であるインターロイキン6の産生を促進することも明らかとなり，その作用もARBの処置により抑制される[11]．これらの結果はRASの活性化が，動脈硬化アテロームでの炎症レベルの亢進につながることを示しているが，さらに代表的な炎症マーカーであるC反応性蛋白質(CRP)がヒト平滑筋細胞でのAT_{1a}受容体の発現増加，酸化ストレス亢進効果を有することも示されている[15]．血管壁でのRASと炎症機転は相互に活性化することによりアテローム増大，プラーク破綻といった悪循環に陥るものと考えられる[16]．

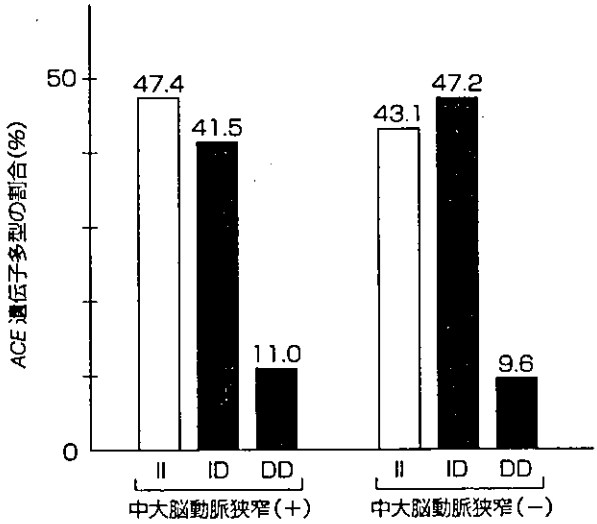
4）頭蓋内アテローム硬化へのRASの関与

頸動脈でのアテローム形成にRASの関与を示すデータが多くあるのに対し，頭蓋内主要動脈のアテローム硬化に関する実験的検討はほとんどなされていない．この理由に，頭蓋内アテローム硬化を作成する実験モデルがないことがあげられる．アテローム硬化の実験モデルとしてはApoE欠損マウスなど高脂血症を介した動脈硬化モデルマウスがよく用いられるが，頭蓋内脳血管では血液脳関門が存在し流血中のLDLをはじめとした脂質成分，血漿成分が脳血管に浸潤しにくく，高脂血症が頭蓋内アテローム硬化の危険因子になりにくいものと考えられる[17]．一方，高血圧自然発症ラットでは中大脳動脈に中膜肥厚を伴う血管リモデリングがみられるが，ARBのカンデサルタン投与はこの血管リモデリングを抑制することから，高血圧に伴う中大脳動脈肥厚へのRASの関与が想定されている[18]．いずれにしても頭蓋内血管のアテローム硬化に関する基礎的な実験はほとんど進んでいない．

臨床的データから得られたlarge vesselのアテローム硬化とRAS

1）large vesselアテローム硬化と危険因子，RAS遺伝子多型との関係

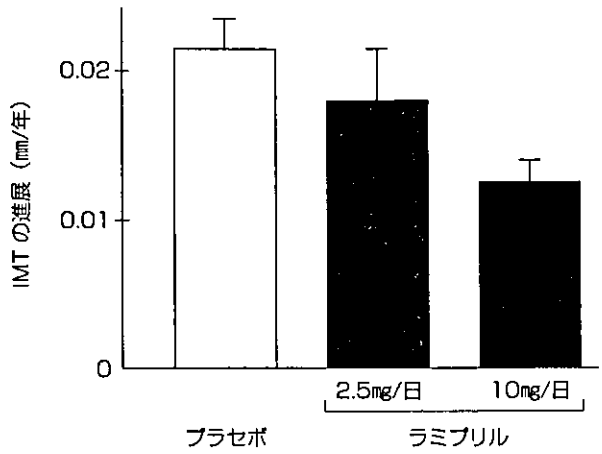
脳血管アテローム硬化へのRASの関与を探る目的で，RAS遺伝子である*ACE*，アンジオテンシノーゲン，AT_1受容体に存在する遺伝子多型の関与が調べられてきた．*ACE*遺伝子には第16イントロンに287 bpの挿入/欠失(I/D)多型があり，ACE活性はDアリルの存在により上昇する．アンジオテンシノーゲン遺伝子には第2エクソンの235番目のアミノ酸残基のメチオニンがスレオニンに置換した変異（M 235 T変異）があり，この変異があると血中アンジオテンシノーゲン濃度が高いことが報告されている．またアンジオテンシノーゲン遺伝子のプロモーター領域にも遺伝子変異が存在する．AT_1受容体遺伝子の遺伝子多型で最もよく調べられているのは3'側非翻訳領域に存在するA 1166 C遺伝子多型である．



図❸ 中大脳動脈狭窄に対するACE遺伝子多型の関与
糖尿病患者に経頭蓋超音波ドプラ検査を施行し，中大脳動脈狭窄を診断し，動脈硬化危険因子，RAS遺伝子多型の関与が検討された．*ACE*遺伝子多型のII，ID，DD各型の割合は，中大脳動脈狭窄217例と血管狭窄を有さない対照490例とのあいだで差はみられなかった．
(Thomas GN *et al*, 2003[24]より引用)

頸動脈分岐部はアテローム硬化の好発部位であり，また頸動脈超音波検査で非侵襲的に評価できる利点があり，頸動脈硬化とRAS遺伝子多型との関連が一般住民を用いて検討されてきている．頸動脈硬化には，年齢，男性，高血圧，喫煙，高脂血症，糖尿病など既知の動脈硬化危険因子はすべて関与するが，RAS遺伝子多型の明らかな関与は認められなかった[19)~22)]．*ACE*の挿入/欠失多型については，Physician's Health Studyで脳卒中発症と関連がないことが示され，RAS遺伝子多型は脳血管のアテローム硬化との関連は少ないと考えられる[23]．

中大脳動脈や脳底動脈といった頭蓋内動脈のアテローム硬化はアジア系人種に多いが，中国で糖尿病患者のな



図❹ ACE阻害薬ラミプリルの頸動脈硬化進展抑制効果
55歳以上で心血管イベントの既往があるか，糖尿病を合併する732例を対象とし，ACE阻害薬ラミプリルまたはプラセボを投与して4.5年間追跡調査した．頸動脈硬化重症度は頸動脈超音波検査で内中膜肥厚（intima-media complex thickness：IMT）を指標として評価した．IMTの進展は，プラセボ群（n=244）では1年間に0.0217 mmであったのに対して，ラミプリル2.5 mg/日投与群（n=244）では0.0180 mm，ラミプリル10 mg/日投与群（n=244）では0.0137 mmとラミプリル群で有意に頸動脈硬化の進展が抑制された．
（Lonn EM *et al*, 2001[28]より引用）

かから中大脳動脈狭窄を合併する217例と血管狭窄を有さない対照490例とのあいだで，*ACE*，AT_1受容体，アンジオテンシノーゲン遺伝子といったRAS遺伝子多型，動脈硬化危険因子が比較されたが，RAS遺伝子多型はいずれも中大脳動脈狭窄と関連がなく（図❸），中大脳動脈狭窄に関連を示したのは高血圧，アルブミン尿の存在であった[24]．

2）RASを抑制する薬剤による介入試験

RASを抑制する薬剤の脳卒中発症予防効果が大規模臨床研究で報告され注目されている．large vesselのアテローム硬化にもとづくものはアテローム血栓性脳梗塞であるが，脳卒中イベントを評価する際には，出血か梗塞かの区別はされていても，脳梗塞の3病型，とくにsmall vesselが原因であるラクナ梗塞とアテローム血栓性脳梗塞は必ずしも区別されていない．しかし，高リスク患者を対象としてACE阻害薬のラミプリルの心筋梗塞・脳卒中発症予防効果を示したHOPE研究[25]，左心室肥大を有する高血圧患者を対象としてβ遮断薬にくらべARBのロサルタンの有意な脳卒中予防効果を示したLIFE試験[26]，高齢者高血圧患者を対象としてARBのカンデサルタンの非致死性脳卒中に対する有意な予防効果を示したSCOPE試験[27]などは，血圧低下効果だけでは説明できない脳卒中予防効果がACE阻害薬またはARBに存在することを示唆している．

一方，large vesselそのものの観察としては頸動脈超音波検査を用いた研究がおこなわれている．前述のHOPE研究のサブスタディとしておこなわれたSECURE研究[28]では732症例を対象としてACE阻害薬のラミプリルの頸動脈硬化進展に対する抑制効果が4.5年間の追跡調査で示されている（図❹）．またII型糖尿病患者を対象としてACE阻害薬のエナラプリルを2年間投与した検討でも，コントロール群にくらべてエナラプリル投与群で頸動脈硬化進展が抑制されていた[29]．われわれの検討では，頸動脈硬化進展には高感度CRP[30]，可溶性細胞間接着因子1濃度（sICAM-1）といった炎症マーカーが既知の危険因子より強く関連していることを示しており，RASを抑制する薬剤の抗動脈硬化作用の一因として抗炎症効果の関与が考えられる．

