessey DA, Kelly M. Purification and partial sequencing of the XL-I form of xenobiotic-metabolizing medium chain fatty acid:CoA ligase from bovine liver mitochondria, and its homology with the essential hypertension protein. *Biochim Biophys Acta*. 1997;1346:231–236.
7. Iwai N, Katsuya T, Mannami T, Higaki J, Ogihara T, Kokame K, Ogata J, Baba S. Association between *SAH*, an acyl-CoA synthetase gene, and hypertriglyceridemia, obesity, and hypertension. *Circulation*. 2002;105:41–47.
8. Fujino T, Takei YA, Sone H, Ioka RX, Kamataki A, Magoori K, Takahashi S, Sakai J, Yamamoto TT. Molecular identification and characterization of two medium-chain acyl-CoA synthetases, MACS1 and Sa gene product. *J Biol Chem*. 2001;276:35961–35966.
9. Matthews DR, Hosker JP, Rudenski AS, Naylor GA, Treacher DF, Turner RL. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. 1985;28:412–419.
10. Hill WG, Robertson A. Linkage disequilibrium in finite populations. *Theor Appl Genet*. 1968;38:226–231.
11. Excoffier L, Slatkin M. Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol Biol Evol*. 1995;12:921–927.
12. Lewontin RC. The interaction of selection and linkage. I. General considerations; heterotic models. *Genetics*. 1964;49:49–67.
13. Atwood LD, Heard-Costa NL, Cupples A, Jaquish CE, Wilson PWF, D'Agostino RB. Genome-wide linkage analysis of body mass index across 28 years of the Framingham Heart Study. *Am J Hum Genet*. 2002;71:1044–1050.
14. Vessey DA, Hu J. Isolation from bovine liver mitochondria and characterization of three distinct carboxylic acid:CoA ligases with activity toward xenobiotics. *J Biochem Toxicol*. 1995;10:329–337.
15. Christensen E, Hagve TA, Gronn M, Christophersen BO. Beta-oxidation of medium chain (C8-C14) fatty acids studied in isolated liver cells. *Biochim Biophys Acta*. 1989;1004:187–195.
16. Bach AC, Ingenbleek Y, Frey A. The usefulness of dietary medium-chain triglyceride in body weight control: fact or fantasy? *J Lipid Res*. 1996;37:708–726.
17. St-Onge MP, Jones PJH. Physiological effects of medium-chain triglycerides: potential agents in the prevention of obesity. *J Nutr*. 2002;132:329–332.

Association of Methylenetetrahydrofolate Reductase Gene Polymorphism With Carotid Atherosclerosis Depending on Smoking Status in a Japanese General Population

Nozomu Inamoto, MD; Tomohiro Katsuya, MD, PhD; Yoshihiro Kokubo, MD, PhD; Toshifumi Mannami, MD, PhD; Takashi Asai, MD; Shunroku Baba, MD, PhD; Jun Ogata, MD, PhD; Hitonobu Tomoike, MD, PhD; Toshio Ogihara, MD, PhD

Background and Purpose**—**The association of the *methylenetetrahydrofolate reductase* gene (*MTHFR*) with carotid atherosclerosis remains inconsistent. This may be due to small sample size and inappropriate analysis. We investigated the association of *C677T/MTHFR* with blood pressure and carotid atherosclerosis in a Japanese general population.

Methods**—**Subjects (30 to 89 years of age; 1693 women, 1554 men) who gave informed consent were randomly selected from a general population in Suita, Japan. *MTHFR* genotypes were determined by TaqMan polymerase chain reaction. Carotid atherosclerosis was evaluated by high-resolution ultrasonography with atherosclerotic indexes of intimal-medial thickness (IMT), maximum IMT in the common carotid artery (CCA), plaque score, and stenosis (>50%).

