

## Pioneering Figures in Dermatology: The Early Career of Professor Howard I. Maibach, M.D.

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Received 19 May 2008, accepted 26 June 2008

Edited by TAKASHI HASHIMOTO

Professor Howard Maibach from the University of California in San Francisco is arguably one of the most well-known and well-published dermatologists in the world today. Born in New York during the late 1920s, Maibach entered his profession via a rather circuitous route however, having originally majored in political science at Tulane University during the late 1940s. After graduating from Tulane with a bachelor's degree, Maibach began a doctorate in anthropology at Columbia University while working part time as a neurology lab technician. As a result of collaborations with Professor Joseph Globus during this period, in 1952 he coauthored what would become the first of many thousands of journal papers, with a manuscript in the *Journal of Neuropathology and Experimental Neurology* [1]. In the same year, Maibach also authored of a book chapter describing the anthropology of hair [2].

After three semesters working on his anthropology doctorate at Columbia however, Maibach concluded that future job prospects for a political science and anthropology graduate were not overly promising. This realization, combined with many summer vacations doing hard physical work, including one summer loading fish onto railway cars, had also indicated that a life of manual labor was equally unappealing. His time spent as a manual worker had not been wasted however, giving Maibach a greater understanding of the difficulties encountered by blue collar employees, as well as a lifelong appreciation and respect for the work they do. Similarly, his one-and-a-half years with Professor Globus had also been fruitful, with Maibach

later recalling that Globus was a major inspiration who instilled a lifelong passion for intellectual curiosity and scientific discovery within the young technician.

In the early 1950s with his sights now firmly set on a medical career, Maibach entered the medical school of his old alumnus at Tulane University. After completing an M.D. degree in 1955, the young medical graduate completed an internship at the William Beaumont Army Hospital in El Paso Texas, before moving on to the Walter Reed Army Hospital, where he majored in Neuropsychiatry in 1956. The dermatology profession was also moving forwards during this time, with clinics investigating contact dermatitis beginning to appear throughout Europe [3], and one of the world's first patch testing clinics being founded by Professor Charles Calnan and colleagues at the St John's Hospital for Diseases of the Skin in London [4]. On the other side of the world where Maibach lived, its opening did not go unnoticed. In 1958, Maibach joined the University of Pennsylvania, where he developed what would become a lifelong and fruitful relationship with Professor Albert Kligman, as well as a particular passion for dermatological research. Maibach's first journal paper as a primary author and dermatologist was published in the prestigious *Archives of Dermatology* in 1960, and described the short-term treatment of onychomycosis with griseofulvin [5]. Kligman was also destined for a long and fruitful career in dermatological research, and even in his 90s still holds the position of Professor Emeritus at the University of Pennsylvania and continues to undertake research.

In 1961, Maibach joined the *University of California, San Francisco* (UCSF) as an Assistant Professor of Dermatology. Although he would later treat thousands of patients in clinical practice, a chance encounter during this formative period changed Maibach's life. Within one month of being appointed at UCSF, a young patient scheduled for amputation of a blackened and necrotic toe of unknown pathology visited the clinic. Somewhat perplexed, Maibach established through questioning that the patient had earlier been prescribed neomycin for a suspected bacterial infection. Rather than using a 0.5% neomycin cream, the patient had actually been using a 100% powder to treat his 'infected' toe. Unbeknownst to the patient, for whom no improvement was seen, repeated application had resulted in a severe case of allergic contact dermatitis and subsequent cutaneous necrosis. This in turn, had led to a nasty looking blue toe, referral for orthopedic surgery and an appointment for radical amputation of the offending digit. Fortunately, Maibach established the cause of the ailment, ceased the 100% neomycin treatment, and thereby solved the problem. The same patient visited Maibach's clinic some years afterwards to acknowledge his efforts, and demonstrate that the previously condemned toe had completely recovered. Maibach later recalled that this interesting case would inspire a lifelong professional interest in toxicology, and an ongoing quest to better understand the toxic effect of chemicals on human skin.

Aside from interesting clinical cases, Maibach's passion for scientific research was also furthered in 1967 when various European groups interested in contact dermatitis amalgamated to form the *International Contact Dermatitis Research Group* (ICDRG), of which he became one of the early members [3]. Maibach was promoted to Associate Professor at UCSF in the same year, and joined the Cancer Research Institute as a Research Associate. Sensing a need for more coordinated contact dermatitis research in his own country, Maibach and others founded a *North American Contact Dermatitis Group* (NACDG) based on the ICDRG, during the late 1960s. He later helped organize regular half-day meetings of the NACDG, in association with annual meetings of the American Academy of Dermatology [3].