文　献

1) Zhuo JL *et al*:Perindopril chronically inhibits angiotensin-converting enzyme in both the endothelium and adventitia of the internal mammary artery in patients with ischemic heart disease. *Circulation* 96:174-182, 1997
2) Ohishi M *et al*:Immunocytochemical localization of angiotensin II, ACE, and chymase in human coronary atherosclerotic lesions. *J Hypertens* 17:547-553, 1999
3) Rakugi H *et al*:Induction of angiotensin converting enzyme in the neointima after vascular injury. Possible role in restenosis. *J Clin Invest* 93:339-346, 1994
4) Fernandez-Alfonso MS *et al*:Early induction of angiotensin I-converting enzyme in rat carotid artery after baloon injury. *Hypertension* 30:272-277, 1997
5) Janiak P *et al*:Role of angiotensin subtype-2 receptor in neointima formation after vascular injury. *Hypertension* 20:737-745, 1992
6) Morishita R *et al*:Antisense oligonucleotide inhibition of vascular angiotensin-converting enzyme expression attenuates neointimal formation. Evidence for tissue angiotensin-converting enzyme function. *Arterioscle Thromb Vasc*

Biol 20:915-922, 2000

7) Harada K *et al*:Vascular injury causes neointimal formation in angiotensin II type 1a receptor knockout mice. *Circ Res* 84:179-185, 1999

8) Faxon DP:Effects of high dose angiotensin-converting enzyme inhibition on restenosis:final results of the MARCATOR study, a multicenter, double-blind, placebo-controlled trial of cilazapril. The Multicenter American Research Trial With Cilazapril After Angioplasty to Prevent Transluminal Coronary Obstruction and Restenosis (MARCATOR) Study Group. *J Am Coll Cardiol* 25:362-369, 1995

9) Diet F *et al*:Increased accumulation of tissue ACE in human atherosclerotic coronary artery disease. *Circulation* 94:2756-2767, 1996

10) Fukuhara M *et al*:Angiotensin-converting enzyme expression in human carotid artery atherosclerosis. Hypertension 35:353-359, 2000

11) Schieffer B *et al*:Expression of angiotensin II and interleukin 6 in human coronary atherosclerotic plaques. Potential implications for inflammation and plaque instability. *Circulation* 101:1372-1378, 2000

12) Hernandez-Presa MA *et al*:ACE inihibitor quinapril reduces the arterial expression of NF-κB-dependent proinflammatory factors but not of collagen I in a rabbit model of atherosclerosis. *Am J Pathol* 153:1825-1837, 1998

13) Keidar S *et al*:Angiotensin II stimulates macrophage-mediated oxidation of low density lipoproteins. *Atherosclerosis* 115:201-215, 1995

14) Scheidegger KJ *et al*：Angiotensin II induces macrophage-mediated modification of low density lipoprotein via a lipoxygenase-dependent pathways. *J Biol Chem* 272:21609-21615, 1997

15) Wang CH *et al*:C-reactive protein upregulates angiotensin type 1 receptors in vascular smooth muscle. *Circulation* 107:1783-1790, 2003

16) Brasier AR *et al*：Vascular inflammation and renin-angiotensin system. *Arterioscler Thromb Vasc Biol* 22: 1257-1266, 2002

17) 桑原敬介ほか:脳血管の動脈硬化ー血管壁の石灰化ー. 最新・分子動脈硬化学, 森崎信尋ほか編, メディカルレビュー社, 東京, 2000, pp. 115-126

18) Ito T *et al*:Protection against ischemia and improvement of cerebral blood flow in genetically hypertensive rats by chronic pretreatment with an angiotensin II AT_1 antagonist. *Stroke* 33:2297-2303, 2002

19) Schmidt R *et al*:Angiotensinogen polymorphism M235T, carotid atherosclerosis, and small-vessel disease-related cerebral abnormality. *Hypertension* 38:110-115, 2001

20) Hung J *et al*:Angiotensin-converting enzyme gene polymorphism and carotid wall thickening in a community population. *Arterioscler Thromb vasc Biol* 19:1969-1974, 1999

21) Mannami T *et al*:Low potentiality of angiotensin-converting enzyme gene insertion/deletion polymorphism as a useful predictive marker for carotid atherogenesis in a large general population of a Japanese city. The Suita Study. *Stroke* 32:1250-1256, 2001

22) Markus H *et al*:Increased common carotid intima-media thickness in UK African caribeans and its relation to chronic inflammation and vascular candidate gene polymorphism. *Stroke* 32:2465-2471, 2001

23) Zee RY *et al*:Prospective evaluation of the angiotensin-converting enzyme insertion/deletion polymorphism and the risk of stroke. *Circulation* 99:340-343, 1999

24) Thomas GN *et al*:Middle cerebral artery stenosis in type II diabetic Chinese patients is associated with conventional risk factors but not with polymorphisms of the rennin-angiotensin system genes. *Cerebrovasc dis* 16:217-223, 2003

25) Bosch J *et al*:Use of ramipril in preventing stroke. Double blind randomized trial. *BMJ* 324:699-702, 2002

26) Dahlöf B *et al*:cardiovascular morbidity and mortality in the losartan intervention for endpoint reduction in hypertensive study (LIFE):a randomized trail against atenolol. *Lancet* 359:995-1003, 2002

27) Lithell H *et al*:The study on cognition and prognosis in the elderly (SCOPE):principal results of a randomized double-blind intervention trial. *J Hypertens* 21:875-886, 2003

28) Lonn EM *et al*:Effects of ramipril and vitamine E on atherosclerosis. The study to evaluate carotid ultrasound changes in patients treated with ramipril and vitamin E (SECURE). *Circulation* 103:919-925, 2001

29) Hosomi N *et al*:Angiotensin-converting enzyme inhibition with enalapril slows progressive intima-media thickening of the common carotid artery in patients with non-insulin-dependent diabetes mellitus. *Stroke* 32:1539-1545, 2001

30) Hashimoto H *et al*:C-reactive protein is an independent predictor of the rate of increase in early carotid atherosclerosis. *Circulation* 104:63-67, 2001

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Cathepsin B and H activities and cystatin C concentrations in cerebrospinal fluid from patients with leptomeningeal metastasis

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Abstract

Background: Cysteine proteases are involved in the extension of cancer into the subarachnoid space. The presence of cathepsins B and H along with their potent inhibitor cystatin C in the cerebrospinal fluid (CSF) was investigated in patients with leptomeningeal metastasis of cancer (LM). *Materials and methods*: CSF samples were obtained in 16 cases of LM (10 solid tumors and 6 leukemia or lymphoma) and compared with 11 cancer cases without involvement of the central nervous system, 12 multiple sclerosis cases and 34 healthy volunteers. The activity of the enzymes was measured, their molecular forms were analyzed by the Western blotting, and the concentration of cystatin C was measured by ELISA. Immunohistochemistry of the leptomeningeal tissues was also performed in six autopsy cases of LM. *Results*: High activities of cathepsins B and H along with decreased cystatin C concentration were observed in CSF of LM as compared with three control groups. Western blot analysis revealed higher concentration of the enzyme protein as well as its active forms in samples with higher enzyme activity. Cells metastasizing leptomeningeal tissue were clearly positive in immunohistochemical staining of cathepsins, indicating active production by tumor cells. *Conclusion*: Production of cathepsins B and H by tumor cells and their high activity along with concomitant decrease of their potent inhibitor, cystatin C, in the CSF might contribute in the process of metastasis and spread of the cancer cells in the leptomeningeal tissues. A high enzyme activity/cystatin C concentration ratio in the CSF could be useful when diagnosing LM in combination with other parameters.
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Keywords: Cerebrospinal fluid; Cystatin C; Cathepsin B; Cathepsin H; Leptomeningeal metastasis

1. Introduction

Leptomeningeal metastasis (LM) is a devastating neurologic complication of cancer. LM occurs in 2.5–25% of patients with solid tumors [1] and more often in the patients with leukemia or lymphoma, especially

Abbreviations: CC, cancer controls; CSF, cerebrospinal fluid; HC, healthy controls; LM, leptomeningeal metastasis; MS, multiple sclerosis.

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in acute lymphocytic leukemia [2,3]. Although the diagnosis is readily established by demonstrating malignant cells in the cerebrospinal fluid (CSF), it is often difficult because of atypical clinical symptoms or negative cytology.

During the process of cancer invasion and metastasis, it is believed that proteases released from the tumor degrade interstitial connective tissue and basement membranes [4]. Cathepsin B, which is a cysteine protease, has been analyzed in several tumors, and its activity and concentrations were found to be significantly higher than in normal tissues in breast, lung, head and neck, and colorectal cancer, and in melanoma [5,6]. Cathepsin H, which is also a cysteine protease, was reported to be significantly increased in the sera of patients with metastatic melanoma, but on the contrary, it was reported to be significantly decreased in head and neck tumor tissues [7].

We recently reported the cathepsin B and H activities and cystatin C concentrations in CSF with inflammatory neurological diseases [8]. Cathepsin B activity, but not H activity, was increased in CSF from patients with Guillain–Barré syndrome and multiple sclerosis (MS). In contrast, cystatin C, a major cysteine protease inhibitor in CSF, was significantly decreased in the same diseases. Since these proteases may also be released during tumor invasion of the central nervous system, there should also be abnormalities in cathepsin B and H activities and cystatin C concentrations in the CSF of patients with LM.

2. Patients and methods

2.1. Patients and CSF samples

All samples and autopsies shown in the present study were obtained with informed consent decided by Shimane Medical University. Multiple CSF samples from 16 patients with LM diagnosed at Shimane Medical University Hospital between July 1993 and December 2001 were obtained. Eleven patients were diagnosed as LM by CSF cytology prior to death and five who were suspected of LM were confirmed to be LM by autopsy. An autopsy was available in 9 of 16 LM patients. The primary tumors in all patients were diagnosed in the disease processes. Solid tumors were found in 10 patients: 1 prostatic cancer, 3 lung cancers, 2 pharyngeal cancers, and 4 gastric cancers. Leukemia and lymphoma were found in the remaining 6 patients, of whom 1 each had acute myelocytic leukemia, acute lymphocytic leukemia, chronic myelocytic leukemia and malignant lymphoma, and 2 had acute monocytic leukemia. In all patients, cytological or pathological findings revealed that the cell types of LM were identical with the primary tumors in the same patients. In the clinical course, the patients with solid tumors survived no more than 3 months because of their illness. Four patients with leukemia or lymphoma had prolonged survival time by effective chemotherapy.