Results**—**Age-adjusted diastolic blood pressure was significantly higher in women with the *TT* genotype than in those with the *CC* genotype. In a recessive model (*CC*+*CT* versus *TT*), all adjusted odds ratios for hypertension and >50% stenosis in women were 1.42 and 3.42 (95% confidence intervals, 1.01 to 1.99 and 1.23 to 9.53), respectively. In women, maximum IMT in CCA for smokers with the *TT* genotype was significantly higher than for smokers with the *CC* genotype and nonsmokers with the *TT* genotype ($P<0.05$).

Conclusions**—**Our study suggests that the *MTHFR TT* genotype is a risk factor for hypertension and carotid stenosis in women. Significant interactions between *C677T/MTHFR* and smoking on maximum IMT in CCA were observed in women but not in men. Smoking cessation for subjects with the *TT* genotype is important in the prevention of cerebrovascular disease. (***Stroke*****. 2003;34:1628-1633.**)

Key Words: amine oxidoreductases ■ blood pressure ■ carotid arteries ■ Japan ■ risk factors

Hyperhomocysteinemia is associated with increased risk of atherosclerotic vascular disease.[1] The association of plasma total homocysteine concentration with atherosclerosis has been the subject of a number of clinical studies that have consistently linked moderate hyperhomocysteinemia with peripheral vascular disease, cerebrovascular disease, and coronary heart disease.[2–5]

Plasma total homocysteine levels are regulated mainly by 5,10-methylenetetrahydrofolate reductase, which is involved in the folate-dependent remethylation of homocysteine to methionine. Frosst et al[6] suggested that the *C677T* polymorphism in the *methylenetetrahydrofolate reductase* gene (*MTHFR*) is a candidate risk factor for vascular disease. The metabolic changes associated with *C677T/MTHFR* are postulated to modify the predisposition to diseases associated with folate deficiency.[7] Particular emphasis has been given to the role of *C677T/MTHFR* in cardiovascular[8] and cerebrovascular disease[9] and venous thrombosis.[10]

On the other hand, technical improvements in carotid ultrasonography have revealed new risk factors for stroke in its wide use. Some studies have demonstrated a close correlation between carotid ultrasound measurement, usually of carotid intimal-medial wall thickness (IMT), and the severity of extracranial carotid atherosclerosis.[11,12] Plasma total homocysteine levels have also been associated with more advanced carotid atherosclerosis in elderly subjects.[3,13] However, there have been controversies among their results. Most studies have failed to show an association between *C677T/MTHFR* and atherosclerotic disease.[14,15] These inconsistencies may be due to small sample size, combined-sex analysis, and lack of consideration of lifestyle. In this study, we examined the effect of *C677T/MTHFR* on carotid atherosclerosis and blood pressure (BP) in a large genetic epidemiological study, the Suita Study.

Received October 16, 2002; final revision received December 25, 2002; accepted January 23, 2003.

From the Department of Preventive Cardiology, National Cardiovascular Center (N.I., Y.K., T.M., S.B., J.O., H.T.), and Department of Geriatric Medicine, Osaka University Graduate School of Medicine (N.I., T.K., T.A., T.O.),Suita, Osaka, Japan.

This article encompasses the doctoral dissertation of Nozomu Inamoto.

Correspondence to Tomohiro Katsuya, MD, PhD, Department of Geriatric Medicine, Osaka University Graduate School of Medicine, 2-2 No. B6, Yamada-Oka, Suita, Osaka 565-0871, Japan. E-mail katsuya@geriat.med.osaka-u.ac.jp

© 2003 American Heart Association, Inc.

