□ CASE REPORT □

Autoimmune Acquired Form of Angioedema that Responded to Danazol Therapy

Shinji HIGA, Haruhiko HIRATA, Seigou MINAMI*, Shouji HASHIMOTO**, Masaki SUEMURA***, Yukihiko SAEKI, Ichiro KAWASE and Toshio TANAKA

Abstract

A 51-year-old man with recurrent episodes of angioedema was diagnosed as having autoimmune acquired angioedema, based on adult onset, lack of apparent family history, decreased activity of C1 esterase inhibitor (C1 INH) and CH50, decreased levels of serum C4 and C1q and the presence of autoantibodies to C1 INH. The danazol treatment relieved the symptoms of angioedema and increased the C1 INH activity and concentration with the normalization of CH50, C1q and C4 levels. To our knowledge, this is the first case of autoimmune acquired angioedema in Japan.

(Internal Medicine 41: 398–402, 2002)

Key words: acquired angioedema, autoantibody, C1 INH

Introduction

Angioedema is a well-demarcated localized edema involving the deep layers of the skin, including the subcutaneous tissue (1). Some forms of angioedema are related to C1 esterase inhibitor (C1 INH) deficiency, due to genetic defects (hereditary angioedema; HAE) or acquired defects (acquired angioedema; AAE) (2–4). HAE, an autosomal dominant disease, is characterized by low levels of C1 INH protein and/or low functional activity of C1 INH. The onset of symptoms usually appears in childhood and early adolescence with a family history. AAE, associated with lymphoproliferative disorders or autoantibody synthesis, has the same clinical manifestations but differs in the lack of family history and usually shows the onset of symptoms in adults. Here we present an adult patient with AAE having autoantibody to C1 INH in whom treatment with danazol appeared to be effective through increased synthesis of C1 INH.

For editorial comment, see p 333.

C1 INH binding ELISA

The existence of autoantibodies binding to C1 INH in the serum was examined by ELISA as described previously (5). Briefly, C1 INH (Sigma-Aldrich Japan, Tokyo) was coated on the ELISA plates (Nunc-Immunoplate, Nunc, Roskilde, Denmark) in carbonate buffer (pH 9.6) at 5 to 10 μg/ml for overnight at 4°C. After washing three times with phosphate-buffered saline (PBS) (pH 7.4) plus 0.1% Tween 20 (Nacalai Tesque Inc., Tokyo), the wells were non-specifically blocked with PBS-Tween 20 containing 1% bovine serum albumin (F-V, Nacalai Tesque Inc.) (blocking buffer) for 2 hours at room temperature. After 5 washes, serially diluted serum (100 μl) with blocking buffer from the patient and from three healthy volunteers was added in duplicate to plates and incubated for 2 hours at room temperature. After five washes, 100 μl of alkaline phosphatase-conjugated anti-human IgG (1:1,000, Sigma), anti-human IgM (1:1,000, BioSource, Camarillo, CA, USA) or anti-human IgA (1:1,000, BioSource) antibody was added to each well and the plates were further incubated for 2 hours at room temperature. After five washes, 100 μl of substrate (p-nitrophenyl phosphate, Sigma Diagnostics, St. Louis, MO, USA) at 1 mg/ml in substrate buffer (0.05 M $NaHCO_3$ and 0.01 M $MgCl_2$, pH 9.8) was added to each well and the plates were incubated at room temperature. Absorption was measured at 450 nm using an Immunoreader (Japan Intermed Inc., Tokyo).

Case Report

A 51-year-old man was admitted to our hospital for further examination after episodes of recurrent angioedema in February 2001. Four years before the admission, he began to have an

Form the Third Department (Molecular Medicine) of Internal Medicine, Osaka University Medical School, Osaka, *the Department of Internal Medicine, Osaka Police Hospital, Osaka, **the Department of Internal Medicine, Toneyama National Hospital, Osaka and ***the Third Department of Internal Medicine, Nissay Hospital, Osaka

Received for publication July 19, 2001; Accepted for publication November 20, 2001

Reprint requests should be addressed to Dr. Toshio Tanaka, the Third Department of Internal Medicine, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565-0871

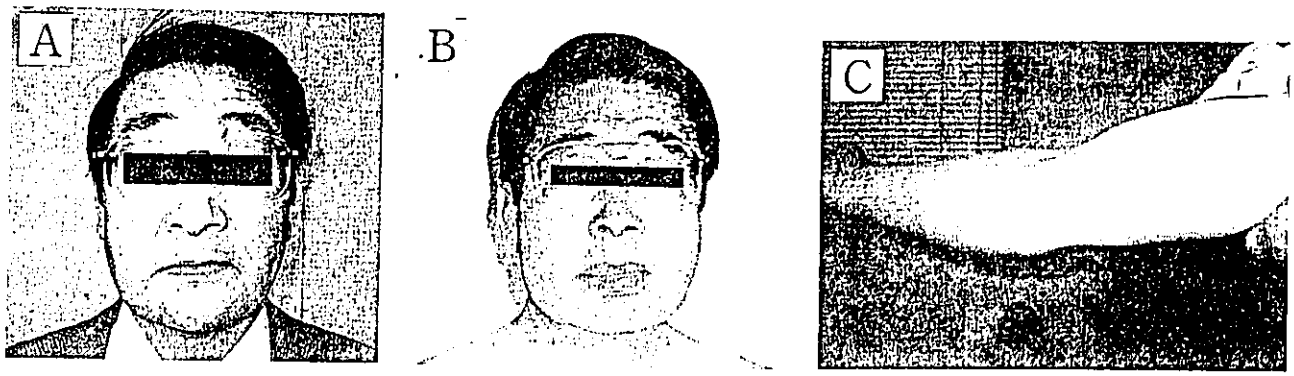

Figure 1. Episode of angioedema in this patient; Facial appearance without attack (A), facial swelling (B) and swelling of the upper limb (C) during the episode.

acute onset swelling of the hands and feet with subclinical fever and general fatigue. The swelling spontaneously resolved within 3 or 4 days. In 1998, episodes of angioedema occurred three or four times a year and localized lesions were expanded to the upper limbs, face and external genitalia. Figure 1 shows swelling of the face and upper extremity in this patient during one episode of angioedema. There was no family history of angioedema. On admission, physical examination did not reveal any remarkable abnormal findings. Chemical analyses of blood showed that the white cell count was 4,820/μl, with 66% neutrophils, 26% lymphocytes, and 2% eosinophils. Serum concentrations of total proteins and albumin, electrolytes, aspartate aminotransferase, creatinine kinase, creatinine, uric acid were normal. Serum immunoglobulin concentrations were within the normal limit (IgG 1,220 mg/dl, IgM 107 mg/dl and IgA 230 mg/dl) but the IgE level (790 IU/ml) was increased. Serum C reactive protein, antinuclear antibody and circulating immune complexes were negative. As for the profile of the complement system shown in Table 1, the C4 and C1q concentration was decreased to 13 mg/dl (17–45 mg/dl at normal value) and less than 2 mg/dl (8.8–15.3 mg/dl), respectively, but the C3 level was normal at 128 mg/dl (86–160 mg/dl). Total hemolytic complement (CH50) was less than 10 U/ml (25–46 U/ml). In addition, the activity of C1 INH was depressed to 50% (80–125%), but the serum concentration of C1 INH measured by immunonephelometry remained at 13 mg/dl (12–23 mg/dl). Thus, the patient was diagnosed as AAE, based on the symptoms, the age at onset, no evidence of family history and the data on complement levels.

AAE is classified into two forms (type I and type II) (2, 6). It has been reported that patients with type I AAE are associated with lymphoproliferative disorders and occasionally with autoimmune or neoplastic diseases (2, 7–11) and that patients with type II AAE have autoantibodies to C1 INH (12–17). After examination, we failed to detect any underlying diseases including lymphoproliferative disorders and subsequently the presence of autoantibodies in the serum of the patient was examined by ELISA. Figure 2 shows that the serum included antibodies reactive to purified C1 INH belonging to both IgM and IgG class but not to IgA class. However, there was no detectable level of antibodies to C1 INH from the three controls. Although the neutralizing activity of these antibodies was not checked, this result strongly suggested that this patient had type II AAE.

Table 1. Complement Profile of the Patient and Its Change by Danazol Therapy

	Serum activity or concentration	
	Pre-treatment	Post-treatment
C1 INH activity (80–125%)	50	145
C1 INH amount (12–23 mg/dl)	13	21
C1q (8.8–15.3 mg/dl)	<2.0	12
C2 (1.6–3.6 mg/dl)	2.3	2.8
C3 (86–160 mg/dl)	128	100
C4 (17–45 mg/dl)	13	30
CH50 (25–46 U/ml)	<10	46

After admission, the patient developed swelling of the upper and lower limbs. The complement profile of the patient showed decreased activity of CH50 (less than 10 U/ml) and decreased concentration of C4 (8 mg/dl) but the serum level of C3 remained at a normal level (97 mg/dl). Then, for the prophylaxis the patient began to receive therapy with danazol 200 mg/day and continued to receive danazol for more than 4 months without adverse effects. During that period, there were no episodes of angioedema. The change in the complement profile of the patient is shown in Fig. 3. One week after the treatment, the activity of C1 INH was elevated to the normal level that was accompanied by normalization of the C4 concentration and CH50 activity. However, serum concentrations of C3 remained unchanged irrespective of treatment. Table 1 demonstrates the change in complement levels before and 3 weeks after danazol therapy. Again, the serum concentrations of C1q and C4 and the activity of CH50 recovered to the normal range. The C1 INH functional activity was also elevated

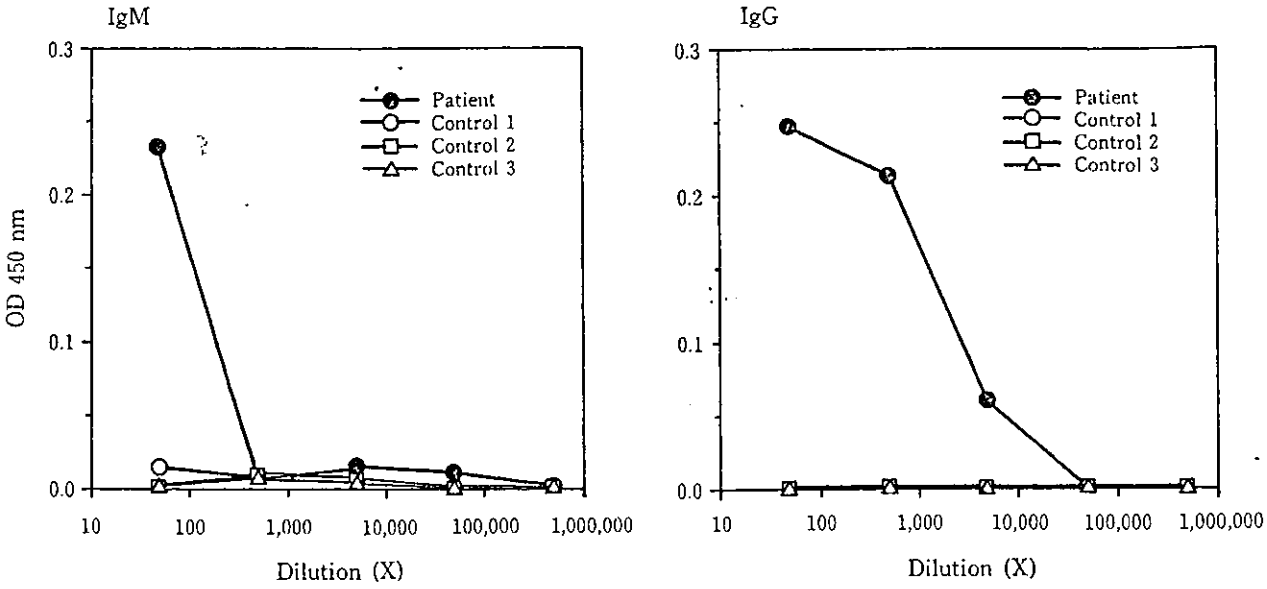

Figure 2. Detection of autoantibodies to C1 INH by ELISA. Serial dilutions of serum from the patient and three controls were added to ELISA microplates that had been coated with C1 INH, as described in Methods.