We established three control groups. Cancer controls (CC): 11 patients with cancer and without metastasis in the central nervous system by autopsy, 6 had solid tumors and 5 had leukemia and lymphoma. Patients with MS: 12 patients of MS matched the criteria of definite MS [9], all in acute relapse. The clinical assessment in these cases was sufficient to prove temporal and spatial dissemination. T_2-weighted images of head and spinal MRI revealed that all MS patients had areas of increased signal predominantly in the white matter. Healthy controls (HC): 34 healthy subjects, from whom CSF was collected with informed consent by conventional lumbar puncture during plastic surgery. The CSF samples were centrifuged to remove the cell fractions and kept frozen at −70 °C until use. In our hospital, the normal CSF protein concentrations ranged from 15 to 45 mg/dl.

2.2. Cathepsin B and H assays

The cathepsin B and H activities were assayed with the established method [8,10], using *N*-carbobenzoxy-arginine-arginine-4-methyl-7-cumaryl-amide (Z-Arg-Arg-MCA) and Arg-MCA as substrates, respectively. One unit of cathepsin B or H was defined as the amount of either enzyme required to release 1 pmol of AMC per hour. The lower limit for the detection of cathepsin B and H activities was 0.01 U/h. The intra- and inter-assay variability of the cathepsin B and H bioassays was < 5%. A standard curve was made in triplicate for each run, and samples were assayed in triplicate.

2.3. Cystatin C concentrations

The cystatin C concentrations in CSF were measured using an established ELISA, as described pre-

viously [8,11]. In brief, monoclonal mouse anti-cystatin C antibody, which was provided by Dr. Anders Grubb, was coated on a 96-well microplate (Nunc, Roskilde, Denmark). Then, 100 μl of the samples or cystatin C standard (Ohtsuka, Tokushima, Japan) was placed in the wells at room temperature for 2 h, and polyclonal rabbit anti-cystatin C antibody (Dako, Copenhagen, Denmark) was added to each well. After peroxidase-labeled goat anti-rabbit IgG antibody (Zymed, San Francisco, CA) was added for 1 h, each sample was reacted with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)diammonium salt. The range of reliable measurements was 20–10,000 ng/ml. The present ELISA system recognizes human cystatin C both in free form and complexed with cysteine protease.

2.4. Western blot analysis for cathepsins

To determine the size of cathepsin in LM CSF, the CSF specimens (10 μl) were analyzed by SDS/PAGE under reducing conditions, and Western blots of the proteins were carried out as described previously [12]. The measured samples were from eight LM patients who had high activities of cathepsin B or cathepsin H and each of four patients randomly chosen from MS, CC, or HC group. After reaction with antibody against cathepsin B (mouse monoclonal antibody, Calbiochem-Novabiochem, Cambridge, MA) or cathepsin H (goat polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA), anti-mouse or anti-goat horseradish peroxidase-conjugated IgG (Medical and Biological Laboratories, Nagoya, Japan) was used as the second antibody, respectively. The reaction products were detected by chemiluminescence (ECL system, Amersham Pharmacia Biotech, Uppsala, Sweden).

2.5. Immunostaining for cathepsins

Paraffin-embedded tissues were obtained from the six autopsied patients. Sections were stained with HE or immunostained against cathepsin B (mouse monoclonal antibody, 1:2000, Calbiochem-Novabiochem) or cathepsin H (goat polyclonal antibody, 1:2000, Santa Cruz Biotechnology). Immunolabeling was detected using the avidin-biotinylated horseradish peroxidase (HRP) complex (ABC) method (ABC kit; Vector, Burlingame, CA) and visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) with 0.03% H_2O_2 in 0.1 mol/l Tris buffer (pH 7.2).

2.6. Statistical analyses

A single-factor ANOVA was used to compare the CSF parameters and the cystatin C concentrations in the different groups, using the StatView-J 4.51.1 statistical analysis package (Abacus Concepts, Berkeley, CA). Data for the cathepsin B and H concentrations were evaluated using the Kruskal–Wallis test. Because there was no difference in the mean cystatin C concentrations or cathepsin B and H activities of patients with solid tumor and leukemia and lymphoma among LM patients, entire LM group was combined together for comparison with the experimental groups. After evaluation with the Kruskal–Wallis test, the data were analyzed by Steel's test for multiple comparison. Pearson's correlation coefficient test was performed to ascertain whether correlations existed between two parameters. A $p<0.05$ were considered to be significant.

3. Results

3.1. Protein concentrations and cell counts in the CSF

The mean protein concentrations were increased in LM compared to MS ($p<0.01$), CC ($p<0.005$), and HC ($p<0.005$) (see Table 1). The mean cell counts in LM were also increased compared to MS ($p<0.005$), CC ($p<0.0005$), and HC ($p<0.0001$) (see Table 1).

Table 1
Characteristics of the study subjects

Patient group	No. of Patients	Sex (F/M)	Age (years)	CSF protein concentrations (mg/dl)*	Cell counts (/3μl)*
LM	16	6:10	61 (28–77)	197.1 ± 224.9**	137.9 ± 170.5**
MS	12	6:6	46 (31–60)	58.8 ± 71.0	4.3 ± 5.5
CC	11	4:7	61 (17–87)	40.1 ± 14.8	2.4 ± 2.2
HC	34	18:16	61 (14–88)	38.5 ± 9.4	1.5 ± 1.2

*Values are mean ± S.D.
**$p<0.05$ compared with MS, CC, or HC.

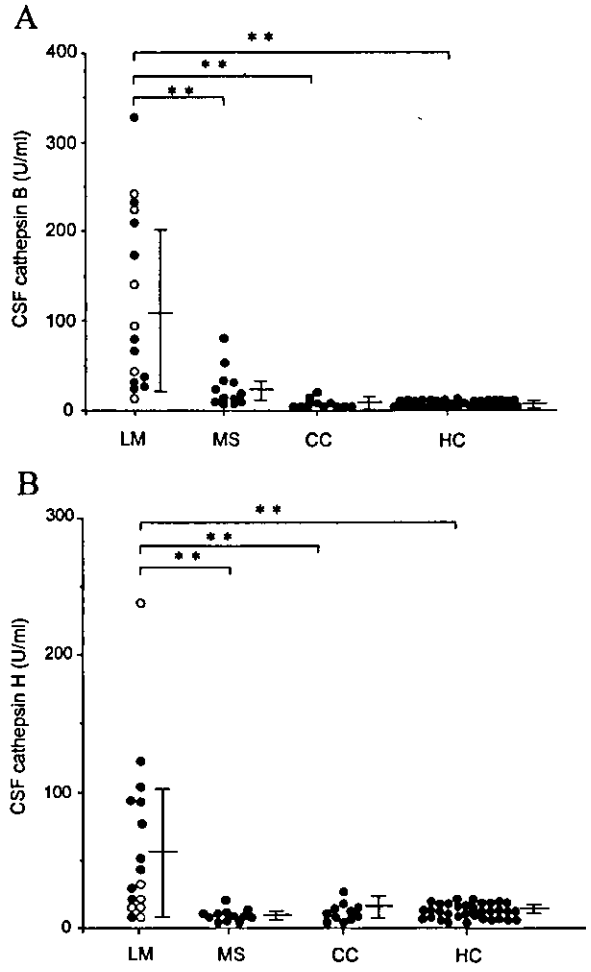

Fig. 1. Cathepsin B and H activities in the CSF of patients with LM, MS, CC, and HC. The vertical bars indicate mean ± S.D. In LM patients, (●) and (○) indicate patients with metastasis due to solid tumor and leukemia or lymphoma, respectively. (A) The mean cathepsin B activity was higher in patients with LM than in MS, CC, and HC. (B) The mean cathepsin H activity was higher in patients with LM than in MS, CC and HC. $^{**}p<0.01$.

Cytology confirmed that the pleocytosis occurred due to tumor cells that were counted as lymphocyte. High pleocytosis with >100 cells/3 μl was seen in seven patients. On the other hand, five patients with LM had no pleocytosis in the CSF. Discrepancy between CSF protein concentration and cell counts was often found in the disease process of LM. It made diagnosis of LM difficult in the present study. Among LM patients, there was no difference in the mean protein concentrations and cell counts between the 10 patients with solid tumors and the 6 patients with leukemia or lymphoma. Furthermore, the cell counts were not correlated with any of the cystatin C concentration, the cathepsin B or H activity.

3.2. Cathepsin B and H activities

Cathepsin B activity was increased in all the patients with LM, and was extremely high in a few of the samples. Two patients with MS had high concentrations of cathepsin B activity in their CSF, but the cathepsin B activity in the remaining 10 was not increased. The mean cathepsin B activity was higher in patients with LM than in MS ($p<0.0001$), CC ($p<0.0001$), or HC ($p<0.0001$) (Fig. 1A). The coefficient of variation (CV) of the cathepsin B activity was 88.1% in LM, 89.0% in MS, 79.4% in CC, and 33.8% in HC. The cathepsin B activity was not correlated with the protein concentration ($r=0.687$) or WBC count ($r=0.375$) in the CSF.