Stroke is available at http://www.strokeaha.org

DOI: 10.1161/01.STR.0000075769.09092.82

Materials and Methods

Subject Population

The Suita Study was based on a random sample of 14 200 Japanese residents of Suita, a city located in the second-largest urban area in Japan, Osaka.[16] These 14 200 residents between 30 and 89 years of age were arbitrarily selected from the municipality population registry, stratified by sex and 10-year age groups. We sent these residents letters to ask if they were willing to participate in this study from 1989 with a cohort base; by February 1007, 51.7% of the subjects (n=7347) had paid an initial visit to the National Cardiovascular Center (NCVC). The participants have visited NCVC every 2 years since then for regular health checkups. In addition to routine blood examinations that included total serum cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, glucose, glycosylated hemoglobin A_{1C} (HbA_{1C}), systolic BP (SBP), and diastolic BP (DBP), DNA was extracted from an extra 5 mL blood withdrawn from those who underwent general examinations at NCVC between May 1996 and February 1998. Ninety percent of the subjects who visited NCVC during this period gave informed consent for genetic analysis of 13 genes including *MTHFR* and storage of a DNA sample and were enrolled in the present study. The study protocol of genetic analysis was approved by the ethics committee of Osaka University. Three physicians performed the carotid ultrasonic examinations. Finally, the subjects in the present study included 1693 women and 1553 men 30 to 89 years of age who attended regular health checkups and subsequently underwent ultrasonic examinations and genetic analysis.

Measurements

The subjects' BPs were measured after at least 10 minutes of rest in the sitting position. The mean value of 2 measurements of SBP or DBP obtained by a physician using a mercury sphygmomanometer (recorded >3 minutes apart) was used for the analysis. Hypertension was defined as a mean SBP of ≥160 mm Hg, a mean DBP of ≥95 mm Hg, or current use of antihypertensive medication.

The subjects were classified as current smokers or drinkers if they smoked or drank. Hypercholesterolemia was defined as serum total cholesterol levels ≥220 mg/dL or current use of antihyperlipidemic medication. Diabetes was defined as fasting plasma glucose levels ≥7.0 mmol/L (126 mg/dL) or nonfasting glucose levels ≥11.1 mmol/L (200 mg/dL), HbA1C ≥6.5%, or current use of antidiabetic medication. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters) squared.

Blood samples drawn from the subjects after 12 hours of fasting were collected in EDTA-containing tubes. Total cholesterol and HDL cholesterol levels were measured with an autoanalyzer (Toshiba TBA-80) in accordance with the Lipid Standardization Program of the US Centers for Disease Control and Prevention through the Osaka Medical Center for Health Science and Promotion, Japan.[17] Among 3247 subjects, 1541 (820 women, 721 men) underwent measurement of fasting total plasma homocysteine levels by high-performance liquid chromatography.[18]

Carotid Ultrasound Measurements

Details of the carotid ultrasonic examination methods have been previously published.[16] We used a high-resolution B-mode ultrasonic machine with a 7.5-MHz transducer yielding an axial resolution of 0.1 mm. The regions from 30 mm proximal to the beginning of the dilation of the bifurcation bulb to 15 mm distal to the flow divider of both common carotid arteries (CCAs) were scanned. All measurements were made at the time of scanning with the electronic caliper and were recorded on photocopies. IMT was measured on a longitudinal scan of the CCAs at a point 10 mm proximal to the beginning of the dilation of each carotid artery bulb. IMT was defined as the mean of the IMT of the proximal and distal walls at the point of measurement. Maximum IMT in the CCA and maximum IMT were defined as the maximum IMT in the scanned CCA area and the maximum IMT in the entire scanned area, respectively. We defined a plaque, a focal IMT thickening, as an area where IMT ≥1.1 mm and calculated plaque score by totaling the maximum thickness of all the plaques in the scanned area. Finally, we defined stenosis as a condition in which a plaque occupied more than half of the lumen circumference of an artery on a cross-sectional scan. We performed color-flow Doppler examination to confirm the presence of stenosis.