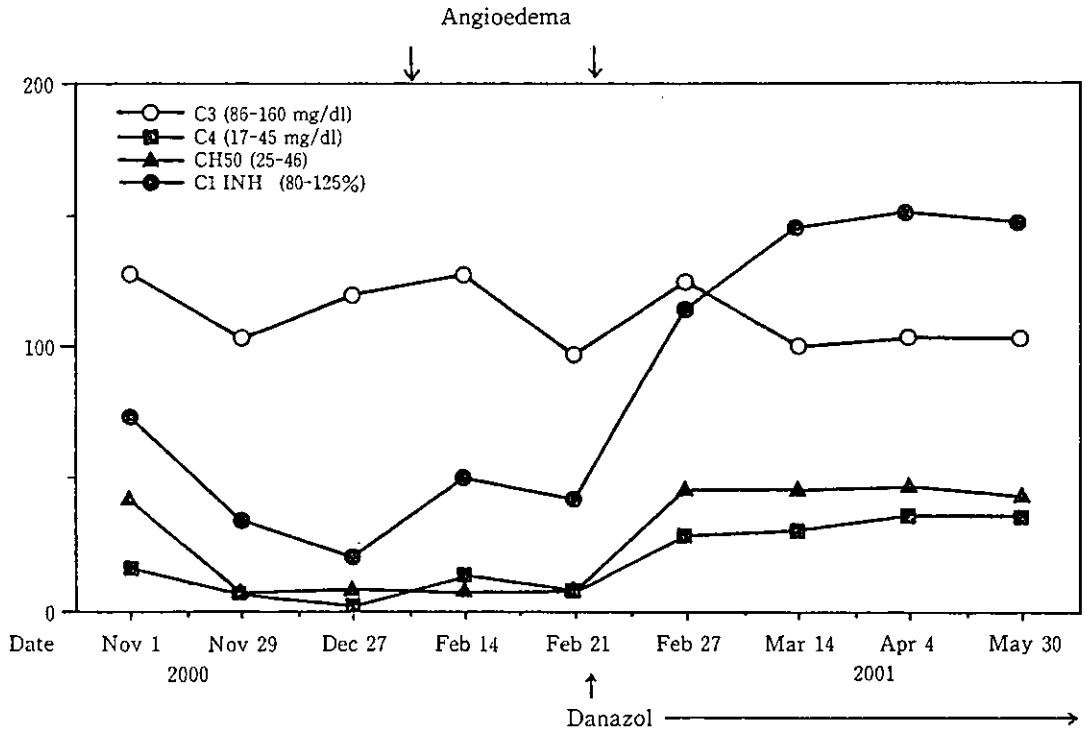

Figure 3. Ameliorative effect of danazol therapy on the complement parameters. Serum concentrations of C3 and C4 and functional activities of CH50 and C1 INH were measured.

from 50 to 145% and its concentration was substantially increased from 13 to 21 mg/dl. Finally, whether the danazol treatment affected the serum titer of autoantibodies to C1 INH was checked by ELISA, as shown in Fig. 4. The titer of IgM or IgG-class antibodies that bound to C1 INH was similar in serum. Thus this ameliorative effect of danazol on the complement levels was not considered to be due to the inhibition of synthesis of autoantibodies but rather due to the increased synthesis of the C1 INH.

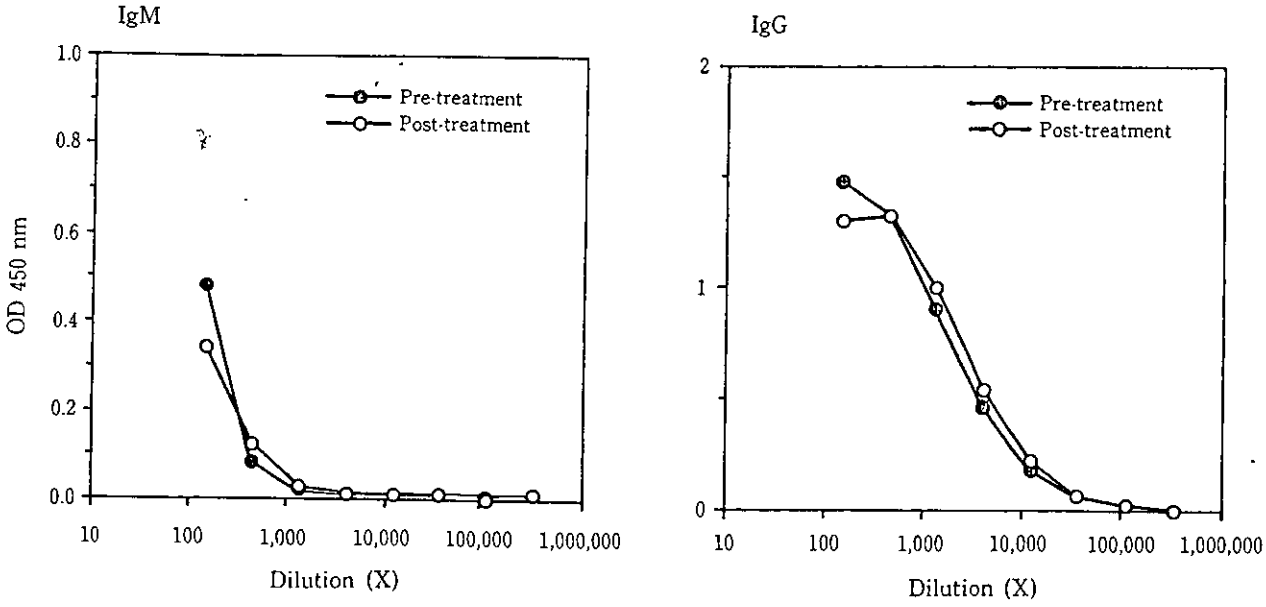

Figure 4. ELISA revealed no alteration in the titer of autoantibodies to C1 INH after danazol treatment.

Discussion

Here, we report one case of the acquired form of angioedema and describe the effectiveness of danazol therapy on the clinical symptoms and on the parameters of the complement. The diagnosis was based on adult onset angioedema, lack of family history, decreased activity of CH50 and C1 INH and depressed concentrations of C1q and C4. Since the protein concentration of C1 INH was not decreased and there were autoantibodies binding to C1 INH, this case was strongly suggested to be autoimmune acquired angioedema, type II AAE. Danazol treatment led to an inhibition of attacks of angioedema for four months and an increase in C1 INH activity, resulting in the normalization of CH50, C4 and C1q. This treatment failed to suppress the titer of autoantibodies of IgM and IgG class to C1 INH but increased the C1 INH concentration, suggesting that the ameliorative effect of danazol was due to its enhancement of the synthesis of C1 INH.

C1 INH is a member of the serpin family of protease inhibitors with a molecular weight of a 105 kd (4, 18). It inactivates C1r and C1s components of the complement system and enzymes of coagulation (factor XIa, XIIa and XIIf), fibrinolytic (plasmin) and kinin systems (kallikrein), thus playing an inhibitory role on the inflammatory response (4). The deficiency of this enzyme was due to the genetic defects or acquired defects. Hereditary forms of angioedema (HAE) are classified into two forms, depending on the C1 INH protein and function levels in serum (2). In type I HAE, both levels are decreased by impaired synthesis and an increased catabolic rate. In type II HAE, the C1 INH activity is defective but the serum concentration of C1 INH remains normal. The molecular basis of these defects was recently characterized (19). As a distinct entity differing from HAE, the acquired form of angioedema (AAE) was described (2). AAE primarily affects adult patients with no family history for the disease. It is generally accepted that measurement of the serum C1q level can distinguish AAE from HAE; a normal level of C1q in HAE and a decreased level of C1q in AAE. Indeed the patient showed a depressed concentration of C1q. AAE is now considered classified into two types; type I and type II (2). In type I AAE mainly associated with lymphoproliferative disorders or other conditions, large numbers of idiotype-anti-idiotype immune complexes consume the available C1q molecules, resulting in consumption of C1 INH (2, 7–11). In type II AAE, there are autoantibodies directed against C1 INH, leading to functional inhibition of this enzyme (12–17). The molecular mechanism through which autoantibodies interfere with C1 INH function was recently characterized (20–22). The autoantibodies mainly bind to C1 INH amino acid residues 438–449 and 448–459 and prevent the formation of the C1s-C1 INH complex. Thus in the latter case, it has been shown that the C1 INH protein level is not decreased. The profile of the patient showed a decreased concentration of C1q, and C4 and a decreased activity of C1 INH and CH50, and a normal protein level of C1 INH, suggesting the patient had type II AAE. The presence of autoantibodies of IgM and IgG class binding to C1 INH confirms the diagnosis, although we have not examined their neutralizing activity. Type II AAE is a rare disorder and the worldwide incidence of type II AAE reported to date is less than 100 cases (12–17, 23–28). To our knowledge, this is the first case of type II AAE in Japan, although two cases of AAE have been reported in the literature (29, 30).

Although the vasopermeability mediator responsible for attacks in C1 INH deficient patients is still undefined, the role of bradykinins and a kinin-like peptide derived from the C2 (C2

kinin) as mediators of angioedema in this disorder has been debated for years. This vasoactive peptide comes from the cleavage of high molecular weight kininogen (HK) by kallikrein during contact system activation. Plasmin, the main component of the fibrinolytic system, can also cleave HK, increasing its susceptibility to kallikrein action. In AAE patients, since the contact and fibrinolytic systems are already activated in basal conditions, such a hyperfibrinolytic state could be the target for the activity of antifibrinolytic agents as tranexamic acid that have been proved to be effective in patients with AAE (28).

According to the recent review (2), the most common treatment for the management of angioedema episodes is androgenic steroids. Androgens are effective in suppressing the frequency and severity of attacks (31). The mechanism of action appears to be related to the increased production of C1 INH. In view of the pathogenesis of type II AAE, immunosuppressive therapy, such as corticosteroids, aimed at decreasing the level of the autoantibody will be of clinical use. But we chose attenuated androgen therapy in consideration of the side effects of corticosteroids and less severe symptoms (lack of laryngeal angioedema) in this case. We confirmed the effectiveness of danazol in this patient. Four months after the initiation of the therapy, there was no episode of angioedema. In addition, the profile of the complement system was normalized. Although it seems likely that the increased production of C1 INH by danazol treatment leads to enhancement of the synthesis of autoantibodies to C1 INH, as the titer of autoantibodies has remained unchanged so far. It will be essential however, to follow-up this patient over a long period of care and to monitor the clinical symptoms, complement levels and the titer of autoantibodies to C1 INH.

References

1) Soter NA. Urticaria and Angioedema. in: Dermatology in General Medicine. 4th ed. Fitzpatrick TB, et al, Eds. McGraw-Hill, New York, 1993.
2) Markovic SN, Inwards DJ, Frigas EA, Phyliky RP. Acquired C1 esterase inhibitor deficiency. Ann Intern Med **132**: 144–150, 2000.
3) Agostoni A, Cicardi M. Hereditary and acquired C1-inhibitor deficiency: biological and clinical characteristics in 235 patients. Medicine (Baltimore) **71**: 206–215, 1992.
4) Davis AE III. C1 inhibitor and hereditary angioneurotic edema. Annu Rev Immunol **6**: 595–628, 1988.
5) Chevailler A, Arlaud G, Ponard D, et al. C-1-inhibitor binding monoclonal immunoglobulins in three patients with acquired angioneurotic edema. J Allergy Clin Immunol **97**: 998–1008, 1996.
6) Frank M. Acquired C1-inhibitor deficiency. Behring Inst Mitt **84**: 161–164, 1989.
7) Costanzi JJ, Coltman CA Jr, Donaldson VH. Activation of complement by a monoclonal cryoglobulin associated with cold urticaria. J Lab Clin Med **74**: 902–910, 1969.
8) Gordon EH, Beall GN, Klaustermeyer WB. Angioedema and multiple myeloma. I. Ann Allergy **51**: 349–350, 1983.
9) Casali P, Borzini P, Pioltelli P, Invernizzi F, Zanussi C. Acquired C1-inhibitor deficiency in essential cryoglobulinemia and macrocryoglobulinemia. Acta Haematol **59**: 277–284, 1978.
10) Wasserfallen JB, Spaeth P, Guillou L, Pecoud AR. Acquired deficiency in C1-inhibitor associated with signet ring cell gastric adenocarcinoma: a probable connection of antitumor-associated antibodies, hemolytic anemia, and complement turnover. J Allergy Clin Immunol **95**: 124–131, 1995.
11) Caldwell JR, Ruddy S, Schur PH, Austen KF. Acquired C1 inhibitor deficiency in lymphosarcoma. Clin Immunol Immunopathol **1**: 39–52, 1972.
12) Jackson J, Sim RB, Whelan A, Feighery C. An IgG autoantibody which inactivates C1-inhibitor. Nature **323**: 722–724, 1986.
13) Alsenz J, Bork K, Loos M. Autoantibody-mediated acquired deficiency of C1 inhibitor. N Engl J Med **316**: 1360–1366, 1987.
14) Frank MM, Malbran A, Simms H, et al. Acquired angioedema type II (AAE-II): a new auto-immune disease. Clin Res **35**: 641A, 1987 (Abstract).
15) Alsenz J, Loos M. The acquired C1-INH deficiencies with autoantibodies (AAE type II). Behring Inst Mitt **84**: 165–172, 1989.
16) Spath PJ, Wuthrich B, Matter L, Loos M, Alsenz J. Acquired angioedema and anti-C1-inhibitor autoantibody. Arch Intern Med **149**: 1213–1216, 1989 (letter).
17) Malbran A, Hammer CH, Frank MM, Fries LF. Acquired angioedema: observations on the mechanism of action of autoantibodies directed against C1 esterase inhibitor. J Allergy Clin Immunol **81**: 1199–1204, 1988.
18) Harrison RA. Human C1 inhibitor; improved isolation and preliminary structural characterization. Biochemistry **22**: 5001–5007, 1983.
19) Tosi M. Molecular genetics of C1 inhibitor. Immunobiology **199**: 358–365, 1998.
20) Jackson J, Sim RB, Whaley K, Feighery C. Autoantibody facilitated cleavage of C1-inhibitor in autoimmune angioedema. J Clin Invest **83**: 698–707, 1989.
21) Mandle R, Baron C, Roux E, et al. Acquired C1 inhibitor deficiency as a result of an autoantibody to the reactive center region of C1 inhibitor. J Immunol **152**: 4680–4685, 1994.
22) He S, Sim RB, Whaley K. Mechanism of action of anti-C1-inhibitor autoantibodies: prevention of the formation of stable C1s-C1-inh complexes. Mol Med **4**: 119–128, 1998.
23) Whaley K, Sim RB, He S. Autoimmune C1-inhibitor deficiency. Clin Exp Immunol **106**: 423–426, 1996 (Editorial).
24) Cicardi M, Bergamaschini L, Cugno M, et al. Pathogenetic and clinical aspects of C1 inhibitor deficiency. Immunobiology **199**: 366–376, 1998.
25) Kleiner GI, Giclas P, Stadtmauer G, Cunningham-Rundles C. Unmasking of acquired autoimmune C1-inhibitor deficiency by an angiotensin-converting enzyme inhibitor. Ann Allergy Asthma Immunol **86**: 461–464, 2001.
26) Bouillet L, Ponard D, Drouet C, et al. Acquired angioneurotic edema. Clinical and biological characteristics in 9 patients. Presse Med **29**: 640–644, 2000.
27) Alsenz J, Lambris JD, Bork K, Loos M. Acquired C1 inhibitor (C1-INH) deficiency type II. Replacement therapy with C1-INH and analysis of patients' C1-INH and anti-C1-INH autoantibodies. J Clin Invest **83**: 1794–1799, 1989.
28) Cicardi M, Bisiani G, Cugno M, Spath P, Agostoni A. Autoimmune C1 inhibitor deficiency: report of eight patients. Am J Med **95**: 169–175, 1993.
29) Nakamura S, Yoshinari M, Saku Y, et al. Acquired C1 inhibitor deficiency associated with systemic lupus erythematosus affecting the central nervous system. Ann Rheum Dis **50**: 713–716, 1991.
30) Seki M, Kojima H, Tochihara T, Ohi H. Acute occlusion of blood access in the patients with C1-inhibitor deficiency undergoing hemodialysis therapy. Touseki-kaishi **31**: 125–130, 1998 (in Japanese).
31) Gelfand JA, Sherins RJ, Alling DW, Frank MM. Treatment of hereditary angioedema with danazol. Reversal of clinical and biochemical abnormalities. N Engl J Med **295**: 1444–1448, 1976.