In 1973, Maibach was promoted to full Professor and Vice Chairperson of Dermatology at UCSF. He

has been head of the Occupational Dermatology Clinic at UCSF since 1984 [6]. Since joining UCSF over 35 years ago, Maibach has constantly lectured and written extensively on dermatopharmacology and dermatotoxicology, being an author or editor for over 40 books and publishing around 2000 scientific journal articles. He currently sits on the editorial board of 30 scientific journals and is a member of 19 academic and professional societies [7]. Professor Maibach has also had a long association with Japan, visiting the country at least 12 times [8]. He has been a teacher, supervisor and mentor to countless dermatology residents and researchers from many countries, all of whom continue to benefit from his clinical talent, personable demeanor and ongoing passion for scientific research and discovery. In 2008, Professor Maibach continues to serve as a Professor in the Department of Dermatology at UCSF [7]. His extensive bibliography is available online via the UCSF website [9].

**ACKNOWLEDGMENTS:** The author acknowledges the kind cooperation of Professor Howard I. Maibach during the preparation of this article.

## REFERENCES

1. Globus JH, Wang SC, and Maibach HI. Radon implantation in the medulla oblongata of the dog; Effects on the degree and extent of cellular reactions. *J Neuropath Exp Neurol* 1952; 11:429-442.
2. Maibach HI. Anthropology of hair. In: *The Scalp Disease*. St. Louis, CV Mosby Co., 1952.
3. American Contact Dermatitis Society Homepage. Available online at: <http://www.contactderm.org/i4a/pages/index.cfm?pageid=3277> (accessed June 2008)
4. Black MM. St John's Hospital for diseases of the skin - Tales of the founder to the present day. *J Eur Acad Dermatol Venereol* 2007; 19(Suppl.2):29.
5. Maibach HI, and Kligman AM. Short-term treatment of onychomycosis with griseofulvin. *Arch Dermatol* 1960; 81:733-734.
6. Proceedings of the 70th Annual Meeting of the Tokyo Division of the Japanese Dermatological Association held in Tokyo, Japan on February 17-18, 2007; p. 56.
7. University of California, San Francisco (UCSF) Website. Available online at: <http://www.dermatology.ucsf.edu/faculty/maibach.aspx> (accessed June 2008)
8. Nakata T. Professor Howard Ira Maibach. *West Jpn J Dermatol* 2008; 70:87-88.
9. Bibliography of Howard I. Maibach, M.D. (UCSF Website). Available online at: <http://www.dermatology.ucsf.edu/faculty/PDF/BiblioHIM.pdf> (accessed June 2008)

# Reduced Pulmonary Function is Associated with Enhanced Inflammation and Tissue Inhibitor of Metalloproteinase 1 Concentration in the Bronchoalveolar Lavage Fluid of Patients with Lung Parenchymal Sarcoidosis

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*Received 31 March 2008, accepted 25 June 2008*

**Summary:** Lung parenchymal disease is associated with reduced pulmonary function in patients with sarcoidosis, however, the underlying pathophysiology of the condition is unclear. The present study was conducted to characterize the association between pulmonary function and bronchoalveolar lavage (BAL) findings in patients with sarcoidosis. Twenty-three patients with lung parenchymal disease (stage 2) and twenty-five patients without lung parenchymal disease (stage 1) underwent pulmonary function tests, including blood gas analysis, spirometry and diffusing capacity for carbon monoxide (DLco) and BAL, to determine the number of inflammatory cells, matrix metalloproteinase (MMP) 9 activity and tissue inhibitor of metalloproteinase (TIMP) 1 concentration in the lower airway. Vital capacity (VC) to its reference value (%VC) and %DLco were significantly reduced in patients with stage 2 disease in comparison with those with stage 1 disease. BAL fluid analysis revealed that the numbers of total inflammatory and CD8 cells, and TIMP-1 concentration were significantly higher in patients with stage 2 disease in comparison with those in patients with stage 1 disease. There were significant correlations between %VC and the numbers of inflammatory cells and TIMP-1 in the BAL fluid. These results suggest that inflammation and enhanced TIMP-1 concentration in the lower airway play critical roles in the impaired pulmonary function in patients with lung parenchymal sarcoidosis.

**Key words** sarcoidosis, lung parenchymal disease

## INTRODUCTION

Lung parenchymal disease is one of the most frequent manifestations in patients with sarcoidosis, and is noted in about half on presentation [1] and in more than half during the course of the disease [2]. Although restrictive impairment in pulmonary function occurs in a significant proportion of patients with lung parenchymal sarcoidosis, the underlying pathophysiology of the condition remains to be elucidated. Pathological studies indicate that factors medi-

ating extracellular matrix (ECM) proteins turnover are likely to be involved in the development of lung parenchymal disease in patients with sarcoidosis. Immunological abnormalities noted in patients with sarcoidosis include intraalveolar and interstitial accumulation of activated inflammatory cells [3], especially cluster of designation (CD) 4 cells polarized to T helper (Th) 1 [4]. Taken together, inflammation and altered ECM proteins homeostasis are likely to play a vital role in pulmonary function impairment in patients with lung parenchymal sarcoidosis.

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Abbreviations: BAL, bronchoalveolar lavage; BHL, bilateral hilar lymphadenopathy; DLco, diffusing capacity for carbon monoxide; ECM, extracellular matrix; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; VC, vital capacity.