The mean cathepsin H activity was higher in patients with LM than in MS ($p<0.0001$), CC ($p<0.0005$), or HC ($p<0.0001$) (Fig. 1B). The CV of the

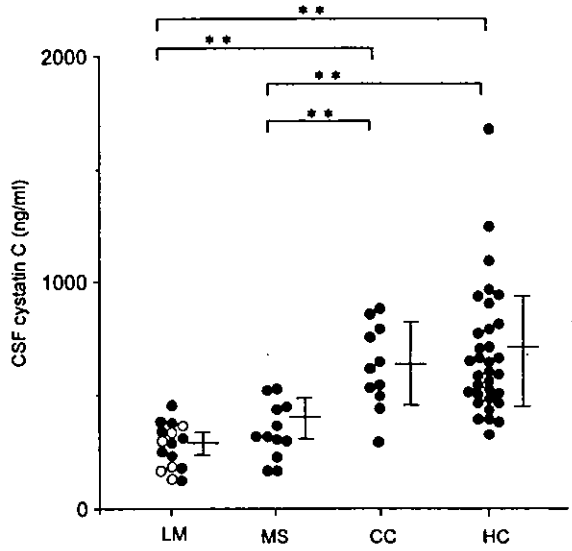

Fig. 2. Cystatin C concentrations in the CSF of patients with LM, MS, CC, and HC. Cystatin C concentrations were measured using the method described in Patients and methods. In LM patients, (●) and (○) indicate patients with metastasis due to solid tumor and leukemia or lymphoma, respectively. The vertical bars indicate mean ± S.D. The mean CSF cystatin C concentrations were decreased in patients with LM compared to CC and HC. The MS patients had low concentrations of cystatin C compared to CC and HC. $^{**}p<0.01$.

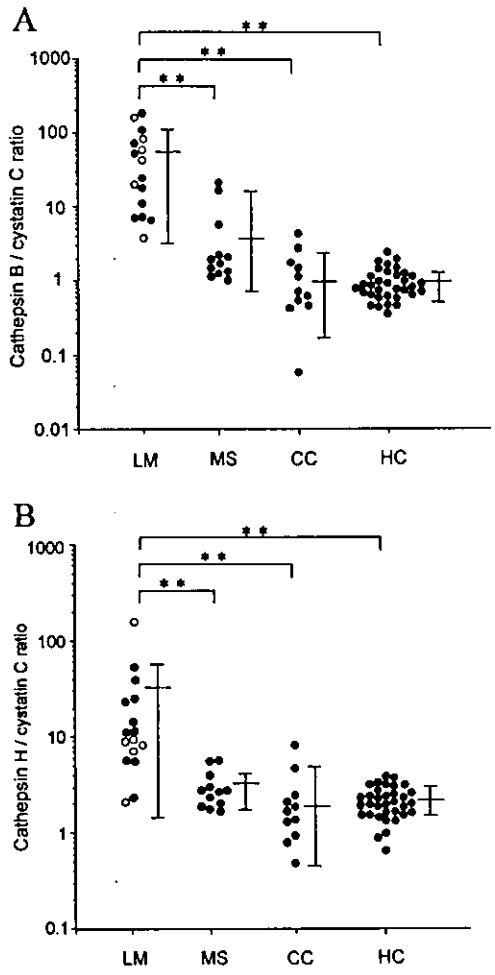

Fig. 3. Relative ratio of cathepsin activities to the cystatin C concentrations in CSF. Results are expressed as cathepsin activities (U/ml)/cystatin C concentration (ng/ml) × 100. The vertical bars indicate mean ± S.D. A log scale was used for the ratio values. In LM patients, (●) and (○) indicate patients with metastasis due to solid tumor and leukemia or lymphoma, respectively. (A) The mean cathepsin B/cystatin C ratio was higher in LM than in MS, CC, and HC. (B) The mean cathepsin H/cystatin C ratio was higher in LM than in MS, CC, and HC. $^{**}p<0.01$.

cathepsin H activity was 97.1% in LM, 29.1% in MS, 76.7% in CC, and 21.6% in HC. Except for one patient who had high concentrations of cathepsin H, LM patients with leukemia or lymphoma seemed to have lower cathepsin H concentrations than patients with solid tumors. No correlation between cathepsin B and H activities was found in the subjects ($r=0.559$), LM ($r=0.329$), or MS ($r=0.694$). Among LM patients, there was no difference in the mean cathepsin B or H activities between patients with solid tumors and those with leukemia or lymphoma.

3.3. Cystatin C concentrations

As shown in Fig. 2, the mean CSF cystatin C concentrations were decreased in the patients with LM compared to CC ($p<0.0005$) and HC ($p<0.0001$). The CV was 34.7% in LM, 31.9% in MS, 30.0% in CC, and 34.7% in HC. As we reported recently, the MS patients had low concentrations of cystatin C compared to CC ($p<0.01$) and HC ($p<0.0001$) [8]. The cystatin C concentrations between LM and MS were not significantly different. The cystatin C concentrations were not correlated with the protein concentrations ($r=0.268$) or WBC counts ($r=0.206$) in the CSF. Among LM patients, the mean CSF cystatin C concentrations in patients with leukemia and lymphoma were slightly lower than in patients with solid tumors ($p<0.05$) (Fig. 2; LM, open circle vs. closed one).

3.4. Ratios of cathepsins B and H to cystatin C in CSF

To estimate the balance between protease and its inhibitor in each disease, we evaluated the relative ratio of the total cathepsin B or H activity against the cystatin C antigen concentration in CSF. As shown in Fig. 3, LM had a higher cathepsin B/cystatin C ratio than MS, CC, or HC (all $p<0.0001$), concomitant with a higher cathepsin H/cystatin C ratio than those groups ($p<0.005$, 0.005, and 0.0005, respectively). Among LM patients, there was no difference in the

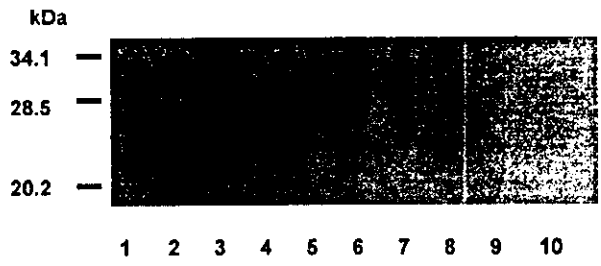

Fig. 4. Western blot analysis of cathepsin B in the CSF of patients with LM, MS, CC, and HC. CSFs (10 μl) collected from patients with HC (lanes 1 and 2), CC (lanes 3 and 4), LM (lanes 5–8), and MS (lanes 9 and 10) were analyzed by Western blot. Prestained SDS-PAGE standards (Bio-Rad Laboratories, Hercules CA) were used as protein MW standards.

mean cathepsin B/cystatin C, and cathepsin H/cystatin C ratios between patients with a solid tumor and patients with leukemia or lymphoma.

3.5. Western blot analysis

According to previous reports, cathepsin B is synthesized as an inactive 43-kDa pro-enzyme that is activated as either a 30-kDa single-chain form or a two-chain form consisting of 25 and 5 kDa subunits [13]. We measured the total cathepsin B activity including pro-enzyme. As shown in Fig. 4, cathepsin B molecule was only detected in the CSF from LM patients by Western blot analysis. In CSF from patients with low cathepsin B activity, only the 25-kDa band was detected, but with high cathepsin B activity, bands at 25 and 30 kDa were detected (Fig. 4, lanes 5–8). The 25-kDa band is likely the heavy chain of double-chain cathepsin B, while the 30-kDa band is single-chain cathepsin B. Western blot analysis for cathepsin H also detected 28-kDa single band only in the CSF from eight LM patients (data not shown).

3.6. Immunostaining against cathepsins

Immunoreactivity for cathepsins B and H was determined immunohistochemically in the cells among the leptmeningeal space. Invaded cells in the six studied cases (three solid tumor patients and three leukemia patients) that occupied almost all the space have positive staining against both cathepsins B (Fig. 5) and H (data not shown).

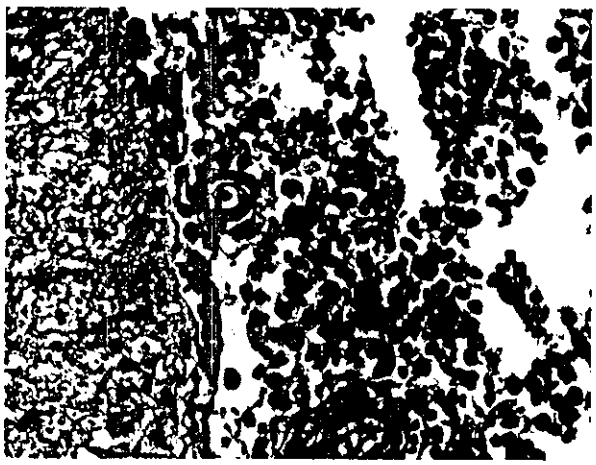

Fig. 5. Immunohistochemical detection of cathepsin B in LM of leukemia. Invaded leukemia cells that occupied the cervical leptomeninges show immunoreactivity for cathepsin B. Original magnification × 200.

4. Discussion

During the invasive processes of cancer, the invasive cells release proteases, penetrate the basal lamina of blood vessels and invade into leptomeninges. Since cathepsins B and H are proteolytic enzymes capable of degrading various constituents of extracellular matrix [14], we hypothesized that cathepsins B and H may be involved in LM of cancer cells. In the present study, cathepsin B and H immunoreactivities were strongly detected in the invaded cells of LM. Many tumor cells occupied the leptomeningeal space, but few inflammatory cells were found there. Moreover, cathepsins B and H were demonstrated in the CSF of LM, but were not detected in the CSF of three control groups by Western blot analysis. These results demonstrate the close relationship between the cathepsins and tumor cells in LM.