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A Mixed Green Vegetable and Fruit Beverage Decreased the Serum Level of Low-Density Lipoprotein Cholesterol in Hypercholesterolemic Patients

HIROHISA SUIDO,*,† TOSHIO TANAKA,‡ TOSHIO TABEI,† AKIRA TAKEUCHI,† MISAKO OKITA,§ TADAMITSU KISHIMOTO,‡ SOJI KASAYAMA,‡ AND KAZUYA HIGASHINO#

Research and Development Center, Sunstar Inc., 3-1 Asahi-machi, Takatsuki, Osaka 569-1195, Japan; Department of Molecular Medicine, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan; Department of Nutritional Science, Faculty of Health and Welfare Science, Okayama Prefectural University, 111 Soja, Okayama 719-1197, Japan; and Department of Internal Medicine III, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8131, Japan

The effects of a canned mixed green vegetable and fruit beverage, containing broccoli (*Brassica oleracea* L. var. *botrytis* L.) and cabbage (*Brassica oleracea* L. var. *capitata* L.) as main materials, on serum lipid levels in hypercholesterolemic patients were investigated. Thirty-one adult subjects were administered two cans of the beverage (160 g/can) per day for 3 weeks. Their serum total cholesterol (TC) levels significantly decreased from 6.7 ± 0.8 to 6.1 ± 0.6 mmol/L, and, more strikingly, the level of low-density lipoprotein cholesterol (LDL-C) significantly decreased from 4.4 ± 0.8 to 3.8 ± 0.7 mmol/L. At 9 weeks after the cessation of the administration, these levels had returned to the preadministration levels. Furthermore, 14 other subjects who were administered one can of the sample for 12 weeks also showed a significant reduction in the levels of serum TC and LDL-C. Thus, daily consumption of this mixed green vegetable and fruit beverage may be useful in lowering serum TC and LDL-C levels in hypercholesterolemic patients.

KEYWORDS: Mixed green vegetable and fruit beverage; broccoli (*Brassica oleracea* L. var. *botrytis* L.); cabbage (*Brassica oleracea* L. var. *capitata* L.); hypercholesterolemia; cholesterol-lowering effect; humans

INTRODUCTION

Although the onset and aggravation of hyperlipidemia are related to genetic factors, environmental factors, especially eating habits, have a strong influence on the disease. Hypercholesterolemia is considered to lead to atherosclerosis; the normalization of serum levels of low-density lipoprotein cholesterol (LDL-C) through diet therapy or administration of drugs decreases the incidence of coronary heart disease (CHD) (*1*, *2*). The clinical approach to primary prevention of CHD calls for lifestyle changes, including (1) reduced intakes of saturated fat and cholesterol, (2) increased physical activity, and (3) weight control (*2*).

The effects of food intake on the body's physiological functions are attracting considerable attention. Fruits and vegetables contain constituents, notably vitamins, minerals, and dietary fiber, essential to a healthy, well-balanced diet. Furthermore, it has been shown that some of the secondary metabolites of fruits and vegetables, such as flavonoids and carotenoids, are beneficial to health in directly combating the onset of cancer and CHD (*3*, *4*). Thus, a diet rich in vegetables and fruits may provide protection against cardiovascular disease, several common cancers, and other chronic diseases (*5*).

The hypocholesterolemic effects of vegetables and fruit and their constituents have been examined in some detail. Isolated dietary fibers from vegetable and fruit sources, especially pectins, have been shown to have hypocholesterolemic action in humans (*6*, *7*). The addition of fiber-containing foods to experimental diets also lowers plasma cholesterol to varying degrees (*8*−*10*).

In our previous studies (submitted) we have found that broccoli and its water extract significantly suppressed the increase of serum cholesterol levels in cholesterol-fed rats, and the hypocholesterolemic activity of broccoli was comparable to that of isolated soy protein (*11*). Also, we have been developing easier methods for serving daily for a long period a fixed amount of certain types of green vegetables and fruits, which may have beneficial health effects on human bodies. By examining the influence of the edible plant beverage on 20 healthy human volunteers in our preliminary study, we found that oral administration daily for 3 weeks of two cans (160

* Author to whom correspondence should be addressed (telephone +81-726-82-5542; fax +81-726-81-8202; e-mail suidou@po.iijnet.or.jp).
† Sunstar Inc.
‡ Osaka University Medical School.
§ Okayama Prefectural University.
# Hyogo College of Medicine.

10.1021/jf0116698 CCC: $22.00
Published on Web 04/27/2002

Table 1. Energy and Known Nutrient Contents in One Can of Test Beverage[a]

energy and nutrient	amount	energy and nutrient	amount
energy, kJ	222	Mg, mg	13.9
protein, g	1.0	Zn, mg	0.395
fat, g	0.32	Cu, mg	0.073
nonfibrous carbohydrate, g	13	vitamin A efficacy, IU	80
		β-carotene, mg	0.133
dietary fiber, g	1.3	vitamin B_1, mg	0.03
cholesterol, mg	0	vitamin B_2, mg	0.05
Na, mg	35	niacin, mg	0.26
K, mg	326	vitamin C, mg	73
Ca, mg	32.2	vitamin D, mg	0
P, mg	24.7	vitamin E efficacy, mg	0.48
Fe, mg	0.24	SMCS,[b] mg	20

[a] One can of the test beverage contained 160 g. [b] SMCS, *S*-methylcysteine sulfoxide.

g/can) of a mixed green vegetable and fruit beverage, containing broccoli (*Brassica oleracea* L. var. *botrytis* L.) and cabbage (*Brassica oleracea* L. var. *capitata* L.) as main materials, significantly decreased the serum level of LDL-C in these subjects from 2.9 ± 0.7 to 2.5 ± 0.7 mmol/L (14% decrease) (*12*). However, there were only three volunteers whose serum levels of total cholesterol (TC) were >6.2 mmol/L included in the study. Thus, in the present study, the effects of the mixed green vegetable and fruit beverage on serum lipid levels in hypercholesterolemic patients whose TC levels were >6.2 mmol/L were investigated.

MATERIALS AND METHODS

Test Beverage. The beverage used in the test is a canned mixed green vegetable and fruit beverage containing a blend of green vegetables and fruits, which are broccoli, cabbage, celery (*Apium graveolens* L.), lettuce (*Lacutuca sativa* L.), spinach (*Spinacia oleracea* L.), parsley (*Petroselinum crispum*), komatsuna (*Brassica campestris* L. var. *komatsuna*), leaves of Japanese radish (*Raphanus sativus* L. var. *acanthiformis* Makino), apples (*Malus pumila* Miller), and lemons (*Citrus limon* Burmann fil.). The beverage was prepared by Sunstar Inc. (Osaka, Japan), briefly described as follows. Broccoli and cabbage, other vegetables, and fruit (juice or puree) were mixed together in the ratio 36:19:45 and poured into cans. The cans were sealed, heated to 97 °C, and immediately cooled to room temperature. **Table 1** shows the nutrients contained in one can (160 g contents). The nutrient contents were analyzed according to *Analytical Manual of Standard Tables of Food Composition in Japan* (*13*). *S*-Methylcysteine sulfoxide (SMCS) was determined by means of HPLC methods (*14*).

Subjects. Forty-five subjects aged 22−59 years with hypercholesterolemia (>6.2 mmol/L of serum TC) were studied after giving their informed consent. The participants had no episodes of ischemic heart disease and did not receive any drug treatment. The patients were randomly divided into two separate study groups.

Study Design. In the first study (study A), 31 patients (20 males and 11 females, average age = 43.3 ± 10.9 years) were administered two cans of the test beverage per day in addition to their regular meals for a period of 3 weeks. The patients were asked to try not to change their lifestyles including eating and exercise habits during the testing period. A fasting blood sample was taken from each patient 12 weeks prior to and just before administration as baselines, and samples were taken again 3 weeks after the start of administration and 9 weeks after the cessation of administration.

In the second study (study B), 14 patients (8 males and 6 females, average age = 45.9 ± 11.1 years) were administered one can of the beverage per day for 12 weeks. A fasting blood sample was taken from each subject just before administration as baseline and a sample was obtained on the last day of the 12-week test period.

These two studies were approved by the institutional ethical committee according to the Helsinki Declaration.

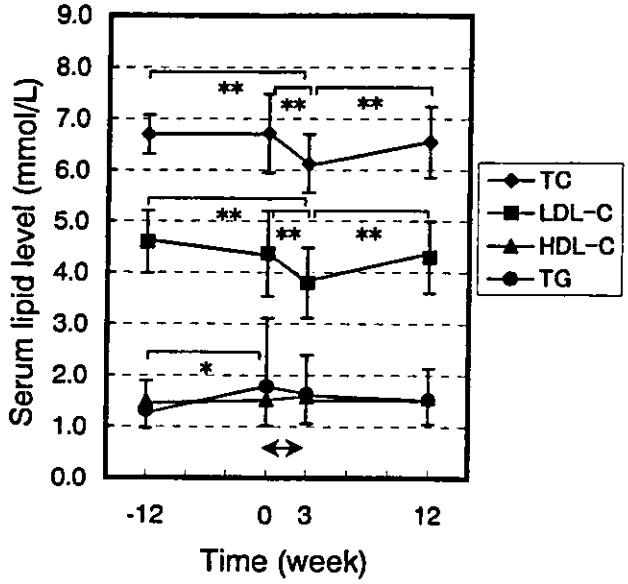

Figure 1. Serum lipid levels in patients who were administered two cans per day of the mixed green vegetable and fruit beverage for 3 weeks (study A): TC, total cholesterol; LDL-C, LDL cholesterol; HLD-C, HD cholesterol; TG, triglyceride. The serum lipid levels were measured at 12 weeks and just before administration, after 3 weeks of administration, and 9 weeks after the cessation of administration. Values shown are means ± SD, $n = 31$. An arrow (↔) shows the administration period. *, **: Significantly different by ANOVA and post-hoc comparison (Scheffe method) with $p < 0.05$ and $p < 0.01$, respectively.

Examination Categories and Method Used. In study A, a questionnaire was filled out by all study patients regarding food intake (*15*), exercise, sleeping patterns, and tobacco and alcohol consumption 1 week before administration and at the last week of the administration period. A physician conducted a medical examination on each patient at the time of the blood sampling, measuring weight and blood pressure. Clinical testing on the blood samples, including TC, high-density lipoprotein cholesterol (HDL-C), and triglyceride (TG), was carried out at Ikagaku Co. (Kyoto, Japan). The serum levels of TC, HDL-C, and TG were enzymatically assayed with commercially available kits, L-type Wako cholesterol (Wako Pure Chemical Industries Ltd., Osaka, Japan) (*16*), Determiner-L HDL-C (Kyowa Medex Co. Ltd., Tokyo, Japan) (*17*), and Auto L (Mizuho) TG-FR (Mizuho Medy Co. Ltd., Saga, Japan) (*18*), respectively. The serum LDL-C levels were calculated using the Friedewald formula (LDL-C = TC − HDL-C − TG/5) (*19*).

Statistical Analyses. Values are expressed as the means ± standard deviation (SD). A paired *t* test or an analysis of variance (ANOVA) and post-hoc comparison were conducted for the data, as appropriate. Statistical significance was considered at $p < 0.05$.