MTHFR Genotype Determination With TaqMan Polymerase Chain Reaction Method

Genomic DNA was extracted from peripheral blood lymphocytes by standard procedures with a QIAamp DNA Blood Kit (Qiangen Inc). To deal with a large number of samples, we introduced the TaqMan polymerase chain reaction (PCR) method (Applied Biosystems). In the current investigation, we prepared 2 probes: C allele–specific probe, 5′ Tet-TCT GCG GGA GcC GAT TTC ATC ATC-Tamra-3′, and T allele–specific probe, 5′-Fam-TCT GCG GGA GtC GAT TTC ATC ATC-Tamra-3′. Primer design for PCR of the flanking region of *C677T/MTHFR* was as follows: forward, 5′-GGC TGA CCT GAA GCA CTT GAA-3′; reverse, 5′-GCG GAA GAA TGT GTC ATC CT-3′. PCR was carried out with a thermal cycler (GeneAmp, PCR System 9700, Applied Biosystems). PCR was performed according to the following conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The fluorescence level of PCR products was measured with an ABI PRISM 7200 and 7900 Sequence Detector (Applied Biosystems), resulting in clear identification of the 3 genotypes of *C677T/MTHFR*.

Statistical Analysis

The number of subjects was restricted to 3247 who had complete data, including *C677T/MTHFR* and carotid ultrasonographic measurements. Analysis of variance was used to compare mean values between groups, and if overall significance was demonstrated, the intergroup difference was assessed by means of a general linear model. Frequencies were compared by χ^2 analysis.

Associations of *C677T/MTHFR* with BP were investigated by sex through logistic regression analysis considering potential confounding risk variables, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), lifestyle (smoking and drinking), and antihypertensive medication. The genotype effect was examined according to a dominant (*TT+CT* versus *CC*) and a recessive (*TT* versus *CT+CC*) model. For multivariate risk predictors, the adjusted odds ratios (ORs) were given with the 95% confidence intervals (CIs). The relationships in men and women between *C677T/MTHFR* and hypertensive risk were expressed in terms of ORs adjusted for possible confounding effects. The association of *C677T/MTHFR* with carotid atherosclerotic index was also investigated by sex through logistic regression analysis considering potential confounding risk variables. Partial correlation coefficients between plasma total homocysteine and carotid atherosclerotic indexes by sex and *C677T/MTHFR* were determined. In addition, gene and environmental interactions were calculated with the following logistic regression model: logit $p=\beta_0+\beta_g x_g+\beta_e x_e+\beta_{ge} x_g x_e$, where x_g and x_e are genetic and environmental data, respectively; β_0 is an intercept term; β_g is the main effect due to genes; and β_e is the main effect of the environment. The coefficient β_{ge} of the product $x_g x_e$ estimates the gene and environmental interaction on the logit scale.[19] All analyses were performed with SAS statistical software (release 6.12, SAS Institute Inc).

Results

Basic Characteristics of Subjects in the Suita Study

As shown in Table 1, age, SBP, DBP, BMI, total cholesterol, HDL cholesterol, IMT, maximum IMT in CCA, plaque score, CCA stenosis (≥50%), percentage of current smokers, percentage of current drinkers, prevalence of hypertension, prevalence of diabetes mellitus, and total plasma homocysteine levels were significantly higher in men than in women.

TABLE 1. Basic Characteristics of Subjects in Suita, a Japanese Urban Population

	Women (n=1693)	Men (n=1554)
Age, y	58.2±12.2	60.4±12.8*
SBP, mm Hg	126.7±21.1	129.5±19.3*
DBP, mm Hg	78.0±11.0	80.7±11.0*
BMI, kg/m²	22.3±3.2	23.0±2.8*
Total cholesterol, mmol/L	5.6±0.9	5.2±0.8*
HDL cholesterol, mmol/L	1.6±0.4	1.4±0.4*
IMT, mm	0.83±0.12	0.88±0.14*
Maximum IMT in CCA, mm	1.02±0.29	1.15±0.45*
Plaque score, mm	2.14±2.99	4.13±4.69*
Stenosis (≥50%), %	1.0	4.6†
Current smokers, %	8.0	39.6†
Current drinkers, %	28.2	70.6†
Present illness		
Hypertension	21.3	25.6†
Hyperlipidemia	48.0	30.2†
Diabetes mellitus	3.3	7.9†
Myocardial infarction	0.5	1.7†
Ischemic stroke	0.8	2.5†
Total plasma homocysteine, μmol/L	10.7±3.0 (n=820)	13.3±4.2* (n=721)

Values are mean±SD or percentage.