We found increased cathepsin B and H activities in CSF with LM. We recently demonstrated high cathepsin B activity in CSF from patients with inflammatory neurological diseases, such as patients with Guillain–Barré syndrome and MS, compared to healthy controls and disease controls with cerebral infarction [8]. However, the mean cathepsin B activity in LM patients was much higher than that in neuroinflammatory diseases. Moreover, the increase in cathepsin H activity was also demonstrated in the CSF of LM. Previous studies reported that several kinds of solid tumors produce cathepsin B in their invasive process [15], while a relationship between cathepsin H activity and tumor invasion has not been clearly determined. Our results of increased activity of cathepsin H as well as cathepsin B in the CSF of LM suggest that cathepsins B and H may both be involved in the pathophysiology of cancer invasion into the leptomeningeal space. In the 16 patients that we examined, CSF in 6 were repeatedly examined to confirm diagnosis of LM. LM in 5 patients (3 solid tumors and 2 leukemia/lymphomas) was revealed by autopsy. If cathepsin activities or cathepsin/cystatin C ratio were used to diagnose them, it might be helpful for accurate diagnosis.

In the present study, two types of cathepsins were not always increased in CSF of same single LM patient. Two LM patients had normal concentrations of both cathepsins. It is possible that different types of tumors produce a distinct spectrum of cathepsins in the process of invasion although we could not find the association between particular solid tumor types and increased concentrations of cathepsins. Furthermore, the role of other important systems associated with tumor invasion, such as the matrix metalloproteinase system should be investigated in the near future.

Our results also indicate that the proteases may play a role in the invasion of leukemia and lymphoma cells into the CNS. Cathepsin B was synthesized in HL-60 leukemia cells in vitro [16], but it is not demonstrated whether the cathepsin B is released. Furthermore, it remains obscure whether leukemia cells produce proteases in vivo to invade organs. The increase of cathepsins B and H in CSF from CNS leukemia and lymphoma suggests that cathepsins B and H may be released from leukemia cells during the extension of leukemia cells and lymphoma cells into leptomeninges.

Invasive potential of cathepsin B was shown in several kinds of carcinoma cells in vitro. Cathepsin B, which is released from cancer cells and acts on the cell surface, degrades extracellular-matrix components and may initiate proteolytic cascades by activating other proteases [17]. Cystatin C, the physiological inhibitor of cathepsin B, can completely abrogate the cathepsin B activity [18], and overexpression of cystatin C has also been shown to diminish the invasiveness of murine melanoma cells [19]. While relative ratio of cathepsin B activity over cystatin C concentration is thought to be one of the invasive properties index [18,20,21], the present study showed high ratio of it in the CSF of LM patients. Since cystatin C is a major cysteine protease inhibitor in CSF [22], the ratio may indicate the proteolytic potential of the cells in LM. Since the ratio in the CSF of LM is significantly higher than the controls, it may be useful in diagnosing LM. Although cathepsins B and H and cystatin C are widely distributed in many kinds of cells, such as macrophages, neurons, and microglia [23], our immunohistochemical examination support that source of increased cathepsins B and H was the cancer cells of LM. It is unknown whether the majority of primary tumor cells had high cathepsin activities and low cystatin C concentrations or a minority subpopulation which had metastatic potential tended to invade in leptomeninges. Metastatic assay using primary tumor of LM patients should be needed to identify the mechanism of leptomeningeal invasion in future.

In clinical studies of breast, lung, head and neck, and colorectal cancers, high concentrations of cathepsin B or H activity in cancer tissue or in sera are associated with reduced patient survival [7]. In the present study, cathepsin B and H activities or the cathepsin/cystatin C ratios were not related with survival periods. This may be ascribed to the serious illness of all the patients with LM, and the patients with solid tumors survived no more than 3 months. Another reason might be due to prolonged survival time in the patients with leukemia or lymphoma by effective chemotherapy. Although those parameters are not useful as prognostic markers, it is noteworthy that measuring cathepsin B and H activities and the cathepsin/cystatin C ratios may diagnose LM easier in combination with other useful tools [24,25] even when cancer cells are not found in CSF.

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References

[1] Balm M, Hammack J. Leptomeningeal carcinomatosis. Presenting features and prognostic factors. Arch Neurol 1996;53: 626–32.
[2] Recht LD. Neurologic complications of systemic lymphoma. Neurol Clin 1991;9:1001–15.
[3] Walker RW. Neurologic complications of leukemia. Neurol Clin 1991;9:989–99.
[4] Aznavoorian S, Murphy AN, Stetler-Stevenson WG, Liotta LA. Molecular aspects of tumor cell invasion and metastasis. Cancer 1993;71:1368–83.
[5] Kos J, Smid A, Krasovec M, Svetic B, Lenarcic B, Vrhovec I, et al. Lysosomal proteases cathepsins D, B, H, L and their inhibitors stefins A and B in head and neck cancer. Biol Chem Hoppe-Seyler 1995;376:401–5.
[6] Budihna M, Strojan P, Smid L, Skrk J, Vrhovec I, Zupevc A,

et al. Prognostic value of cathepsins B, H, L, D and their endogenous inhibitors stefins A and B in head and neck carcinoma. Biol Chem Hoppe-Seyler 1996;377:385–90.
[7] Lah TT, Kos J. Cysteine proteinases in cancer progression and their clinical relevance for prognosis. Biol Chem 1998;379: 125–30.
[8] Nagai A, Murakawa Y, Terashima M, Shimode K, Takeuchi H, Kobayashi S. Cystatin C and cathepsin B in CSF from patients with inflammatory neurologic diseases. Neurology 2000;55:1828–32.
[9] Poser CM, Paty DW, Scheinberg L, McDonald WI, Davis FA, Ebers GC, et al. New diagnostic criteria for multiple sclerosis: guidelines for research protocols. Ann Neurol 1983;13: 227–31.
[10] Barrett AJ, Kirschke H. Cathepsin B, cathepsin H, and cathepsin L. Methods Enzymol 1981;80:535–61.
[11] Shimode K, Fujihara S, Nakamura M, Kobayashi S, Tsunematsu T. Diagnosis of cerebral amyloid angiopathy by enzyme-linked immunosorbent assay of cystatin C in cerebrospinal fluid. Stroke 1991;22:860–6.
[12] Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979;76:4350–4.
[13] Keppler D, Sloane BF. Cathepsin B: multiple enzyme forms from a single gene and their relation to cancer. Enzyme Protein 1996;49:94–105.
[14] Buck MR, Karustis DG, Day NA, Honn KV, Sloane BF. Degradation of extracellular-matrix proteins by human cathepsin B from normal and tumour tissues. Biochem J 1992;282:273–8.
[15] Yan S, Sameni M, Sloane BF. Cathepsin B and human tumor progression. Biol Chem 1998;379:113–23.
[16] Burnett D, Crocker J, Vaughan AT. Synthesis of cathepsin B by cells derived from the HL60 promyelocytic leukaemia cell line. J Cell Physiol 1983;115:249–54.
[17] Kobayashi H, Ohi H, Sugimura M, Shinohara H, Fujii T, Terao T. Inhibition of in vitro ovarian cancer cell invasion by modulation of urokinase-type plasminogen activator and cathepsin B. Cancer Res 1992;52:3610–4.
[18] Corticchiato O, Cajot JF, Abrahamson M, Chan SJ, Keppler D, Sordat B. Cystatin C and cathepsin B in human colon carcinoma: expression by cell lines and matrix degradation. Int J Cancer 1992;52:645–52.
[19] Sexton PS, Cox JL. Inhibition of motility and invasion of B16 melanoma by the overexpression of cystatin C. Melanoma Res 1997;7:97–101.
[20] Coulibaly S, Schwihla H, Abrahamson M, Albini A, Cerni C, Clark JL, et al. Modulation of invasive properties of murine squamous carcinoma cells by heterologous expression of cathepsin B and cystatin C. Int J Cancer 1999;83:526–31.
[21] Rozhin J, Gomez AP, Ziegler GH, Nelson KK, Chang YS, Fong D, et al. Cathepsin B to cysteine proteinase inhibitor balance in metastatic cell subpopulations isolated from murine tumors. Cancer Res 1990;50:6278–84.
[22] Olafsson I, Gudmundsson G, Abrahamson M, Jensson O, Grubb A. The amino terminal portion of cerebrospinal fluid cystatin C in hereditary cystatin C amyloid angiopathy is not truncated: direct sequence analysis from agarose gel electropherograms. Scand J Clin Lab Invest 1990;50:85–93.
[23] Katunuma N, Kominami E. Distribution and localization of lysosomal cysteine proteinases and cystatins. In: Turk V, editor. Cysteine proteinases and their inhibitors. New York: Gruyter Wd & Co; 1986. p. 219–27.
[24] Freilich RJ, Krol G, DeAngelis LM. Neuroimaging and cerebrospinal fluid cytology in the diagnosis of leptomeningeal metastasis. Ann Neurol 1995;38:51–7.
[25] Gleissner B, Siehl J, Korfel A, Reinhardt R, Thiel E. CSF evaluation in primary CNS lymphoma patients by PCR of the CDR III IgH genes. Neurology 2002;58:390–6.