RESULTS

Figure 1 shows the change in serum lipid levels in study A. TC values significantly changed from 6.7 ± 0.8 mmol/L at baseline (0 week) to 6.1 ± 0.6 mmol/L (9% decrease) after 3 weeks of the administration of two cans of the test beverage per day, while LDL-C values significantly changed from 4.4 ± 0.8 to 3.8 ± 0.7 mmol/L (13% decrease). At 9 weeks after the cessation of the administration, TC levels increased to 6.5 ± 0.7 mmol/L and LDL-C levels also returned to 4.3 ± 0.7 mmol/L, which were not significantly different from the preadministration levels. No significant change was observed in the levels of HDL-C (from 1.5 ± 0.5 to 1.6 ± 0.5 mmol/L) and TG (from 1.8 ± 1.3 to 1.6 ± 0.8 mmol/L) during the course of the study.

Table 2. Body Weights, Body Mass Index, and Nutrient Intakes in Study A Patients before and during Administration Periods[a,b]

	before	during
body weight, kg	58.5 ± 11.2	58.5 ± 11.0
BMI[c]	22.1 ± 3.1	22.1 ± 3.0
energy, MJ	8.4 ± 3.1	7.7 ± 2.1
protein, g	69.9 ± 21.5	66.0 ± 18.9
fat, g	46.3 ± 14.0	41.8 ± 11.3
cholesterol, mg	231 ± 86	225 ± 74
nonfibrous carbohydrate, g	300 ± 148	273 ± 83
dietary fiber, g	9.2 ± 3.2	11.2 ± 3.3
potassium, g	1.7 ± 0.6	2.3 ± 0.6

[a] Values are means ± SD, $n = 31$. [b] Energy and nutrient intakes per day including the test beverage were calculated from 7-day food intake records at 1 week before administration (before) and at the last week of the administration period (during). [c] BMI, body mass index.

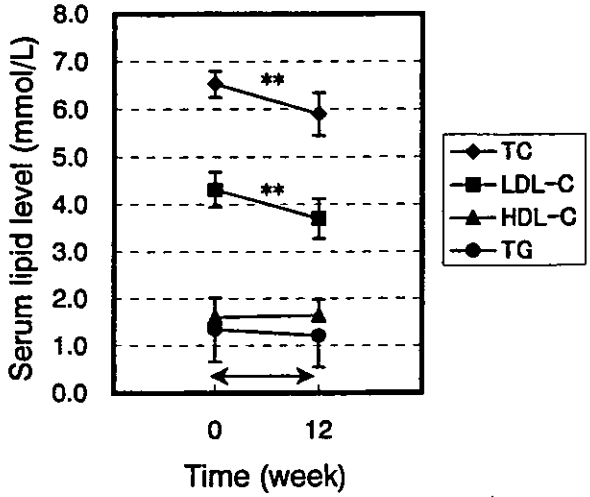

Figure 2. Serum lipid levels in patients who were administered one can per day of the mixed green vegetable and fruit beverage for 12 weeks (study B): TC, total cholesterol; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol; TG, triglyceride. The serum lipid levels were measured just before administration and after 12 weeks of administration. Values shown are means ± SD, $n = 14$. An arrow (↔) shows the administration period. **: Significantly different by paired t test with $p < 0.01$.

Body weight, body mass index (BMI), and daily nutrient intakes including the test beverage before and during administration periods, determined using a questionnaire in study A, are shown in **Table 2**. There were no significant differences in body weight or BMI before versus during the administration periods. Examination of nutrient intakes showed significant increases in intakes of dietary fibers (~2 g/day) and potassium (~0.6 g/day) during the administration period. There were no significant differences in other nutrients.

Figure 2 shows the serum lipid levels before and after administration of one can of the test beverage per day for 12 weeks in study B. TC values changed significantly from 6.5 ± 0.3 to 5.9 ± 0.4 mmol/L after the administration (10% decrease), while LDL-C values changed significantly from 4.3 ± 0.4 to 3.7 ± 0.4 mmol/L (14% decrease). No significant change was observed for HDL-C (from 1.6 ± 0.4 to 1.7 ± 0.3 mmol/L) or TG (from 1.3 ± 0.7 to 1.2 ± 0.7 mmol/L).

In addition to serum lipids, blood laboratory tests before and after the study revealed no significant changes in the number of peripheral blood cells and biochemical values, as well as blood pressure, with all measurements being within normal ranges. No subjective symptoms or abnormal physical observations were witnessed at the conclusion of the study.

With regard to compliance measured by administration records, 71% of the subjects in studies A and B completely drank all of the assigned samples, and the rest of them forgot to drink at most 3 cans of the samples during the administration period. Ninety-seven percent of the assigned samples were drunk on the average. No patients dropped out during the test periods.

DISCUSSION

Vegetables, fruits, and their constituents are potent effectors of biological systems in humans (*5*). The cholesterol-lowering effects of vegetables, fruits, and their constituents have been examined in detail. Daily doses of these food agents that showed cholesterol-lowering effects in humans have been reported as follows: a variety of vegetables (570 g/day) and fresh apples (600 g/day), fresh carrots (200 g/day), apples (350−400 g/day), guava fruit (0.5−1 kg/day), garlic (10−20 g/day), and a low saturated fat diet combined with a vegetable (soy) protein (33 g/day) and a high soluble fiber (18 g/day) (*5*, *20*).

The U.S. Food and Drug Administration recommends including four servings of at least 6.25 g each (25 g/day) of soy protein into a diet low in saturated fat and cholesterol to reduce the risk of heart disease. A meta-analysis found that soy protein consumption achieved an average 9.3% decrease in TC and a 12.9% decrease in LDL-C (*21*).

In study A, we have concluded through a test on subjects with hypercholesterolemia that serum TC and LDL-C levels were significantly reduced (9 and 13%, respectively) by drinking two cans (320 g) of the mixed green vegetable and fruit beverage every day for 3 weeks. These lipid levels returned to baseline within 9 weeks after cessation of the administration of the test beverage. In study B, we have also shown that serum TC and LDL-C levels were significantly reduced (10 and 14%, respectively) by drinking 1 can (160 g) of the test beverage every day for 12 weeks. The reduction rates in TC and LDL-C levels by drinking the test beverage are almost equal to those by soy protein consumption described above.

The cholesterol responses to the two doses of the supplemental beverage tested in this study were similar. The similar clinical effects in these separate studies imply that the mechanism contributing to the hypocholesterolemic effect is saturable or the length of the intervention is contributing in part to the response.

On the other hand, there was no significant change in serum TG or HDL-C levels in either study. Thus, the serum lipid lowering effect of the test sample on TC level was due to that on the LDL-C level, leading to a decrease in the atherogenic index (TC/HDL-C ratio) (*22*−*24*).

It is not clear in this study which constituents in the mixed green vegetable and fruit beverage showed hypocholesterolemic action. Generally, the effects of vegetables and fruits on cholesterol metabolism are attributed to their fiber content. Isolated dietary fibers from these plants, such as pectins (12−50 g/day) (*25*−*27*), guar gum (10−20 g/day) (*6*, *28*, *29*), and psyllium (6−15 g/day) (*30*, *31*), have been shown to lower cholesterol levels in humans. The mechanisms for the cholesterol-lowering in humans remain unclear. Results from in vitro and animal studies that used isolated fibers suggested that the reductions in cholesterol are probably due to different mechanisms specific to each fiber source and to different dietary fiber intake amounts (*32*−*34*). Possible mechanisms include (1) increased fecal excretion of bile acids and neutral steroids, (2) altered ratios of primary to secondary bile acids, (3) increased fecal excretion of cholesterol and fatty acids, and (4) indirect effects, such as high-fiber foods replacing fat- and cholesterol-

containing foods in a diet (*35*). However, the amount of dietary fiber (1.3−2.6 g/day) provided by the consumption of one or two cans of the test beverage is far out of proportion with the doses (6−50 g/day) of isolated dietary fibers described above to obtain a significant reduction in serum cholesterol levels in humans.

As other potent constituents in the test beverage, cabbage leaf proteins (*36*) and *S*-methylcysteine sulfoxide (SMCS) (*37, 38*) have been shown to have cholesterol-lowering effects on animals. The total protein content in the beverage is 1 g/160 g. This protein amount seems to be far less than the daily dose of soy protein recommended for obtaining a significant reduction in serum cholesterol levels in humans. On the other hand, SMCS, a naturally occurring S-containing amino acid, is contained at high concentrations in *Brassica* vegetables such as broccoli and cabbage and *Allium* vegetables such as onion (*39, 40*). The mechanism of SMCS for cholesterol-lowering effects has been hypothesized to enhance fecal bile acid excretion and cholesterol 7α-hydroxylase activity, the rate-limiting enzyme of bile acid biosynthesis, in the microsomal fraction of the liver (*37, 38*). Indeed, the test beverage contained broccoli and cabbage as main materials, and the amount of SMCS derived from these *Brassica* vegetables in the beverage was at least 20 mg/160 g of juice.

Therefore, the dietary fibers and other potent constituents including SMCS in the beverage might have shown synergistic effects to lower cholesterol levels. Research on the contribution of these potent constituents is now being conducted.

In conclusion, we have shown for the first time that the canned mixed green vegetable and fruit beverage tested significantly lowered serum TC and LDL-C levels. This kind of sample made from natural products may become an alternative way to treat patients with hypercholesterolemia.

ABBREVIATIONS USED

LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride; BMI, body mass index; SMCS, *S*-methylcysteine sulfoxide.

LITERATURE CITED

(1) Keys, A. Coronary heart disease, serum cholesterol, and the diet. *Acta Med. Scand.* **1980**, *207*, 153−160.

(2) National Cholesterol Education Program, National Institutes of Health. *Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adult (Adult Treatment Panel III), Executive Summary*; NIH Publication: Bethesda, MD, 2001; No. 01-3670.

(3) Hertog, M. G. L.; Poppel, G. V.; Verhoeven, D. Potentially anticarcinogenic secondary metabolites from fruit and vegetables. In *Phytochemistry of Fruit and Vegetables*; Tomas-Barberan, F. A., Robins, R. J., Eds.; Oxford Science Publications: Oxford, U.K., 1997; pp 313−329.

(4) Leake, D. S. The possible role of antioxidants in fruit and vegetables in protecting against coronary heart disease. In *Phytochemistry of Fruit and Vegetables*; Tomas-Barberan, F. A., Robins, R. J., Eds.; Oxford Science Publications: Oxford, U.K., 1997; pp 287−311.

(5) Lampe, J. W. Health effects of vegetables and fruit: assessing mechanisms of action in human experimental studies. *Am. J. Clin. Nutr.* **1999**, *70* (Suppl.), 475S−490S.

(6) Jenkins, D. J. A.; Reynolds, D.; Leeds, A. R.; Waller, A. L.; Cummings, J. H. Hypocholesterolemic action of dietary fiber unrelated to fecal bulking effect. *Am. J. Clin. Nutr.* **1979**, *32*, 2430−2435.

(7) Jenkins, D. J. A.; Popovich, D. G.; Kendall, C. W. C.; Vidgen, E.; Tariq, N.; Ransom, T. P. P.; Wolever, T. M. S.; Vuksan, V.; Mehling, C. C.; Boctor, D. L.; Bolognesi, C.; Huang, J.; Patten, R. Effect of a diet high in vegetables, fruit and nuts on serum lipids. *Metabolism* **1997**, *46*, 530−537.

(8) Stasse-Wolthuis, M.; Albers, H. F. F.; van Jeveren, J. G. C.; Wil de Jong, J.; Hautvast, J. G. A. J.; Hermus, R. J. J.; Katan, M. B. K.; Brydon, W. G.; Eastwood, M. A. Influence of dietary fiber from vegetables and fruits, bran or citrus pectin on serum lipids, fecal lipids, and colonic function. *Am. J. Clin. Nutr.* **1980**, *33*, 1745−1756.

(9) Robertson, J.; Brydon, W. G.; Tadesse, K.; Wenham, P.; Wall, A.; Eastwood, M. A. The effect of raw carrot on serum lipids and colon function. *Am. J. Clin. Nutr.* **1979**, *32*, 1889−1892.

(10) Sablé-Amplis, R.; Sicart, R.; Agid, R. Further studies on the cholesterol-lowering effect of apple in humans. Biochemical mechanisms involved. *Nutr. Res.* **1983**, *3*, 325−328.

(11) Nagata, Y.; Tanaka, K.; Sugano, M. Further studies on the hypocholesterolemic effect of soya-bean protein in rats. *Br. J. Nutr.* **1981**, *45*, 233−241.

(12) Tabei, T.; Tanaka, T.; Suido, H.; Fukuhara, K.; Kotani, M.; Takeuchi, A.; Okita, M.; Kasayama, S. The effects of a mixed green vegetable and fruit beverage on serum lipid levels and the composition of red blood cell membrane phospholipids in healthy subjects. *Jpn. Med. J.* **2000**, *3985*, 46−49 (in Japanese).

(13) Science and Technology Agency, Japan. *Analytical Manual of the 5th Standard Tables of Food Composition in Japan*; Printing Bureau, Ministry of Finance: Tokyo, Japan, 1997 (in Japanese).

(14) Marks, H. S.; Hilson, J. A.; Leichtweis, H. C.; Stoewsand, G. S. *S*-Methylcysteine sulfoxide in *Brassica* vegetables and formation of methyl methanethiosulfinate from Brussels sprouts. *J. Agric. Food Chem.* **1992**, *40*, 2098−2101.