Hypertension indicates SBP ≥160 mm Hg and/or DBP ≥95 mm Hg or antihypertensive medication; hyperlipidemia, total cholesterol ≥5.68 mmol/L (220 mg/dL) or antihyperlipidemia medication; diabetes, fasting plasma glucose ≥7.0 mmol/L (126 mg/dL), nonfasting plasma glucose ≥11.1 mmol/L (200 mg/dL), or antidiabetic medication.

*$P<0.05$ between female and male by Student's *t* test.

†$P<0.05$ between women and men by χ^2 test.

Only the frequency of hyperlipidemia was significantly higher in women than in men.

C677T/MTHFR, Hypertension, and Plasma Homocysteine Levels

The frequencies of *C677T/MTHFR* in women were 37.5% for *CC*, 47.2% for *CT*, and 15.3% for *TT* genotypes, whereas those in men were 36.2% for *CC*, 47.8% for *CT*, and 16.0% for *TT* genotypes. There was no significant difference in allele frequencies between age groups ($\chi^2=1.07$, $df=2$, $P=0.59$). The genotype distribution of *C677T/MTHFR* was not significantly deviated from Hardy-Weinberg's expectation in men or women. In women, SBP and DBP increased according to the number of *T677* alleles of *MTHFR*, but the association was not statistically significant. Only DBP in *TT* women was significantly higher in those with the *C677* allele after age adjustment. In the recessive model (*CT*+*CC* versus *TT*), however, *C677T/MTHFR* was significantly associated with the prevalence of hypertension, and the all adjusted OR for hypertension was 1.42 (95% CI, 1.01 to 1.99) in women (Table 2).

Figure 1 shows plasma total homocysteine levels according to genotype of *C677T/MTHFR* in men and women. Mean plasma total homocysteine levels in subjects with the *TT* genotype was significantly higher than that in subjects with the *CC* or *CT* genotype.

TABLE 2. ORs of Presence of Hypertension in Men and Women by *C677T/MTHFR*

	Dominant Model		Recessive Model	
	CC	*CT+TT*	*CC+CT*	*TT*
Women (n=1693)				
Hypertensive, %	19.5	22.4	20.7	24.7
All adjusted OR*	1	1.15 (0.88–1.49)	1	1.42 (1.01–1.99)†
Men (n=1554)				
Hypertensive, %	25.9	25.3	25.5	25.7
All adjusted OR*	1	0.93 (0.73–1.20)	1	1.00 (0.72–1.40)

*Conditional logistic analysis, adjusted for age, BMI, SBP, smoking, drinking, antihypertensive drug use, hypercholesterolemia, and diabetes.

†$P<0.05$ vs *CC* or *CC+CT* subjects.

Carotid Atherosclerotic Index and *C677T/MTHFR*

Carotid atherosclerotic indexes (IMT, maximum IMT in CCA, maximum IMT, and plaque score) were evaluated in men and women separately, according to *C677T/MTHFR* genotype (Table 3). In women with the *CT* genotype, age-adjusted IMT, maximum IMT in CCA, and all adjusted maximum IMT in CCA were significantly thicker than in those with the *CC* genotype. However, there was no difference between subjects with the *TT* and *CC* genotypes in any atherosclerotic indexes.

In contrast, *C677T/MTHFR* gave a significantly increased risk for stenosis (>50%) of CCA in women. In a recessive model (*CC*+CT versus *TT*), the all adjusted OR for stenosis (>50%) was 3.42 (95% CI, 1.23 to 9.53) in women and 1.41 (95% CI, 0.76 to 2.63) in men.