Original Paper

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Effect of a Novel Free Radical Scavenger, Edaravone (MCI-186), on Acute Brain Infarction

Randomized, Placebo-Controlled, Double-Blind Study at Multicenters

The Edaravone Acute Brain Infarction Study Group (Chair: Eiichi Otomo, MD)[1]

Key Words
Clinical trial · Free radical · Neuroprotection · Acute stroke

Abstract
Edaravone, a novel free radical scavenger, demonstrates neuroprotective effects by inhibiting vascular endothelial cell injury and ameliorating neuronal damage in ischemic brain models. The present study was undertaken to verify its therapeutic efficacy following acute ischemic stroke. We performed a multicenter, randomized, placebo-controlled, double-blind study on acute ischemic stroke patients commencing within 72 h of onset. Edaravone was infused at a dose of 30 mg, twice a day, for 14 days. At discharge within 3 months or at 3 months after onset, the functional outcome was evaluated using the modified Rankin Scale. Two hundred and fifty-two patients were initially enrolled. Of these, 125 were allocated to the edaravone group and 125 to the placebo group for analysis. Two patients were excluded because of subarachnoid hemorrhage and disseminated intravascular coagulation. A significant improvement in functional outcome was observed in the edaravone group as evaluated by the modified Rankin Scale ($p = 0.0382$). Edaravone represents a neuroprotective agent which is potentially useful for treating acute ischemic stroke, since it can exert significant effects on functional outcome as compared with placebo.

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Introduction

Data have been accumulated which demonstrate that free radicals play a crucial role in brain injury following ischemia [1–3]. In the ischemic state, the metabolism of arachidonic acid is accelerated within the brain tissue [4, 5], including the brain microvessels [6], leading to an increase in free radical production. Free radicals cause membrane injury through peroxidation of unsaturated fatty acids in the phospholipids constituting the cell membrane, and such injury progresses sequentially, leading to ischemic brain injury as represented by neuronal death and brain edema [7].

A novel free radical scavenger, edaravone (MCI-186, 3-methyl-1-phenyl-2-pyrazolin-5-one, MW: 174.20), has been shown to inhibit lipid peroxidation [8] and vascular endothelial cell injury [9] in vitro. In rat brain ischemic models, edaravone can ameliorate brain edema [10, 11],

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Table 1. The grade of neurological deficits

Neurological deficit	Categories					
	0	1	2	3	4	5
Aphasia	normal	no problems in daily conversation, but aphasic symptoms present	slight disturbance in daily conversation	difficulty to understand, but possible	almost impossible to understand	
Dysarthria	normal	very slight	slurs in at least some words, but could be understood with some difficulty	moderate	unable to be understood	
Dysphagia	normal	very slight	choking occasionally	unable to swallow occasionally with difficulty	unable to swallow	
Sensory disturbance	normal	very slight	tolerable sensory paralysis and/or abnormal sensation	moderate	loss of sensation and/or intolerable abnormal sensation	
Muscle strength of the legs and arms with motor weakness	normal	able to move against resistance	able to move against gravity	able to move when gravity was removed	presence of muscle contraction	absence of muscle contraction

tissue injury [8, 12, 13], delayed neuronal death [14] and neurological deficits [11, 12]. Additionally, it can prevent cerebral vasospasm in canine subarachnoid hemorrhage models [15].

Edaravone was therefore expected to have a neuroprotective effect in cases of acute brain infarction. It was first evaluated in an early phase 2 study [16] at doses of 20, 30 and 60 mg, twice a day, in 85 patients with acute brain infarction, and subsequently in a late phase 2 double-blind study [17] at doses of 10, 30 and 45 mg, twice a day, in 356 patients within 72 h of onset. The results of these clinical studies demonstrated improvement in neurological deficits without serious safety problems. On the basis of the above investigations, the appropriate dosage was considered to be 30 mg (i.v., b.i.d.) for 14 days.

The present multicenter, randomized, placebo-controlled double-blind study was carried out in order to verify the efficacy of edaravone (30 mg, i.v., b.i.d.) in terms of functional outcome in patients with acute ischemic stroke.

Materials and Methods

Patients

The study was conducted at institutions throughout Japan from December 1993 through March 1996 (see Appendix).

The criteria for inclusion were as follows: (1) inpatients within 72 h after the onset of ischemic stroke including patients that were both thrombotic and embolic in nature, and (2) patients with a level of consciousness between 0 (alert) and 30 (able to be aroused with mechanical or verbal stimuli) according to the Japan Coma Scale [18]. Patients who could not be aroused with forceful mechanical stimuli (levels 100–300) were excluded.

Prior to enrollment in the study, informed consent was obtained from each patient or the patient's next-of-kin, if the patient was not competent to take this responsibility. The protocol and consent form employed during the trial were approved by each participating center's institutional review board. The study was performed in accordance with Good Clinical Practice (Ministry of Health and Welfare of Japan).

Test Drugs and Random Allocation

The active drug was provided in 20-ml ampoules containing 30 mg of edaravone. Physiological saline, indistinguishable from the active drug, was used as the placebo. Mitsubishi Pharma Corporation supplied the test drugs. The controller, who randomly allocated the test drugs, confirmed the indistinguishability.

Dosage and Administration

One ampoule was diluted in 100 ml of physiological saline and administered by intravenous drip infusion over a period of 30 min, every 12 h for 14 days. Intravenous infusion of 400–600 ml of 10% glycerol was allowed to give if needed. The use of fibrinolytic agents (urokinase and recombinant tissue plasminogen activator), citicoline, and ozagrel sodium was avoided throughout the study period.

Observation Parameters

The patient characteristics, including sex, age, stroke subtype, time to treatment after onset, level of consciousness according to the Japan Coma Scale, neurological deficits evaluated according to table 1, associated diseases, and CT or MRI findings, were recorded. Thrombotic and embolic infarctions were diagnosed according to the

Table 2. Baseline characteristics

Characteristics	Edaravone (n = 125)	Placebo (n = 125)	Statistics[4]
Sex (M/F)	82/43	84/41	χ^2: p = 0.893
Age, years			
≤65/65<	42/83	48/77	χ^2: p = 0.510
Mean ± SD	66.3 ± 8.0	66.1 ± 8.5	t: p = 0.836
Stroke subtype			
Thrombotic	97	101	χ^2: p = 0.809
Embolic	24	21	
Not determined	4	3	
Time to treatment after stroke onset, h			
≤24	42	39	χ^2: p = 0.218
25–48	41	56	
48<	42	30	
Mean ± SD	37.3 ± 22.6	35.2 ± 26.6	t: p = 0.488
Level of consciousness before treatment[1]			
Alert (0)	79	81	χ^2: p = 0.132
Grade I (1, 2, 3)[2]	37	27	
Grade II (10, 20, 30)[3]	9	17	
Aggregate score for neurological deficits before treatment			
Median (Q1, Q3)	6 (4, 11)	6 (4, 10)	W: p = 0.628
Associated diseases			
Hypertension	75	72	
Diabetes mellitus	26	29	
CT or MRI findings before treatment			
Infarction (+/–)	94/31	86/39	χ^2: p = 0.324
Middle cerebral artery/others	75/19	67/19	χ^2: p = 0.900
Perforator/cortex	57/37	56/29 (not determined: 1)	χ^2: p = 0.568
Use of 10% glycerol	111	111	χ^2: p = 1.000

[1] Japan Coma Scale.
[2] Grade I: patient is awake without any stimuli, and is: (1) almost fully conscious; (2) unable to recognize time, place, and person; (3) unable to recall name or date of birth.
[3] Grade II: patient can be aroused (then reverts to previous state after cessation of stimulation): (10) easily by being spoken to (or is responsive with purposeful movements, phrases, or words); (20) with loud voice or shaking of shoulders (or is almost always responsive to very simple words like 'yes' or 'no', or to movements); (30) only by repeated mechanical stimuli.
[4] χ^2 = chi-square test; t = t test; W = Wilcoxon's rank sum test.

criteria proposed by Minematsu et al. [19], in which the mode of onset, presence or absence of underlying heart diseases and other factors were included.

The outcome was assessed according to the modified Rankin Scale [20] at discharge within 3 months or at 3 months after onset. Additionally, we collected outcome data at 3, 6 and 12 months after onset.

Physical examinations and routine clinical laboratory tests (hematology, blood chemistry and urinalysis) were performed before the treatment and on days 7 and 14 of the treatment.

Handling of the Patients' Data

The handling of patients with incomplete data was discussed by the Steering Committee (see Appendix) before the key break. The data were frozen, until the key codes were broken, and the data analysis was then performed subsequently. The data were subjected to intent-to-treat analysis.

Statistical Analysis

Comparisons of the modified Rankin Scale were undertaken by Wilcoxon's rank sum test, with the criterion for significance set at 5% (two-tailed).

Table 3. Functional outcome assessed at discharge within 3 months or at 3 months after onset, using the modified Rankin Scale

Functional outcome	Edaravone (n = 125)	Placebo (n = 125)
0 No symptoms at all	27	12
1 No significant disability despite symptoms	36	35
2 Slight disability	29	40
3 Moderate disability	12	12
4 Moderately severe disability	10	15
5 Severe disability	7	6
Death	4	5
Wilcoxon's rank sum test	p = 0.0382	

Table 4. Modified Rankin Scale assessed at 3 months, 6 months and 12 months after onset

Grade	3 Months		6 Months		12 Months	
	edaravone (n = 115)	placebo (n = 113)	edaravone (n = 105)	placebo (n = 103)	edaravone (n = 100)	placebo (n = 94)
0	26	10	27	9	27	8
1	34	39	35	37	31	35
2	24	26	15	23	14	19
3	10	11	11	10	8	7
4	9	14	3	9	4	11
5	7	7	8	7	6	5
Death	5	6	6	8	10	9
p[1]	0.0481		0.0112		0.0248	

[1] Wilcoxon's rank sum test.