(15) Suzuki, K.; Sasagawa, T.; Okita, M.; Fujiwara, Y.; Mori, K.; Saito, M.; Abe, Y. Development of new simplified-food frequency method for use in the surveillance on life style in order to promote healthy condition in Okayama prefecture, computed by Microsoft Excel. *Bull. Fac. Health Welfare Sci., Okayama Pref. Univ.* (in Japanese); **1996**, *3*, 143−153.

(16) Allain, C. C.; Poon, L. S.; Chan, C. S.; Richmond, W.; Fu, P. C. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **1974**, *20*, 470−475.

(17) Sugiuchi, H.; Uji, Y.; Okabe, H.; Irie, T.; Uekama, K.; Kayahara, N.; Miyauchi, K. Direct measurement of high-density lipoprotein cholesterol in serum with polyethylene glycol-modified enzymes and sulfated alpha-cyclodextrin. *Clin. Chem.* **1995**, *41*, 717−723.

(18) Abe, A.; Iida, H.; Kawade, M. Automated enzyme assay of triglyceride with none effective free glycerol by use of mono channel analyzer. *Japanese Journal of Clinical Laboratory Automation* (in Japanese); **1981**, *6*, 192−195.

(19) Friedewald, W. T.; Levy, R. I.; Frederickson, D. S. Estimation of the concentration of low-density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. *Clin. Chem.* **1972**, *18*, 499−502.

(20) Jenkins, D. J. A.; Kendall, C. W. C.; Mehling, C. C.; Parker, T.; Rao, A. V.; Agarwal, S.; Novokmet, R.; Jones, P. J. H.; Raeini, M.; Story, J. A.; Furumoto, E.; Vidgen, E.; Griffin, L. C.; Cunnane, S. C.; Ryan, M. A.; Connelly, P. W. Combined effect of vegetable protein (soy) and soluble fiber added to a standard cholesterol-lowering diet. *Metabolism* **1999**, *48*, 809−816.

(21) Dunn, A. V. Incorporating soy protein into a low-fat, low-cholesterol diet. *Cleveland Clin. J. Med.* **2000**, *67*, 767−771.

(22) Kannel, W. B.; Castelli, W. P.; Gordon, T.; McNamara, P. M. Serum cholesterol, lipoproteins, and the risk of coronary heart disease. The Framingham study. *Ann. Intern. Med.* **1971**, *74*, 1−12.

(23) Martin, M. J.; Hulley, S. B.; Browner, W. S.; Kuller, L. H.; Wentworth, D. Serum cholesterol, blood pressure, and mortality: implications from a cohort of 361,662 men. *Lancet* **1986**, *2* (8513), 933−936.

(24) NIH Consensus Conference. Triglyceride, high-density lipoprotein, and coronary heart disease. *JAMA—J. Am. Med. Assoc.* **1993**, *263*, 505−510.

(25) Durrington, P. N.; Manning, A. P.; Bolton, C. H.; Hartog, M. Effect of pectin on serum lipids and lipoproteins, whole gut transit time and stool weight. *Lancet* **1976**, *2* (7982), 394−396.

(26) Kay, R. M.; Truswell, A. S. Effect of citrus pectin on blood lipids and fecal steroid excretion in man. *Am. J. Clin. Nutr.* **1977**, *30*, 171−175.

(27) Miettinen, T. A.; Tarpila, S. Effect of pectin on serum cholesterol, faecal bile acids and biliary lipids in normolipidemic and hyperlipidemic individuals. *Clin. Chim. Acta* **1977**, *79*, 471−477.

(28) Haskell, W. L.; Spiller, G. A.; Jensen, C. D.; Ellis, B. K.; Gates, J. E. Role of water-soluble dietary fiber in the management of elevated plasma cholesterol in healthy subjects. *Am. J. Cardiol.* **1992**, *69*, 433−439.

(29) Kawatra, A.; Kapoor, A. C.; Seghal, S. Hypocholesterolemic effect of guar gum in overweight adults. *Plant Foods Hum. Nutr.* **1991**, *41*, 241−245.

(30) Dennison, B. A.; Levine, D. M. Randomized, double-blind, placebo-controlled, two-period crossover clinical trial of psyllium fiber in children with hypercholesterolemia. *J. Pediatr.* **1993**, *123*, 24−29.

(31) Everson, G. T.; Daggy, B. P.; McKinley, C.; Story, J. A. Effects of psyllium hydrophilic mucilloid on LDL-cholesterol and bile acid synthesis in hypercholesterolemic men. *J. Lipid Res.* **1992**, *33*, 1183−1192.

(32) Fernandez, M. L. Distinct mechanisms of plasma LDL lowering by dietary fiber in the guinea pig: specific effects of pectin, guar gum, and psyllium. *J. Lipid Res.* **1995**, *36*, 2394−2404.

(33) Story, J. A.; Kritchevsky, D. Comparison of binding of various bile acids and bile salts in vitro by several types of fiber. *J. Nutr.* **1976**, *106*, 1292−1294.

(34) Tsuji, K. Healthy effects of dietary fiber. *Bifidus* **1995**, *8*, 125−134.

(35) Glore, S. R.; Van Treeck, D.; Knehans, A. W.; Guild, M. Soluble fiber and serum lipids: a literature review. *J. Am. Diet. Assoc.* **1994**, *94*, 425−436.

(36) Igarashi, K.; Satoh, A.; Numazawa, S.; Takahashi, E. Effects of cabbage leaf protein concentrate on the serum and liver lipid concentrations in rats. *J. Nutr. Sci. Vitaminol.* **1997**, *43*, 261−270.

(37) Fujiwara, M.; Itokawa, Y.; Uchino, H.; Inoue, K. Anti-hypercholesterolemic effects of a sulfur containing amino acid, *S*-methyl-L-cysteine sulfoxide, isolated from cabbage. *Experientia* **1972**, *28*, 254−255.

(38) Komatsu, W.; Miura, Y.; Yagasaki, K. Suppression of hypercholesterolemia in Hepatoma-bearing rats by cabbage extract and its component, *S*-methyl-L-cysteine sulfoxide. *Lipids* **1998**, *33*, 499−503.

(39) Morris, C. J.; Thompson, J. F. The identification of (+)*S*-methyl-L-cysteine sulfoxide in plants. *J. Am. Chem. Soc.* **1956**, *78*, 1605−1608.

(40) Tsuno, S.; Murakami, F.; Tazoe, K.; Kikumoto, S. Studies on the nutritional value of allium plants. XXX. Isolation of methiin. *Vitamins (Kyoto)* **1960**, *20*, 93−96.

Received for review December 17, 2001. Revised manuscript received March 8, 2002. Accepted March 8, 2002.

JF0116698

Efficacy of Glimepiride for the Treatment of Diabetes Occurring During Glucocorticoid Therapy

Approximately 5–25% of patients receiving glucocorticoids exhibit overt diabetes (1). Glucocorticoids may precipitate diabetes in individuals with impaired insulin secretion by reducing insulin sensitivity (2). The relative importance of β-cell dysfunction and insulin resistance for the pathophysiology of the glucocorticoid-induced diabetes is not well defined. Therefore, there is no consensus treatment for glucocorticoid-induced diabetes. Glimepiride is a sulfonylurea that lowers blood glucose levels by stimulating insulin secretion from pancreatic β-cells and secondarily by increasing glucose uptake in peripheral tissues (3). Such action mechanisms might be suitable for the treatment of glucocorticoid-induced diabetes. Here we examined the effects of glimepiride on patients with newly diagnosed diabetes during glucocorticoid therapy.

Three Japanese female patients who had been taking oral glucocorticoids were newly diagnosed with diabetes. Patient 1 (aged 68 years) had systemic lupus erythematodes, patient 2 (aged 65 years) had Behcet's disease, and patient 3 (aged 48 years) had angiolymphoid hyperplasia with eosinophilia. They had been initially given 20–40 mg/day prednisolone. The dosage of prednisolone was tapered and maintained at 5–10 mg/day. The status of these diseases was well controlled with the glucocorticoid treatment. At 1–2 years after starting the glucocorticoid therapy, they showed overt diabetes, with mean fasting blood glucose 12.6 ± 0.7 mmol/l and HbA_{1c} 9.5 ± 1.5% (means ± SE). Their index for pancreatic β-cell function (HOMA-%β), as determined by the correct homeostasis model assessment evaluation (4), was 27 ± 8%, significantly ($P < 0.001$) lower than that (72 ± 4% [range 44–111%]) in 24 healthy Japanese control subjects (mean age 47 ± 2 years) who had normal glucose tolerance by 75-g oral glucose tolerance test. The index for insulin sensitivity (HOMA-%S) (4) was 56 ± 15% in the patients, significantly ($P < 0.01$) lower than in healthy control subjects (144 ± 10% [81–273%]). We administered glimepiride to these patients (1 mg/day for patient 1 and 3 mg/day for patients 2 and 3). The dosage of prednisolone was unchanged throughout the observation period (24 weeks). Fasting blood glucose declined 4 weeks after the glimepiride administration and was kept below 7 mmol/l until 24 weeks. HbA_{1c} significantly decreased 4 weeks after the treatment, decreasing to 6.7 ± 0.6% after 8 weeks and maintaining that level until 24 weeks. HOMA-%β and HOMA-%S increased to the control levels (76 ± 9% and 108 ± 54%, respectively) 8 weeks after the treatment, remaining within the control ranges at 24 weeks (60 ± 3% and 99 ± 55%).

In three patients with glucocorticoid-induced diabetes, HOMA-%β and HOMA-%S were lower than in healthy control subjects. After the treatment with glimepiride, HOMA-%β and HOMA-%S increased to control ranges, in association with remarkable improvement of glycemic controls persisting until 24 weeks. This is suggested to be attributable to the dual effects of glimepiride on β-cell function and insulin sensitivity. It has been shown that a thiazolidinedione has a potential for the glucocorticoid-induced diabetes (5). From our study, glimepiride is also a strong candidate for the treatment of the glucocorticoid-induced diabetes.

Soji Kasayama, MD[1]
Toshio Tanaka, MD[1]
Kunihiko Hashimoto, MD[2]
Masafumi Koga, MD[2]
Ichiro Kawase, MD[1]

From the [1]Department of Molecular Medicine, Osaka University Graduate School of Medicine, Osaka, Japan; and the [2]Department of Internal Medicine, Kinki Central Hospital, Itami, Japan

Address correspondence to Soji Kasayama, MD, Department of Molecular Medicine, Osaka University Graduate School of Medicine (C-4), 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. E-mail: kasayama@imed3.med.osaka-u.ac.jp.

References

1. Hirsch IB, Paauw DS: Diabetes management in special situations. *Endocrinol Metab Clin North Am* 26:631–645, 1997
2. Henriksen JE, Alford F, Ward GM, Beck-Nielsen H: Risk and mechanism of dexamethasone-induced deterioration of glucose tolerance in non-diabetic first-degree relatives of NIDDM patients. *Diabetologia* 40:1439–1448, 1997
3. Clark HE, Matthews DR: The effects of glimepiride on pancreatic β-cell function under hypoglycaemic clamp and hypoinsulinaemic, englycaemic clamp conditions in non-insulin-dependent diabetes mellitus. *Horm Metab Res* 28:445–450, 1996
4. Levy JC, Matthews DR, Hermans MP: Correct homeostasis model assessment (HOMA) evaluation uses the computer program. *Diabetes Care* 21:2191–2192, 1998
5. Fujibayahi K, Nagasaka S, Itabashi N, Kawakami A, Nakamura T, Kusaka I, Ishikawa S, Saito T: Troglitazone efficacy in a subject with glucocorticoid-induced diabetes. *Diabetes Care* 22:2088–2089, 1999

Brief communication

Activation of TGF-β/Smad2 signaling is associated with airway remodeling in asthma

Hironori Sagara, MD,[a] Takanori Okada, MD,[a] Ko Okumura, MD,[b] Hideoki Ogawa, MD,[b] Chisei Ra, MD,[b] Takeshi Fukuda, MD,[a] and Atsuhito Nakao, MD[b] *Tochigi and Tokyo, Japan*

Background: Transforming growth factor β (TGF-β) has been suggested to play an important role in the development of airway remodeling in asthma; this suggestion is based on evidence that expression levels of TGF-β are correlated with unique parameters of airway remodeling, such as thickness of basement membrane. However, the relevant studies were inconclusive because they were unable to demonstrate active signaling mediated by the cytokine in the airways of asthmatic individuals.
Objective: We sought to determine whether TGF-β signaling was active in the airways of asthmatic subjects and, if so, whether it was correlated with clinicopathologic features associated with the development of airway remodeling in asthma.
Methods: We examined the phosphorylation status of Smad2 in bronchial biopsy samples obtained from 40 asthmatic subjects as a marker of active TGF-β signaling, and we studied its correlation with basement membrane thickness.
Results: Expression levels of phosphorylated Smad2 in bronchial biopsy specimens from asthmatic subjects were higher than those in specimens from normal subjects, and they were correlated with basement membrane thickness in asthma.
Conclusion: The findings provide evidence that TGF-β signaling was active in asthmatic airways and that the activity was associated with the development of airway remodeling in asthma. (J Allergy Clin Immunol 2002;110:249-54.)