Partial correlation coefficients between plasma total homocysteine levels and carotid atherosclerotic index by *C677T/MTHFR* genotype are shown in Table 4. Positive relationships were found between plasma total homocysteine levels and IMT in men with the *CC* genotype and maximum IMT in CCA for men. These associations were stronger in men than in women.

Interaction Between *C677T/MTHFR* and Lifestyle on Carotid Atherosclerotic Index According to Sex

Figure 2 shows the association of IMT and maximum IMT in CCA with *C677T/MTHFR* according to smoking and drink-

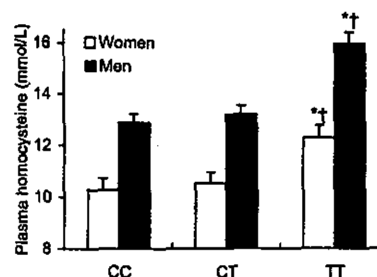


Figure 1. Plasma total homocysteine levels according to *C677T/MTHFR* by sex. Values are least-square mean±SE adjusted for age, BMI, smoking, drinking, antihypertensive drug use, hyperlipidemia, and diabetes. Bars indicate SE. *$P<0.0001$ vs CC subjects; †$P<0.0001$ vs CT subjects.

TABLE 3. Carotid Atherosclerotic Index in Men and Women by *C677T/MTHFR*

	MTHFR Genotype			
	CC	*CT*	*TT*	χ^2 *P*
Women (n=1693)				
IMT, mm				
Age adjusted	0.825±0.004	0.842±0.003‡	0.832±0.006	0.004
All adjusted*	0.861±0.009	0.874±0.009‡	0.866±0.010	0.030
Maximum IMT in CCA, mm				
Age adjusted	1.004±0.011	1.035±0.010†	1.023±0.018	0.122
All adjusted	1.075±0.026	1.100±0.025	1.094±0.029	0.231
Maximum IMT, mm				
Age adjusted	1.274±0.021	1.311±0.018	1.307±0.032	0.383
All adjusted	1.415±0.050	1.441±0.049	1.444±0.056	0.586
Plaque score, mm				
Age adjusted	1.990±0.108	2.259±0.096	2.141±0.169	0.178
All adjusted	2.915±0.262	3.114±0.254	3.026±0.293	0.369
Men (n=1554)				
IMT, mm				
Age adjusted	0.886±0.005	0.882±0.004	0.889±0.007	0.668
All adjusted	0.892±0.007	0.890±0.006	0.898±0.009	0.586
Maximum IMT in CCA, mm				
Age adjusted	1.162±0.020	1.140±0.017	1.144±0.030	0.713
All adjusted	1.173±0.027	1.163±0.026	1.165±0.036	0.916
Maximum IMT, mm				
Age adjusted	1.642±0.034	1.627±0.030	1.721±0.051	0.273
All adjusted	1.653±0.047	1.638±0.044	1.75±0.060	0.145
Plaque score, mm				
Age adjusted	4.201±0.178	4.010±0.155	4.308±0.268	0.550
All adjusted	4.413±0.246	4.215±0.232	4.625±0.316	0.363

*Values are least-square mean±SE adjusted for age, SBP, BMI, smoking, drinking, and medication (for hypertension, hyperlipidemia, or diabetes).

†$P<0.05$ vs *CC* subjects; ‡$P<0.005$ vs *CC* subjects.

ing status. In women with the *CC* or *CT* genotype, IMT in smokers was significantly higher than in nonsmokers. In women with the *TT* genotype, maximum IMT in CCA in smokers and drinkers was significantly higher than that in nonsmokers and nondrinkers, respectively (Figure 2–A2, $P<0.05$ for interaction; Figure 2–B2). In men with the *CC* or *TT* genotype, IMT and maximum IMT in CCA were significantly higher in smokers than in nonsmokers (Figure 2–A1).