Results

A total of 252 patients were registered, of whom 125 were assigned to the edaravone group and 127 were assigned to the placebo group. Among them, 1 patient in the placebo group who had suffered subarachnoid hemorrhage and 1 patient in the placebo group who turned out to have disseminated intravascular coagulation (DIC) due to ovarian tumors immediately after the start of treatment and subsequently died as a result of brain hernia following hemorrhagic infarction were excluded from the analysis. Thus, 250 patients, comprising 125 in the edaravone group and 125 in the placebo group, were eligible for intent-to-treat analysis.

The patient characteristics of the two groups were comparable as shown in table 2. There was no difference between both groups.

Efficacy

Table 3 lists the results for the analysis using the modified Rankin Scale performed at discharge within 3 months or at 3 months after onset. There was a significant difference (p = 0.0382, Wilcoxon's rank sum test) between the groups in favor of the edaravone group. The average time from onset to assessment was 49 days in the edaravone group and 50 days in the placebo group. Additionally, based on the outcome data at 3, 6 and 12 months after onset, the above benefit was sustained for relatively longer as shown in table 4. When subset analysis was undertaken for the patients treated within 24 h, the difference between the two groups was clearer than that in the whole patient analysis, as shown in table 5.

Safety

Adverse reactions were observed in 9 patients (7%) in the edaravone group and in 14 patients (11%) in the pla-

Table 5. Modified Rankin Scale assessed at discharge within 3 months or at 3 months after onset, in patients treated within 24 h after onset

Grade	Edaravone (n = 42)	Placebo (n = 39)
0	14	1
1	10	6
2	8	13
3	5	3
4	2	8
5	2	4
Death	1	4
	p = 0.0001[a]	

[a] Wilcoxon's rank sum test.

cebo group. Such reactions in the edaravone group consisted of skin rash in 4 patients, abnormal liver function in 3, itching and nausea in 1, and fever and abnormal liver function in 1, but recovery was achieved during or after the treatment. In the placebo group, the adverse reactions that occurred included skin rash in 2 patients, abnormal liver function in 6, diarrhea, fever and acute renal failure in 1, a bleeding tendency and DIC in 1, increases in white blood cell, lactate dehydrogenase and serum amylase in 1, epigastric discomfort in 1, anxiety attack and dyspnea in 1, and anemia and abnormal liver function in 1. Abnormal changes in laboratory tests were observed mainly to involve parameters of the liver functions (aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, γ-glutamyl transpeptidase), and the proportions of patients with such changes amounted to about 10% in each group.

Four patients in the edaravone group and 5 patients in the placebo group died. The causes of death in the edaravone group were exacerbation of brain infarction, sudden cardiac arrest, pneumonia, and suicide due to mental depression in 1 patient each, and the relation to the test drug was judged to be nil. In the placebo group, the causes of death comprised exacerbation of brain infarction in 1 patient, advanced brain edema or tonsillar herniation in 2, pneumonia in 1, and DIC assumed to be due to large infarct and liver cirrhosis in 1.

Discussion

Neuroprotective drugs are expected to extend the therapeutic time window after stroke with fibrinolytic therapy by inhibiting cell death and blocking reperfusion injury, and a combination of both drugs could provide new weapons for effective treatment of stroke in the future [21]. Several studies have indicated the presence of an area of constrained blood flow with partially preserved energy metabolism, the so-called ischemic penumbra, around the ischemic core within the brain of animal models as well as stroke patients, allowing drugs to reach and prevent the progress to infarct in this area [22–24]. Drug treatments for acute ischemic stroke can be roughly divided into antithrombotic agents, such as thrombolytics, anticoagulants and antiplatelets, and neuroprotective agents, such as free radical scavengers.

Edaravone has been reported to inhibit vascular endothelial cell injury [9], brain edema [10, 11], tissue injury [8, 12, 13] and delayed neuronal death [14], and consequently lessens neurological deficits [11, 12]. Additionally, preservation of N-acetyl-aspartate, a neuron-specific amino acid, in the ischemic brain of edaravone-treated patients as revealed by sequential MR spectroscopic examinations has been reported [25]. In terms of its chemical characteristics, edaravone can inhibit peroxidation of the phosphatidylcholine liposomal membrane initiated by water-soluble as well as lipid-soluble radicals, which makes it comparable to ascorbic acid and α-tocopherol, which are well-known antioxidants [26]. Contrary to superoxide dismutase, another free radical scavenger, which has difficulty in penetrating the blood-brain barrier (BBB), edaravone is a low-molecular-weight radical scavenger, of which the BBB permeability has been estimated to be around 60% [27], eliminating highly cytotoxic hydroxyl radicals in the brain following intravenous administration [14]. In fact, edaravone can diminish increases in the levels of hydroxyl radicals after infusion which appear to be preferentially produced in the perifocal ischemic area, possibly the ischemic penumbra, resulting in reducing the extent of brain damage [13]. Edaravone does not affect blood coagulation, platelet aggregation, fibrinolysis or bleeding time [28, 29], so that there is no additional risk of bleeding. Edaravone is therefore regarded as a readily utilizable neuroprotective drug with a free radical scavenging action. The effective concentration of this drug in in vitro experiments was found to be 10^{-6}–10^{-5} mol/l, and the plasma concentration at an effective dose in animal experiments was 988–1,729 ng/ml (5.7–9.9 × 10^{-6} mol/l) [30]. The pharmacokinetics

have been investigated in healthy volunteers, and the C_{max} and AUC were found to increase in proportion to the dose [31]. Further, the plasma concentration of the drug when administered to the elderly at the same dose as that employed in the present study was reported to be 1,041 ng/ml (6×10^{-6} mol/l) [32]. The effective concentration in nonclinical studies and the plasma concentration encountered in actual clinical practice agree well.

As regards the time to treatment after the onset of stroke, we set the inclusion criterion as within 72 h, since the time did not appear to influence the efficacy so much in the late phase 2 study [17]. In general, the duration of brain edema in humans is several times longer than that in the rat which shows a tendency to decrease after 3 days of infarction [33]. Recently, Matsui et al. [34] indicated that the infarct volume slowly increased from 24 h until as late as 168 h after permanent focal ischemia in rats, and Peters et al. [35] suggested prolonged oxidative stress after cerebral infarct, which can trigger intraparenchymal neutrophil infiltration leading to oxidative damages of BBB and neuronal cells persisting for at least 5 days or longer in patients [36].

The data obtained in the present study demonstrated that the effects of edaravone on the functional outcome were significantly superior to those of the placebo. During 12 months of follow-up, the outcome data indicated a sustained benefit for edaravone in acute ischemic stroke patients. As regards the time to treatment after stroke onset, the results for the patients treated within 24 h were clearer than those for the patients treated within 72 h after onset.

The major adverse reactions were skin rash and abnormal liver function, but their incidence did not differ significantly between the groups, or was rather more frequent in the placebo group when considering other adverse reactions. There were thus no serious problems in terms of safety.

These findings suggest that edaravone exerts a neuroprotective effect in humans, and has a promising potential for clinical use. Besides the neuroprotective action evident in the single treatment described above, edaravone, due to the absence of a bleeding risk, can also be expected to offer future advantages in combination therapy with fibrinolytic agents and antithrombotics by scavenging the free radicals associated with reperfusion injury, leading to an expansion of the therapeutic time window. We therefore conclude that edaravone represents a promising neuroprotective agent in the treatment of acute ischemic stroke.