Key words: Transforming growth factor β, Smad, asthma, airway remodeling, subepithelial thickness

Chronic inflammation of the airways and airway tissue remodeling are the most common histopathologic features of bronchial asthma.[1] Airway remodeling is defined by a collection of chronic structural changes, including subepithelial fibrosis, myofibroblast hyperplasia, airway smooth muscle hypertrophy/hyperplasia, mucous gland and goblet cell hyperplasia, and epithelial disruption, and it is thought to lead to irreversible airway obstruction, which is one of the factors that makes treatment of asthmatic patients difficult.[2] Among the features of airway remodeling, subepithelial fibrosis that might result in basement membrane thickening has attracted much attention,[3] inasmuch as it has been suggested to be associated with disease severity and correlated with a decline of FEV_1.[4,5] Although the presence of airway remodeling in asthma is generally accepted, the cellular and molecular events underlying the remodeling process are poorly understood.

Abbreviation used
TGF-β: Transforming growth factor β

Previous studies have suggested that a fibrogenic cytokine, transforming growth factor β1 (TGF-β), might be a relevant molecule, given that TGF-β was shown to be expressed in the tissue of asthmatic airways[6,7] and its expression level is correlated with basement membrane thickness and fibroblast number/and or disease severity.[7,8] Most of the relevant studies evaluated the levels of TGF-β1 expression in asthmatic airways by using immunohistochemistry with anti–TGF-β1 antibody or in situ hybridization with a TGF-β1 probe. However, because TGF-β1 is secreted as latent complexes[9] and anti–TGF-β1 antibody and because detection of mRNA with a TGF-β1 probe is not generally able to discriminate the latent form from the active form (unless one specifically uses antibody against the active form of TGF-β1), the actual activity of TGF-β1 in asthmatic airways and its association with airway remodeling remain uncertain.

Recent identification of the Smad family of proteins has advanced our understanding how TGF-β signals from the membrane to the nucleus.[10] The activated TGF-β receptors induce phosphorylation of Smad2 and Smad3, which form hetero-oligomeric complexes with Smad4. The complexes then translocate to the nucleus and regulate transcriptional responses, together with DNA binding cofactors. Therefore, phosphorylation of Smad2 (or Smad3) is a key step in the initiation of TGF-β signal transduction and serves as an indicator of active TGF-β signaling.

From [a]the Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Tochigi, and [b]the Atopy (Allergy) Research Center, Juntendo University School of Medicine, Tokyo.
Supported in part by a grant from the Ministry of Education, Science, Sports, and Culture of Japan.
Received for publication September 17, 2001; revised April 19, 2002; accepted for publication April 20, 2002.
Reprint requests: Atsuhito Nakao, MD, Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan.

0091-6749/2002 $35.00 + 0 1/86/126078
doi:10.1067/mai.2002.126078

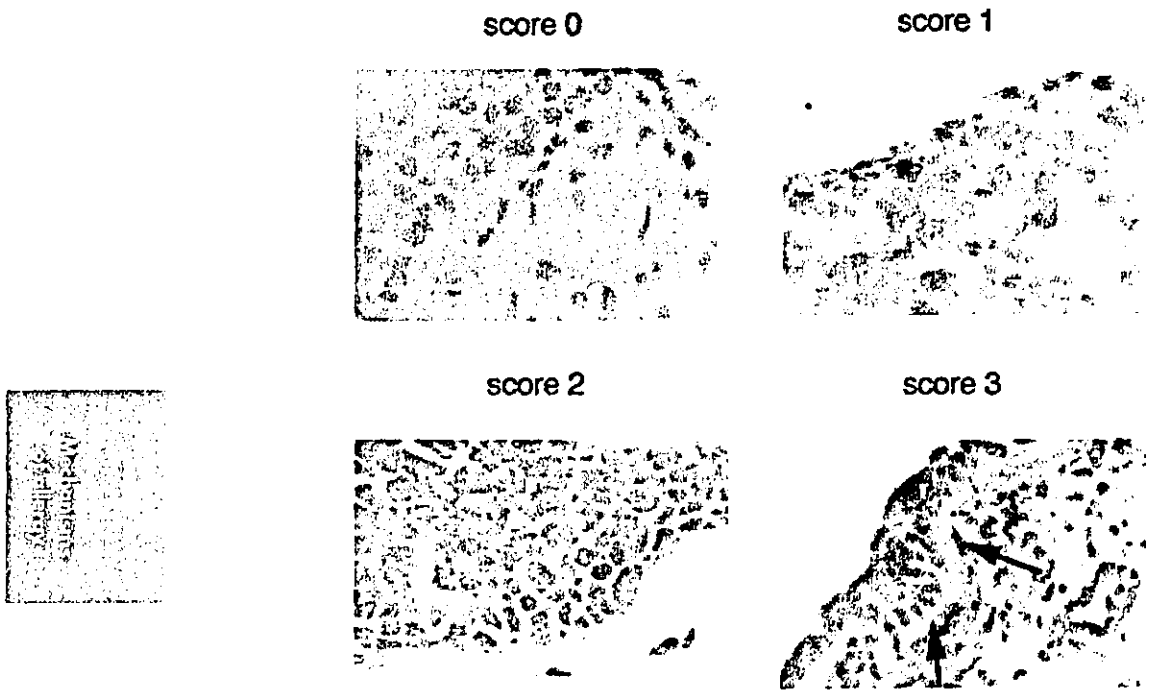

FIG 1. Photomicrographs show scoring for phosphorylated Smad2 staining in bronchial biopsy specimens from asthmatic and control subjects. Note the basement membrane thickness *(arrows)* in a sample derived from a severely asthmatic patient (score 3).

In this study, we examined the expression of phosphorylated Smad2 in the airways of asthmatic individuals and its correlation with basement membrane thickness in asthma to determine whether TGF-β1 signaling was active in the process of airway remodeling.

METHODS

Subjects

Eight normal control subjects without asthma (mean [± SE] age, 43.0 ± 3.8 years) and 40 asthmatic subjects (20 with mild asthma, a mean [± SE] age of 41.4 ± 4.4 years, and a mean [± SE] $FEV_{1.0}$ of 2441 ± 113.8 mL; 16 with moderate asthma, a mean [± SE] age of 43.0 ± 3.8 years, and a mean [± SE] $FEV_{1.0}$ of 2518 ± 174.5 mL; and 4 with severe asthma, a mean [± SE] age of 54 ± 5.0 years, and a mean [± SE] $FEV_{1.0}$ of 1990 ± 330.3 mL), as defined by a combination of asthma symptom grade and frequency of symptoms (based on the criteria of the Japanese Society of Allergology[11]) were studied (Table I). The investigation was approved by the Ethics Committee of Dokkyo University School of Medicine, and all subjects gave written informed consent. The thickness of total basement membrane in each asthmatic subject and in each control subject was assessed as previously described.[4] Airway responsiveness was measured as the minimal cumulative dose of acetylcholine at which respiratory resistance began to increase during continuous inhalation of acetylcholine in stepwise incremental concentrations.[12]

Bronchial biopsy

Tissue samples of asthmatic patients were taken from subcarina between the right lower lobe and middle lower lobe bronchi (the origin of the right B6 bronchus) through use of standard forceps under fiberoptic bronchoscopic examination, as previously described.[4] Each biopsy specimen was placed immediately in OCT medium, snap-frozen in liquid nitrogen, and stored at –80°C until cryostat sectioning.

Immunohistochemistry

Sections of respiratory mucosa from asthmatic subjects were stained with affinity-purified antiphosphorylated Smad2 antibody[13] (kindly provided by Drs Peter ten Dijke and Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden) through use of ABC kits (Vector Laboratories, Burlingame, Calif) on 3-μm consecutive serial sections. Briefly, slides were quenched in 3% H_2O_2 for 10 minutes to block endogenous peroxidase and then washed in PBS. Next, sections were incubated with the primary antibody for 1 hour and then with biotinylated secondary antibody followed by ABC reagents. Color development was achieved by incubating diaminobenzidine as a substrate. Slides were counterstained with Mayer's hematoxylin. Preincubation of the primary antibody with specific blocking peptides or substitution of the primary antibody with an irrelevant IgG served as negative controls. Phosphorylated Smad2-positive cells were counted in at least 6 high power fields in each sample by 3 independent observers (H.S., T.O., and A.N.). A minimum of 500 cells were counted. In each instance, the percentage of positive cells was calculated as follows:

$$\frac{\text{no. of positive cells}}{\text{total no. of cells}} \times 100\%$$

Scoring was as follows: 0, no staining found; 1, staining seen in 0% to 40% of cells; 2, staining seen in 40% to 70% of cells; 3, staining seen in >70% of cells. The average of the scores of the 3 investigators for each sample was calculated; these averages were used as

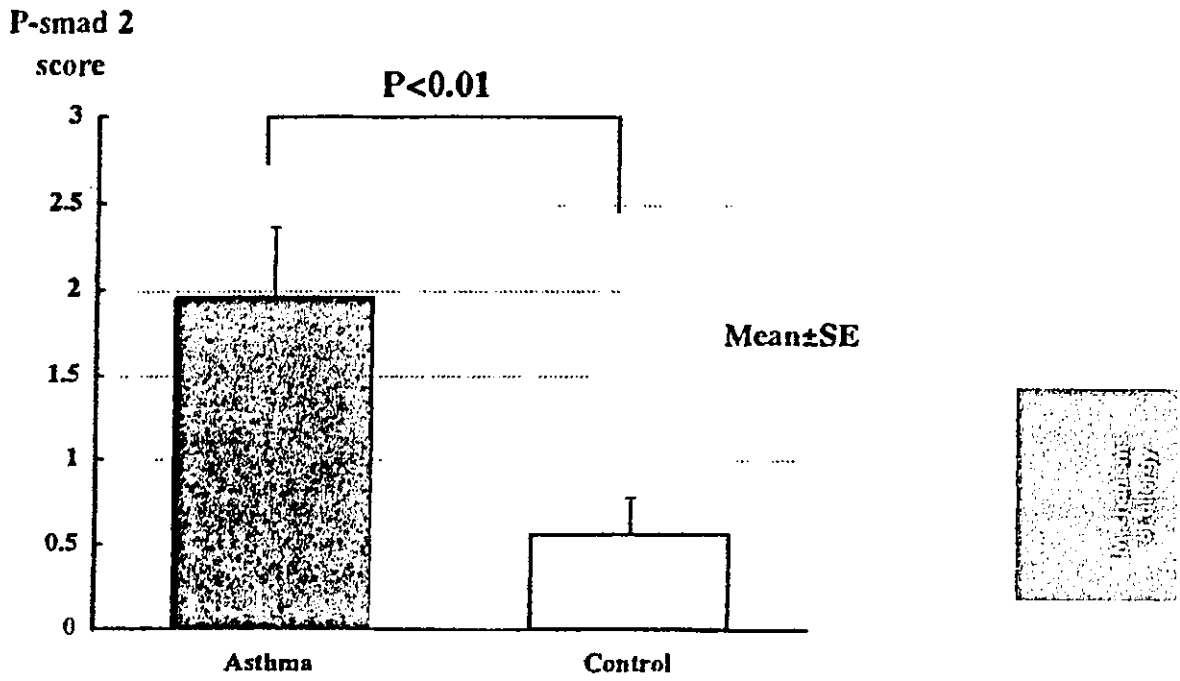

FIG 2. Higher scoring for phosphorylated Smad2 in bronchial biopsy samples from asthmatic subjects than in samples from normal subjects.

TABLE I. Characteristics of asthmatic subjects studied

	Subjects with asthma			
	Mild	Moderate	Severe	Healthy subjects
n	20	16	4	6
Age (y)*	41 ± 4.4	43 ± 3.8	54 ± 5.0	59 ± 16
Age at onset (y)*	34.0 ± 4.7	32.5 ± 5.1	27.5 ± 7.4	57.2 ± 16.4
$FEV_{1.0}$ (mL)*	2241 ± 113.8	2518 ± 174.5	1990 ± 330.3	2410 ± 690.0

Values are means ± SEs.

data. Sections used in immunohistochemistry series were stained with hematoxlyin-eosin.

Data analysis

Data are summarized as means ± SEs. Statistical analysis of the results was based on the amount of variance; we used Fisher's least significant difference test for multiple comparisons. Relationships were estimated through use of the Spearman rank correlation coefficient (rs). A P value of <.05 was considered significant.

RESULTS

To determine whether TGF-β signaling was active in the airways of asthmatic individuals, we examined the phosphorylation status of Smad2 in bronchial biopsy samples obtained from 40 asthmatic subjects and 6 control subjects by immunohistochemistry. Smad2 is a downstream effector for TGF-β and thus serves as indicator of active TGF-β signaling in situ.

Bronchial biopsy samples derived from healthy control subjects showed little immunoreactivity of phosphorylated Smad2. In contrast, biopsy samples derived from asthmatic subjects showed clearly positive immunoreactivity of phosphorylated Smad2 in bronchial epithelial cells, fibroblastlike cells, and vascular endothelial cells. Although substantial proportions (30%) of infiltrating cells, including mononuclear cells, also appeared to be positively stained, accurate quantification (scoring) of the staining was difficult because of their scattered presence. We confirmed that the immunoreactivity was efficiently blocked by a 10-fold excess of the phosphorylated Smad2 peptide used to pro-

We then examined the relationships between the scoring and clinicopathologic features associated with airway remodeling. As shown in Fig 3, *A*, the scoring of phosphorylated Smad2 was significantly correlated with basement membrane thickness in asthmatic subjects. Interestingly, phosphorylated Smad2 scoring was also correlated with airway hypersensitivity to acetylcholine in asthmatic subjects (Fig 3, *B*). These findings indicate that TGF-β signaling was active in asthmatic airways, and the activity was associated with the degree of airway remodeling and airway hyperresponsiveness in asthma.