TABLE 4. Partial Correlation Coefficient Between Plasma Total Homocysteine and Carotid Atherosclerotic Index by Sex and *C677T/MTHFR*

	CC	*CT*	*TT*
IMT			
Women	0.056 (0.334)	0.014 (0.784)	−0.027 (0.758)
Men	0.167 (0.001)	0.056 (0.300)	0.111 (0.253)
Maximum IMT in CCA			
Women	0.058 (0.398)	0.005 (0.935)	−0.098 (0.355)
Men	0.218 (0.003)	0.146 (0.016)	0.363 (0.002)

Figures in parentheses indicate *P* value adjusted for age, BMI, drinking, smoking, SBP, and medication for hypertension, hyperlidemia, and diabetes mellitus.

Discussion

The present study showed that the *TT* genotype of *C677T/MTHFR* was significantly associated with the prevalence of hypertension (OR, 1.15) and carotid stenosis (<50%) in women but not in men. In addition, the specific genotype of *C677T/MTHFR* affected maximum IMT in CCA in the interaction with smoking in women. These results show an association of *C677T/MTHFR* with BP and carotid atherosclerosis on the basis of gene and environmental interaction, which has not been previously reported.

Although previous studies showed that subjects with the *TT* genotype of *C677T/MTHFR* are associated with an increased risk of cardiovascular disease via an increase in plasma homocysteine levels,[2,6,20] the conclusion is still controversial.[7,14,15,21,22] The inconsistencies may be attributed to small sample size, combined-sex analysis, and no inclusion of lifestyle factors such as smoking and drinking. One should be aware that detecting gene and environmental interactions

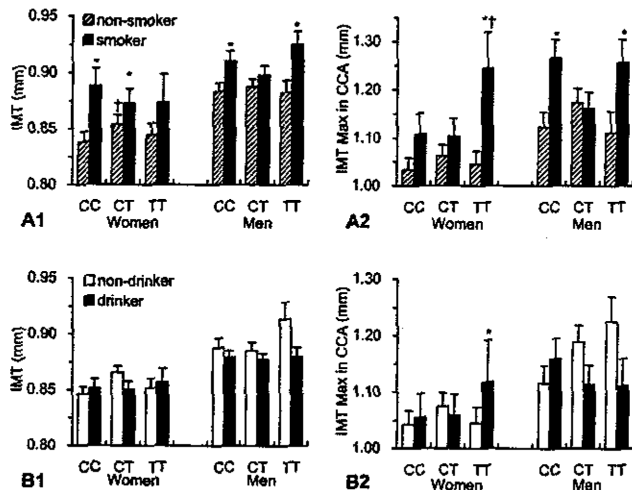


Figure 2. Association between *C677T/MTHFR* and carotid atherosclerotic indexes (IMT and maximum IMT in CCA) according to smoking (A) and drinking (B) status in men and women. Data are shown as the least-square mean±SE adjusted for age, BMI, SBP, smoking, drinking, and medication (for hypertension, hyperlipidemia, and diabetes). **P*<0.05 vs nonsmokers (or nondrinkers) in subjects with the same genotype; †*P*<0.05 vs *CC* subjects with the same lifestyle (for smoking and drinking).

could require a substantially larger sample size than the sample size necessary for detecting genetic or environmental effects alone.[23] Thus, we examined the effect of *C677T/MTHFR* in a large general population with various phenotypes that included plasma homocysteine levels, atheromatous indexes, smoking and drinking status, and relevant basic characteristics.