Appendix

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References

1 Kontos HA: George E Brown memorial lecture. Oxygen radicals in cerebral vascular injury. Circ Res 1985;57:508–516.
2 Schmidley JW: Free radicals in central nervous system ischemia. Stroke 1990;21:1086–1090.
3 Halliwell B: Reactive oxygen species and the central nervous system. J Neurochem 1992;59:1609–1623.
4 Gaudet RJ, Levine L: Transient cerebral ischemia and brain prostaglandins. Biochem Biophys Res Commun 1979;86:893–901.
5 Moskowitz MA, Kiwak KJ, Hekimian K, Levine L: Synthesis of compounds with properties of leukotrienes C4 and D4 in gerbil brains after ischemia and reperfusion. Science 1984;224:886–889.
6 Asano T, Gotoh O, Koide T, Takakura K: Ischemic brain edema following occlusion of the middle cerebral artery in the rat. II. Alteration of the eicosanoid synthesis profile of brain microvessels. Stroke 1985;16:110–113.
7 Chan PH, Fishman RA, Longar S, Chen S, Yu A: Cellular and molecular effects of polyunsaturated fatty acids in brain ischemia and injury. Prog Brain Res 1985;63:227–235.
8 Watanabe T, Yuki S, Egawa M, Nishi H: Protective effects of MCI-186 on cerebral ischemia: Possible involvement of free radical scavenging and antioxidant actions. J Pharmacol Exp Ther 1994;268:1597–1604.
9 Watanabe T, Morita I, Nishi H, Murota S: Preventive effect of MCI-186 on 15-HPETE induced vascular endothelial cell injury in vitro. Prostaglandins Leukot Essent Fatty Acids 1988;33:81–87.
10 Abe K, Yuki S, Kogure K: Strong attenuation of ischemic and postischemic brain edema in rats by a novel free radical scavenger. Stroke 1988;19:480–485.
11 Nishi H, Watanabe T, Sakurai H, Yuki S, Ishibashi A: Effect of MCI-186 on brain edema in rats. Stroke 1989;20:1236–1240.
12 Kawai H, Nakai H, Suga M, Yuki S, Watanabe T, Saito K: Effects of a novel free radical scavenger, MCI-186, on ischemic brain damage in the rat distal middle cerebral artery occlusion model. J Pharmacol Exp Ther 1997;281:921–927.
13 Mizuno A, Umemura K, Nakashima M: Inhibitory effect of MCI-186, a free radical scavenger, on cerebral ischaemia following rat middle cerebral artery occlusion. Gen Pharmacol 1998;30:575–578.
14 Yamamoto T, Yuki S, Watanabe T, Mitsuka M, Saito K, Kogure K: Delayed neuronal death prevented by inhibition of increased hydroxyl radical formation in a transient cerebral ischemia. Brain Res 1997;762:240–242.
15 Nakagomi T, Yamakawa K, Sasaki T, Saito I, Takakura K: Effect of edaravone on cerebral vasospasm following experimental subarachnoid hemorrhage. J Stroke Cerebrovasc Dis, in press.
16 Otomo E, Tohgi H, Kogure K, Hirai S, Terashi A, Gotoh F, Tazaki Y, Ito E, Sawada T, Kobayashi S, Fujishima M, Nakashima M: Clinical efficacy of a free radical scavenger, MCI-186, on acute cerebral infarction: Early phase II clinical trial. Ther Res 1998;19:1311–1332.
17 MCI-186 Acute Cerebral Infarction Study Group: Late phase 2 clinical trial of MCI-186 on acute cerebral infarction: A dose-finding double-blind study (in Japanese). J Clin Exp Med 1998;185:841–863.
18 Ohta T, Kikuchi H, Hashi K, Kudo Y: Nizofenone administration in the acute stage following subarachnoid hemorrhage: Results of a multi-center controlled double-blind clinical study. J Neurosurg 1986;64:420–426.
19 Minematsu K, Yamaguchi T, Omae T: Spectacular shrinking deficit: Rapid recovery from a major hemispheric syndrome by migration of an embolus. Neurology 1992;42:157–162.
20 van Swieten JC, Koudstaal PJ, Visser MC, Schouten HJA, van Gijn J: Interobserver agreement for the assessment of handicap in stroke patients. Stroke 1988;19:604–607.
21 Barinaga M: Finding new drugs to treat stroke. Science 1996;272:664–666.
22 Astrup J, Siesjo BK, Symon L: Thresholds in cerebral ischemia: The ischemic penumbra. Stroke 1981;12:723–725.
23 Hossmann KA: Viability thresholds and the penumbra of focal ischemia. Ann Neurol 1994;36:557–565.
24 Fisher M: Characterizing the target of acute stroke therapy. Stroke 1997;28:866–872.
25 Houkin K, Nakayama N, Kamada K, Noujou T, Abe H, Kashiwaba T: Neuroprotective effect of the free radical scavenger MCI-186 in patients with cerebral infarction: Clinical evaluation using magnetic resonance imaging and spectroscopy. J Stroke Cerebrovasc Dis 1998;7:1–9.
26 Yamamoto Y, Kuwahara T, Watanabe K, Watanabe K: Antioxidant activity of 3-methyl-1-phenyl-2-pyrazolin-5-one. Redox Rep 1996;2:333–338.
27 Takamatsu Y, Watanabe T: Studies on the concentrations of 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186) in dog plasma and cerebral spinal fluid. Jpn Pharmacol Ther 1997;25(suppl):1793–1797.

28 Ando K, Nishi H, Watanabe T, Watanabe Y, Yuki S, Sugasawa K, Horii D, Ishibashi A, Kimura A, Nakano D, Morinaga T: General pharmacological studies of 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), a novel radical scavenging agent. Jpn Pharmacol Ther 1997; 25(suppl):1723–1753.
29 Watanabe Y, Watanabe T, Ando K, Yuki S, Saito K, Ishibashi A: Drug interaction between radical scavenger, 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186), and stroke-medicating agents. Jpn Pharmacol Ther 1997;25(suppl): 1709–1718.
30 Takamatsu Y, Yuki S, Watanabe T: Studies on the concentrations of 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186) in MCA occlusion and reperfusion model of rats. Jpn Pharmacol Ther 1997;25(suppl):1785–1791.
31 Shibata H, Arai S, Izawa M, Murasaki M, Takamatsu Y, Izawa O, Takahashi C, Tanaka M: Phase I clinical study of MCI-186 (edaravone, 3-methyl-1-phenyl-2-pyrazoline-5-one) in healthy volunteers: Safety and pharmacokinetics of single and multiple administrations. Jpn J Clin Pharmacol Ther 1998;29:863–876.
32 Yokota S, Kumagai Y, Uchiumi M, Isawa S, Murasaki M, Akimoto K, Iwamoto M, Yuasa T, Iwano M: A pharmacokinetic study of MCI-186, a novel drug for cerebrovascular disease in elderly and young healthy subjects. Jpn J Clin Pharmacol Ther 1997;28:693–702.
33 Gotoh O, Koide T, Asano T, Takakura K, Tamura A, Sano K: A model to study ischemic brain edema in rats and the influence of drugs; in Go KG, Baethmann A (eds): Recent Progress in the Study and Therapy of Brain Edema. Plenum Press, 1984, pp 499–508.
34 Matsui T, Mori T, Tateishi N, Kagamiishi Y, Satoh S, Katsube N, Morikawa E, Morimoto T, Ikuta F, Asano T: Astrocytic activation and delayed infarct expansion after permanent focal ischemia in rats. I. Enhanced astrocytic synthesis of s-100β in the periinfarct area precedes delayed infarct expansion. J Cereb Blood Flow Metab 2002;22:711–722.
35 Peters O, Back T, Lindauer U, Busch C, Megow D, Dreier J, Dirnagl U: Increased formation of reactive oxygen species after permanent and reversible middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 1998;18: 196–205.
36 Wang PY, Kao CH, Mui MY, Wang SJ: Leukocyte infiltration in acute hemispheric ischemic stroke. Stroke 1993;24:236–240.

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ORIGINAL COMMUNICATION

Chitosan decreases total cholesterol in women: a randomized, double-blind, placebo-controlled trial

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Background: Hypercholesterolemia is an important risk factor for cardiovascular disease. Orally administered chitosan binds lipids in the small intestine and reduces their absorption. Chitosan has been shown to decrease serum cholesterol in animal and human studies. This study investigated the effectiveness of chitosan in reducing serum cholesterol without concomitant diet therapy.
Methods: Ninety female volunteers (age 34–70 y) with confirmed mild to moderate hypercholesterolemia were enrolled into the study. They were randomly assigned to receive chitosan (1.2 g per day) or placebo in a double-blind manner. Serum lipids, body weight and adverse events were assessed at baseline and after 28 and 56 days of treatment. Subjects maintained their usual diet and documented the type and gross amount of food consumed.
Results: Eighty-four subjects (41 chitosan, 43 placebo) were included in the analysis. Chitosan significantly ($F=3.19$, $P=0.04$) reduced total cholesterol compared to placebo. In a subgroup of subjects with over 60 y of age, chitosan group significantly reduced total and LDL cholesterol ($F=4.21$, $P=0.02$, and $F=3.46$, $P=0.04$, respectively) compared with placebo. Adverse effects were few; no serious events were reported.
Conclusion: Our results demonstrate that chitosan is safe and effective for lowering cholesterol. However, the effect of chitosan for decreasing cholesterol is mild.
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Keywords: chitosan; total cholesterol; LDL cholesterol; humans; females

Introduction

In recent years, many clinicians have pointed out that overweight and hypercholesterolemia are important risk factor for cardiovascular disease (Rose *et al*, 1977; Martin *et al*, 1986) (Pekkanen *et al*, 1990; Kannel, 1995). Furthermore, cholesterol-lowering therapy reduces the risk of coronary heart disease and cerebrovascular accidents in hyperlipidemic patients who are free of previous heart disease or stroke (Shepherd *et al*, 1995; Bucher *et al*, 1998). Regular physical exercise or decreased consumption of total energy and fat are effective in reducing serum cholesterol. Unfortunately, daily exercise or dieting frequently fails, due to poor compliance. HMG-CoA reductase inhibitors have been shown to reduce the risk of coronary events or cerebrovascular disease by lowering cholesterol levels (Sacks *et al*, 1996; Bucher *et al*, 1998). However, HMG-CoA reductase inhibitors have side effects such as myopathy with or without rhabdomyolysis (Lee & Maddix, 2001; Moghadasian *et al*, 2000).

Chitosan, a natural polysaccharide of β-1,4-linked glucosamine residues, is a biopolymer obtained primarily from the exoskeletons of crustaceans. Hydrolysis of chitin from shrimps and crabs results in deacetylation of the aminoacetyl groups and yields chitosan (Muzzarelli *et al*, 1994). When ingested, chitosan develops an HCl-layer in the stomach. As capsulated particles of chitosan move into the duodenum, the HCl-layer becomes diluted and the chitosan particles form agglomerates with fatty acids and cholesterol, thus reducing lipid absorption from the gastrointestinal tract. Studies in primates have shown that chitosan can increase the amount of fat eliminated in the stool (Sugano *et al*, 1980; Ebihara & Schneeman, 1989). This finding led to the use of chitosan as a dietary supplement for weight loss or serum cholesterol reduction. Studies in rats also demonstrated the cholesterol-reducing activity of dietary chitosan (Sugano *et al*, 1980; Gallaher *et al*, 2000). However, a double-blind, randomized, placebo-controlled clinical study did not find

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