DISCUSSION

In this study, we showed that expression levels of phosphorylated Smad2 in bronchial biopsy samples were clearly correlated with basement membrane thickness and airway hypersensitivity to acetylcholine in asthmatic individuals. The findings suggest that TGF-β signaling was active in asthmatic airways, and the activity was associated with the extent of airway remodeling and airway hyperresponsiveness in asthma.

Immunoreactivity of phosphorylated Smad2 in bronchial samples derived from healthy control subjects was weak or very weak in contrast to that in samples derived from asthmatic subjects. In previous studies it had been reported that TGF-β1 was expressed in bronchial biopsy specimens or bronchoalveolar lavage fluid in normal subjects as well as in asthmatic subjects.[14,15] Our findings thus suggest that though TGF-β1 was expressed in the airways of normal subjects, it might not be the active form of TGF-β1 and did not mediate signaling in situ. Inflammation is a factor in conversion of the latent form of TGF-β1 to the active form[9] and might thus contribute to activation of TGF-β1 at the site of asthmatic airways.

We found that immunoreactivity of phosphorylated Smad2 was similar in mild, moderate, and severe asthma for different cell types—bronchial epithelial cells, fibroblastlike cells, and endothelial cells. This was consistent with recent findings by Rosendahl et al[16] in a murine model of asthma. They showed that expression of phosphorylated Smad2 in bronchial epithelial cells, fibroblasts, endothelial cells, and inflammatory cells was dramatically induced by inhalation of ovalbumin in the airways of ovalbumin-sensitized Balb/c mice. Little expression of phosphorylated Smad2 was observed without the inhaled challenge. Thus, TGF-β signaling might become active in almost all residential and inflammatory cells in the airways on allergen challenge and subsequent airway inflammation in asthma.

TGF-β affects functions of airway residential cells, such as bronchial epithelial cells, lung fibroblasts, and smooth muscle cells, and it might contribute to several features of airway remodeling, including subepithelial fibrosis and smooth muscle cell hyperplasia.[2] Our results suggest that active TGF-β signaling indeed occurs in these cell types, though activity of TGF-β signaling in smooth muscle cells remains unknown.

It is not yet clear whether TGF-β signaling was active in infiltrating cells in our study (in contrast to what was seen in a study involving an acute model of asthma conducted by Rosendahl et al[16]). Substantial fractions of infiltrating cells appeared to show positive immunoreactivity for phosphorylated Smad2, but most of the cells appeared to show negative staining. TGF-β inhibits immune cell functions in general; we thus speculate that inflammatory cells might express inhibitory molecules for TGF-β signaling and escape from the suppression by TGF-β in situ. Abnormal expression of Smad7 (an intracellular antagonist of TGF-β signaling) in the mucosa in inflammatory bowel disease was recently reported; this might result in the deterioration of TGF-β signaling.[17]

In summary, we have provided evidence that TGF-β signaling was active in respiratory mucosa in asthmatic patients and that the activity correlated well with basement membrane thickness, a feature of airway remodeling. To our knowledge, this is the first description of intracellular molecular events underlying the remodeling process in asthma. Smad2 might become a therapeutic target for airway remodeling in asthma.

We thank Drs Hiroko Ushio, Keiko Maeda, Chiharu Nishiyama, Toshiro Takai, and Yutaka Kanamaru for support.

REFERENCES

1. Holgate ST. What does inflammation and airway remodeling mean? Clin Exp Allergy Rev 2001;1:59-61.
2. Elias JA, Zhu Z, Chupp G, Homer RJ. Airway remodeling in asthma. J Clin Invest 1999;104:1001-6.
3. Roche WR, Easley R, Williams J, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. Lancet 1989;1:520-4.
4. Chetta A, Foresse A, Donno MD, Bertorelli G, Pesci A, Olivieri D. Airway remodeling is a distinctive feature of asthma and is related to severity of desease. Chest 1997;111:852-7.
5. Hoshino M, Nakamura Y, Sim J, Shimojo J, Isogai S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. J Allergy Clin Immunol 1998;102:783-8.
6. Ohno I, Nitta Y, Yamauchi K, Hoshi H, Honma M, Woolley K, et al. Transforming growth factor-β1 gene expression by eosinophils in asthmatic airway inflammation. Am J Respir Cell Mol Biol 1996;15: 404-9.
7. Minshall EM, Leung DY, Martin RJ, Song YL, Cameron L, Ernst P, et al. Eosinophil-associated TGF-β1 mRNA expression and airways fibrosis in bronchial asthma. Am J Respir Cell Mol Biol 1997;17:326-33.
8. Vignola A, Chanez P, Chiappara G, Merendino A, Pace E, Rizzo A, et al. Transforming growth factor-β expression in mucosal biopsies in asthma and chronic bronchitis. Am J Respir Crit Care Med 1997;156:591-9.
9. Massague J. The transforming growth factor-β family. Annu Rev Cell Biol 1990;6:597-641.
10. Moustakas A, Souchelnytskyi S, Heldin CH. Smad regulation in TGF-β signal transduction. J Cell Sci 2001;14:4359-69.
11. Makino S, Furusho K, Miyamoto T, Ohta K, editors. Asthma prevention and management guidelines 1998, Japan (JGL1998). Report of the research group for asthma prevention and management guidelines, supported by the Immunology & Allergy Research Study Project, Ministry of Health and Welfare. Jpn Int Arch Allergy Immunol 2000; 121(Suppl 1):4-14.
12. Makino S, Ikemori R, Fukuda T, Motojima S, Yukawa T, Yamada G, et al. Clinical evaluation of standard method of acetylcholine inhalation test in bronchial asthma. Jpn J Allergol 1984;33:167-75.
13. Brodin GP, ten Dijke P, Funa K, Heldin CH, Landstrom M. Increased Smad expression and activation are associated with apoptosis in normal and malignant prostate after castration. Cancer Res 1999;59:2731-8.

14. Redington AE, Roche WR, Holgate ST, Howarth PH. Co-localization of immunoreactive TGF-β1 and decorin in bronchial biopsies from asthmatics and normal subjects. J Pathol 1998;186:410-5.
15. Redington AE, Madden J, Frew AJ, Djukanovic R, Roche WR, Holgate ST, et al. Transforming growth factor-β1 in asthma. Measurement in bronchoalveolar lavage fluid. Am J Respir Crit Care Med 1997;156:642-7.
16. Roseadahl A, Checchin D, Fehniger TE, ten Dijke P, Heldin CH, Sideras P. Activation of the TGF-β/activin-Smad2 pathway during allergic airway inflammation. Am J Respir Cell Mol Biol 2001;25:60-8.
17. Fiocchi C. TGF-β/Smad signaling defects in inflammatory bowel disease: mechanisms and possible novel therapies for chronic inflammation. J Clin Invest 2001;108:523-6.

Research News

Smad7: a new key player in TGF-β-associated disease

Atsuhito Nakao, Ko Okumura and Hideoki Ogawa

Smad7 is a major inhibitory regulator of transforming growth factor (TGF)-β signaling. Smad7 expression is induced by TGF-β itself and other signaling pathways, indicating a key role for Smad7 in feedback or cross-talk control of TGF-β signaling. Recent reports have implicated Smad7 as a crucial regulator of TGF-β activity in human disease; aberrant expression of Smad7 is involved in inflammatory bowel disease and scleroderma. Thus, modulation of Smad7 expression could provide a novel therapeutic basis for TGF-β-associated disorders.

Published online: 27 June 2002

TGF-β is a multifunctional cytokine, capable of regulating the growth, differentiation and apoptosis of virtually all cell types, and has been implicated in numerous disease states including atherosclerosis, immune-mediated diseases, fibrotic diseases, angiogenic diseases and cancer [1]. In general, abnormal production of TGF-β or genetic mutations in TGF-β signaling components, resulting in dysregulation of TGF-β activity, have been linked to disease states. Now, a new example of dysregulated TGF-β signaling associated with disease states has been revealed by two publications showing that aberrant expression of Smad7, an intracellular regulator of TGF-β signaling, resulting in uncontrolled TGF-β activity, is involved in the pathology of inflammatory bowel disease (IBD) and sclerosis [2,3]. Regulation of Smad7 expression is thus critical for balanced TGF-β activity, and dysregulated Smad7 activity can lead to the development of TGF-β-associated human disease in certain cases.

Smad7 is a major inhibitory regulator for TGF-β/Smad signaling

The identification of Smad proteins has advanced our understanding of how TGF-β signals from the membrane to the nucleus [4]. The activated TGF-β receptors phosphorylate Smad2 and Smad3, which form heteromeric complex with Smad4 and enter the nucleus, bind to DNA in a sequence-specific manner, and regulate gene transcription in cooperation with various transcriptional factors and coactivators and/or corepressors.

Recent studies have identified several molecules that control the signal transduction of TGF-β, thereby regulating TGF-β activity [4–6]. Smad7 is one of these molecules and inhibits TGF-β-induced transcriptional responses [7–9]. Smad7 associates with the activated TGF-β receptor and interferes with the activation of Smad2 and Smad3 by preventing their receptor interaction and phosphorylation. In addition, Smad7 interacts with a group of ubiquitin ligases, termed Smurf [5]. After recruitment of the Smad7–Smurf1 complex to the activated TGF-β receptors, Smurf1 induces their degradation through proteasomal and lysosomal pathways. Thus, the expression level of Smad7 is a major determinant for TGF-β transcriptional responses, which could regulate intensity and/or duration of TGF-β signals.

The mechanisms that regulate the expression of Smad7 are not fully understood. Smad7 expression is strongly and rapidly induced by TGF-β itself [9],

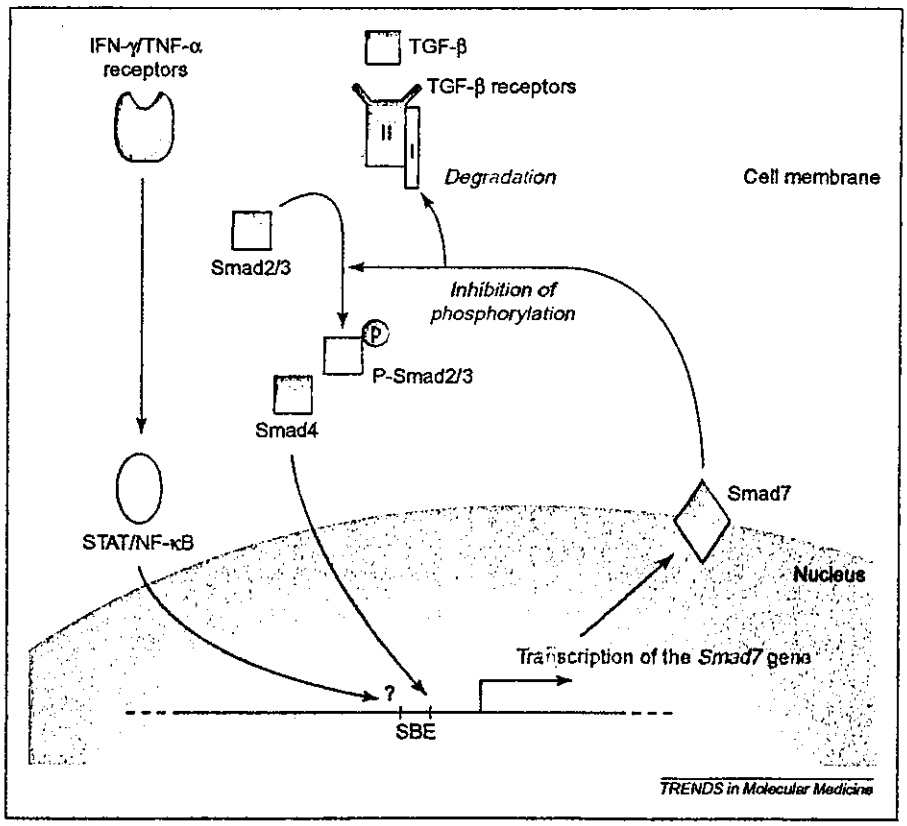

Fig. 1. Smad7 is an inhibitory regulator in feedback or cross-talk control of transforming growth factor (TGF)-β signal transduction. TGF-β receptors consist of type I and type II receptors with intrinsic serine/threonine kinase activity. Activated TGF-β receptors phosphorylate Smad2 and Smad3, leading to hetero-oligomeric complexes with Smad4. The complexes then translocate to the nucleus, where they regulate the transcription of target genes including the Smad7 gene. Smad7 associates with the activated type I receptor and interferes with the activation of Smad2 and Smad3 by competing with their receptor interaction. In addition, Smad7 interacts with ubiquitin ligases, termed Smurf, and binds to the activated TGF-β receptors, inducing degradation of the receptors, through proteasomal and lysosomal pathways. Other signaling pathways, such as interferon (IFN)-γ/tumor necrosis factor (TNF)-α pathways, are reported to induce Smad7 expression in certain cell types. Thus, the expression level of Smad7 is a major determinant for TGF-β transcriptional responses. Abbreviations: NF-κB, nuclear factor κB; SBE, Smad binding element.