It can be questioned why the *TT* genotype of *C677T/MTHFR* is not unequivocally associated with increased cardiovascular risk,[5] based on the argument that the gene is a strong predictor of hyperhomocysteinemia in general populations.[6,24,25] It could be attributed to the close relationship between plasma homocysteine levels and folate metabolism. Several reports revealed that plasma total homocysteine levels become elevated only in folate-deficient subjects with the *TT* genotype[7,25,26] and that the slope of regression lines relating total homocysteine to folate increases in the order of *CC*, *CT*, and *TT* genotypes.[15,24] In other words, if folate intake is sufficient, subjects with the *TT* genotype would not have increased risk of cardiovascular disease via hyperhomocysteinemia.

Under stratification by sex, we observed that the *TT* genotype was independently associated with DBP and carotid stenosis in women and showed a greater disadvantage in female smokers and drinkers. Even though homocysteine would injure the endothelium of small arteries at an early stage[27] and endothelial dysfunction plays a critical role in the early events of atherosclerosis,[28] we currently have no definitive answer to explain the results. However, it seems to be an important finding that most of the positive results in the present study were obtained only in women. As supporting data of our results, a female-specific significant association with the *TT* genotype was also reported in the predisposition to ischemic stroke[29] and asymptomatic carotid atherosclerosis.[30] Motti et al[31] reported that sex differentiation is independently associated with homocysteine. Plasma homocysteine levels are significantly higher in healthy men than in women, which is consistent with our results (Table 1). In addition, homocysteine levels are reported to be lower in premenopausal women than in men and postmenopausal women. Furthermore, a recent report suggested that total homocysteine levels were significantly correlated with fat-free mass and testosterone and inversely with estradiol. The sex difference with regard to total homocysteine levels was explained primarily by differences in fat-free mass but also by estradiol concentration. Those results might be a feasible explanation for the lack of association in men.[32] However, there was no association between *C677T/MTHFR* and carotid atherosclerosis in premenopausal and postmenopausal women (data not shown). This result suggests that estrogen might have a protective effect against homocysteinemia but not atherosclerosis via *C677T/MTHFR*. Indeed, previous reports did not find such a specific advantage in the relationship between *C677T/MTHFR* and coronary artery disease in young woman in a small Caucasian population.[33,34]

Disadvantages of our study design were that only half of the subjects had their total plasma homocysteine levels analyzed. This is not a serious limitation, however, because the association between *C677T/MTHFR* and plasma homocysteine levels has already been demonstrated in several large studies.[7,26] Another disadvantage was that we had no data on the physical activity and nutrition of the subjects, but these data were also supported by previous studies. The dietary intake of folate, vitamin B_6, and B_{12} is inversely (negatively) correlated with plasma homocysteine[35,36]; physical activity is also inversely associated with plasma homocysteine.[37] There is a need for additional prospective studies with data on relevant confounders that have sufficient power to examine the association between homocysteine concentration and stroke risk, whether linear or threshold, and to study interactions between homocysteine, other dietary markers, and established stroke risk factors such as smoking and hypertension. Similarly, the evidence linking hyperhomocysteinemia with hypertension is limited and inconsistent. Ultimately, the case for a causal role of elevated homocysteine levels in vascular disease, including hypertension and stroke, will depend on data from randomly controlled trials of homocysteine-lowering interventions.

In summary, the present study shows that the homozygous *T677* allele of *C677T/MTHFR* is a risk factor for hypertension and carotid stenosis in women. In addition, smoking increased IMT in CCA in women with the *TT* genotype. In the near future, physicians might use the genotypic data of *C677T/MTHFR* to modify their patients' lifestyles to prevent cardiovascular disease.

Acknowledgments

This study was supported by a grant-in-aid (H14-027) from the Japanese Ministry of Health, Labor, and Welfare; grants-in-aid for scientific research (12557063, 13770349, 13204050, 13670709) from the Ministry of Education, Science, Sports and Culture of Japan; and research grants from the Takeda Medical Foundation, Salt Science Research Foundation, Osaka Medical Research Foundation for Incurable Diseases, Yokoyama Foundation for Clinical Pharmacology and Therapeutics, Kanae Foundation for Life and Socio-