  PII: S1471-4914(02)02376-6

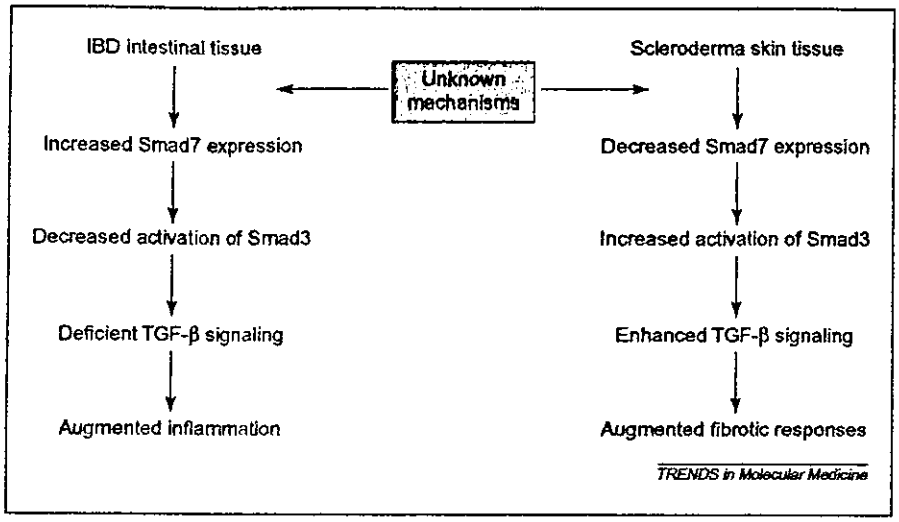

Fig. 2. Effect of aberrant expression of Smad7 in the intestinal lesions of inflammatory bowel disease (IBD) or skin lesions of scleroderma. Transforming growth factor (TGF)-β has two properties in inflammation: anti-inflammatory and pro-fibrotic. Aberrant Smad7 expression occurs at the target sites of IBD or sleroderma by as yet undefined mechanisms. The increased or decreased Smad7 expression in IBD or scleroderma, respectively, leads to decreased or increased activity of a major signal transducing Smad, Smad3, respectively, and then suppresses or enhances TGF-β signaling, respectively. As a consequence, aberrant TGF-β activity results in sustained inflammation in IBD or enhanced fibrotic responses in sclerosis.

and the promoter region of the Smad7 gene contains a consensus Smad3–Smad4 binding element (SBE), a palindromic sequence of GTCTAGAC, to which the Smad3–Smad4 complex binds [10–12]. Efficient expression of Smad7 appears to require cooperation of Smad, Sp1, and AP-1 transcription factors [13].

However, in some cell types, Smad7 expression is induced by other signaling pathways, for example, by the Jak1/Stat1 pathway following stimulation with IFN-γ [14], by activated nuclear factor (NF)-κB following stimulation with tumor necrosis factor (TNF)-α [15], and by fluid shear stress acting on endothelial cells [8]. How Smad7 expression is differentially regulated dependent on cell type remains to be determined. In any case, the resulting surge in Smad7 expression levels interferes with activation of Smad2 and Smad3 or accelerates degradation of TGF-β receptors, inhibiting TGF-β/Smad signaling. Thus, Smad7 has a potential central role as an effector in an autoregulatory feedback loop in TGF-β/Smad signaling and as a mediator of inhibitory signaling cross-talk between various pathways and the TGF-β–Smad pathway (Fig. 1).

Aberrant expression of Smad7 impairs efficient TGF-β signaling in IBD and scleroderma

Recent studies have implicated Smad7 as an important molecule for regulating TGF-β activity in human disease (Fig. 2). Monteleone *et al.* reported that Smad7 was overexpressed in IBD mucosa and purified mucosal T cells [2]. Both whole tissue and isolated cells exhibited defective TGF-β signaling as measured by phospho-Smad3 immunoreactivity. Importantly, antisense oligonucleotides for Smad7 restored TGF-β signaling and enabled TGF-β to inhibit pro-inflammatory cytokine production such as interferon (IFN)-γ and TNF-α in cells isolated from IBD patients and also in inflamed tissue explants from patients with Crohn's disease. Thus, Smad7 blockade of TGF-β signaling helps maintain the chronic production of pro-inflammatory cytokines that drives the inflammatory process in IBD.

In a separate study, Dong *et al.* reported deficient Smad7 expression in the skin lesions of scleroderma [3]. TGF-β induces fibroblast growth and stimulates the synthesis of extracellular-matrix proteins including collagen. It has been shown that fibroblasts from skin lesion of patients with scleroderma show enhanced responses to TGF-β [16]. Dong *et al.* showed that basal level and TGF-β-inducible expression of Smad7 were selectively decreased both in scleroderma skin and in explanted scleroderma fibroblasts in culture. They also showed enhanced TGF-β signaling in scleroderma skin as judged by increased phospho-Smad3 immunoreactivity. Importantly, *in vitro* adenoviral gene transfer of Smad7 restored normal TGF-β signaling in scleroderma fibroblasts. Thus, in the case of scleroderma, suppression of Smad7 expression appeared to be a key to enhanced responses to TGF-β in fibroblasts and development of the sclerotic skin lesions.

In both reports, it remains unclear how aberrant expression of Smad7 occurs at the site of the diseases. It is possible that pro-inflammatory cytokines such as IFN-γ and TNF-α, usually elevated in IBD tissue [2], upregulate Smad7 expression, but this has not been demonstrated. Because little is known about the negative transcriptional regulation of the Smad7 gene, how Smad7 expression decreases in scleroderma skin remains unexplained at the moment. Most recently, another example of the effect of decreased Smad7 expression was shown by Wang *et al.* in a cardiac infarction model in rats [17]. Thus, positive and negative regulation of Smad7 expression needs to be thoroughly investigated in future studies.

TGF-β has been shown to effectively suppress inflammation in several animal models including IBD [18,19], which could be in contrast to the human study [2]. In general, animal studies deal with acute models of inflammation. It might therefore require a chronic inflammatory state to impair regulatory mechanisms of Smad7 expression and to cause abnormal responses to TGF-β observed in human IBD [2]. It is possible that persistent production of TGF-β or pro-inflammatory cytokines at the site of chronic inflammation induces Smad7 expression, making disease cells unresponsive to TGF-β.

Perspectives

Many *in vitro* and *in vivo* animal studies have implicated Smad7 as a major inhibitory regulator of the TGF-β–Smad pathway. It is well known that signal-transduction pathways have their own intracellular regulators and there is much cross-talk signaling regulation through regulatory molecules such as Smad7 [4]. This regulatory role for Smad7 is supported by the findings in the IBD and scleroderma studies in humans [2,3].

Upregulation of TGF-β has been documented in certain inflammatory disorders such as IBD and asthma, and it seems paradoxical that elevated TGF-β levels fail to control the diseases through its anti-inflammatory activity [2,20]. As seen in the IBD study, overexpression of

Smad7 in inflammatory cells can dampen their responses to TGF-β produced at the site of chronic inflammation. Thus, Smad7 could be a key molecule for the mechanisms of chronic inflammation in these disorders, and downregulation of Smad7 expression (e.g. by the use of antisense Smad7 oligonucleotides) might restore normal control of TGF-β signaling in inflammatory cells and be beneficial for the treatment of chronic inflammatory disorders associated with high TGF-β production and its resistance.

Th observed reduced expression of Smad7 in scleroderma should encourage us to analyze the transcriptional regulation of the promoter region of the Smad7 gene in more detail; for example, by searching for single nucleotide polymorphisms (SNPs) that might affect Smad7 transcription in patients with scleroderma. Such studies might also provide clues as to why certain population are susceptible to tissue fibrosis or scar formation after skin injury. In addition, exploring mechanisms of Smad7 degradation should be another important area of investigation for scleroderma study.

Acknowledgements
We thank Hiroko Ushio, Keiko Maeda, Chiharu Nishiyama, Toshiro Takai, Yutaka Kanamaru, Koji Sumiyoshi, Toshinari Funaki, Nobuyuki Ebihara, Yasuhiro Setoguchi, and Ryoji Tsuboi for helpful discussion and support. This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

References

1 Blobe, G.C. *et al.* (2000) Role of transforming growth factor-β in human disease. *New Engl. J. Med.* 342, 1350–1358
2 Monteleone, G. *et al.* (2001) Blocking Smad7 restores TGF-β1 signaling in chronic inflammatory bowel disease. *J. Clin. Invest.* 108, 601–609
3 Dong, C. *et al.* (2002) Deficient Smad7 expression: a putative molecular defect in scleroderma. *Proc. Natl. Acad. Sci. U. S. A.* 99, 3908–3913
4 Massague, J. (2000) How cells read TGF-β signals. *Nat. Rev. Mol. Cell Biol.* 1, 169–178
5 Moustakas, A. *et al.* (2001) Smad regulation in TGF-β signal transduction. *J. Cell Sci.* 114, 4359–4369
6 Wrana, J. (2000) Regulation of Smad activity. *Cell* 100, 189–192
7 Hayashi, H. *et al.* (1997) The Mad-related protein Smad7 associates with the TGF-β receptor and functions as an antagonist of TGF-β signaling. *Cell* 89, 1165–1173
8 Topper, J.N. *et al.* (1997) Vascular MADs: two novel MAD-related genes selectively induced by flow in human vascular endothelium. *Proc. Natl. Acad. Sci. U. S. A.* 94, 9314–9319
9 Nakao, A. *et al.* (1997) Identification of Smad7, a TGF-β-inducible antagonist of TGF-β signaling. *Nature* 389, 631–635
10 von Gersdorff, G. *et al.* (2000) Smad3 and Smad4 mediate transcriptional activation of the human Smad7 promoter by transforming growth factor-β. *J. Biol. Chem.* 275, 11320–11326
11 Nagarajan, R.P. *et al.* (1999) Regulation of Smad7 promoter by direct association with Smad3 and Smad4. *J. Biol. Chem.* 274, 33412–33418
12 Denissova, N.G. *et al.* (2000) Transforming growth factor-β-inducible independent binding of SMAD to the Smad7 promoter. *Proc. Natl. Acad. Sci. U. S. A.* 97, 6397–6402
13 Brodin, G. *et al.* (2000) Efficient TGF-β induction of the Smad7 gene requires cooperation between AP-1, Sp1, and Smad proteins on the mousse Smad7 promoter. *J. Biol. Chem.* 275, 29023–29030
14 Ulloa, L. *et al.* (1999) Inhibition of transforming growth factor-β/SMAD signaling by the interferon-γ/STAT pathway. *Nature* 397, 710–713
15 Bitzer, M. *et al.* (2000) A mechanism of suppression of TGF-β/SMAD signaling by NF-κB/RelA. *Genes Dev.* 14, 187–197
16 Cotton, S.A. *et al.* (1998) TGF-β-a role in systemic sierosis? *J. Pathol.* 184, 4–6
17 Wang, B. *et al.* (2002) Decreased Smad7 expression contributes to cardiac fibrosis in the infacted rat heart. *Am. J. Physiol. Heart Circ. Physiol.* 282, H1685–H1696
18 Wahl, S.M. (1994) Transforming growth factor-β: the good, the bad, and the ugly. *J. Exp. Med.* 180, 1587–1590
19 Kitani, A. *et al.* (2000) Treatment of experimental colitis by intranasal administration of TGF-β1 plasmid: TGF-β1-mediated suppression of T helper cell type1 response occurs by IL-10 induction and IL-12 receptor β2 chain downregulation. *J. Exp. Med.* 192, 41–52
20 Ling, E.M. *et al.* (2002) Transforming growth factor-β1: its anti-inflammatory and pro-fibrotic effects. *Clin. Exp. Allergy* 32, 175–178

Atsuhito Nakao*
Ko Okumura
Hideoki Ogawa
Atopy (Allergy) Research Center, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan.
*e-mail: anakao@med.juntendo.ac.jp

Techniques & Applications

Encapsulation of pancreatic islets for transplantation in diabetes: the untouchable islets

Paul de Vos and Piero Marchetti

The aim of encapsulation of pancreatic islets is to transplant in the absence of immunosuppression. It is based on the principle that transplanted tissue is protected from the host immune system by an artificial membrane. Encapsulation allows for application of insulin-secreting cells of animal or other surrogate sources, to overcome human islet shortage. The advantages and pitfalls of the approaches developed so far are discussed and compared, together with some recent progress, in view of applicability in clinical islet transplantation.

Published online: 1 July 2002

Recent successes [1] have increased the optimism and interest in clinical application of pancreatic islet transplantation in type 1 diabetes on a large scale. Unfortunately, present approaches involve the use of high-dose and strict immunosuppressive protocols, which is associated with serious side-effects. Therefore, it is still doubtful whether clinical islet transplantation in combination with immunosuppression will ever be a sound alternative to insulin therapy for the majority of diabetic patients [2]. By encapsulation (i.e. immunoisolation) of the islets, chronic administration of immunosuppressants can be eliminated, as the hostile host-immune system cannot reach the physically protected pancreatic islet cells.

Immunoisolation is based on the principle that transplanted cells are separated from the host immune system by a biocompatible, semipermeable membrane [2,3]. The membrane does not allow the entry of inflammatory cells and large molecules such as antibodies,

 1471-4914/02/$ – see front matter PII: S1471-4914(02)02381-X