Therefore, the present study was conducted to analyze the association between pulmonary function and inflammation and representative mediators of ECM proteins turnover, i.e. matrix metalloproteinase (MMP) 9 and tissue inhibitor of metalloproteinase (TIMP) 1, in the lung in patients with sarcoidosis.

## MATERIALS AND METHODS

### *Subjects*

Patients with sarcoidosis were consecutively recruited at Kurume University Hospital between 2000 and 2005. The diagnosis of sarcoidosis was based on clinical and pathological criteria [3], and recruited patients were categorized based on chest radiographic findings as follows; stage 0, normal chest radiograph; stage 1, bilateral hilar lymphadenopathy (BHL); stage 2, BHL plus pulmonary infiltrations; stage 3, pulmonary infiltrations only; stage 4, pulmonary fibrosis. Patients underwent arterial blood gas determination under room air and lung function tests including spirometry [5] and diffusing capacity for carbon monoxide (DLco) [6]. Since vital capacity (VC) and DLco were frequently affected in pulmonary sarcoidosis [7], these indices, expressed as percentages of reference values [8], were considered to represent lung function impairment. The DLco adjustments for alveolar volume (VA) have been applied in the evaluations of carbon monoxide uptake properties and alveolar gas volumes in lung diseases. However, the adjustment has not been validated in lung diseases where lung pathology has reduced carbon monoxide uptake properties, and the ratio of pathological reductions in DLco and VA may be quite variable and of unclear physiological or clinical significance in many disease states [9]. Thus, the diffusing capacity was not adjusted for VA in the present study.

The study protocol was reviewed and approved by the ethics committee of Kurume University, and written informed consent was obtained from each participant in the study.

### *Bronchoalveolar lavage*

Bronchoalveolar lavage was performed using fiberoptic bronchoscopy (BF240, Olympus, Tokyo) under local anesthesia with lidocaine following premedication with intramuscular atropine and hydroxyzine as a sedative. The bronchoscope was wedged in the right middle lobar bronchus and three 50 ml aliquots of sterile 0.9% saline were gently instilled and recovered by suction. The recovered fluid was collected and

stored on ice and processed within 1 hr after retrieval. Recovered fluid was strained through a layer of sterile gauze and centrifuged at 400 g for 10 min at 4°C. Supernatant was stored at -70°C until used.

### *Differential cell count*

Cell pellets were washed twice with phosphate buffered saline (PBS) (Gibco, NY, USA), resuspended in PBS supplemented with 10% heat-inactivated fetal calf serum. Total cell count was determined with a hemocytometer and viability (median=96%) was assessed by trypan blue exclusion. Cyto centrifugation (Cytospin, Shandon, UK) was performed using part of the cell suspension stained with May-Grünwald-Giemsa, and differential cell counts were determined by counting more than 200 cells. Another part of the cell suspension was incubated with phycoerythrin-labeled anti-CD4 antibody (Becton Dickinson, Mountain View, CA, USA), and fluorescein isothiocyanate-labeled anti-CD8 antibody (Becton Dickinson, Mountain View, CA, USA) for 20 min, washed twice, and resuspended for flow cytometry. The stained cells were analyzed on a flow cytometer (Becton Dickinson, Mountain View, CA, USA). Lymphocytes were gated on forward and side scatter, and the percentages of positively stained cells were scored to determine the number of CD4 and CD8 cells.

### *Measurement of MMP-9 specific activity*

The MMP-9 specific activity was quantified using a calorimetric assay with a modified detection proenzyme, which is activated by active MMP-9 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The proenzyme is activated by cleavage of an amino acid sequence recognized specifically by active MMP-9 and, in turn, digests a chromogenic peptide substrate. The system detects a minimum of 0.125 ng/ml MMP-9 and does not crossreact with MMP-1, MMP-2, MMP-3, MMP-8, TIMP-1, or TIMP-2.

### *Determination of TIMP-1 concentrations*

The concentrations of TIMP-1 were determined using a commercially available EIA based assay system (Fuji Chemical Industries, Takaoka, Japan). Assays were performed using the protocols recommended by the manufacturer. The sensitivity of the assay was 1.25 ng/ml. The assay does not crossreact with TIMP-2.

### *Statistical analysis*

Statistical analysis was performed using SPSS 13.0J for Windows software (SPSS Inc., Chicago, IL-

linois). The Mann-Whitney U test or chi-square test was used to compare the two groups. Spearman's rank correlation analysis was used to assess the correlation. A *p* value of <0.05 was considered statistically significant.

RESULTS

There were 48 patients with sarcoidosis (25 patients with stage 1 disease and 23 with stage 2, 16 males, mean age: 47). There were no patients with stage 3 or 4 disease in this recruitment period. There were no significant differences in age, gender, smoking status, extrapulmonary manifestations, and blood gases (PaO<sub>2</sub> and PaCO<sub>2</sub>) between the patients with stage 1 sarcoidosis and those with stage 2 disease. Pulmonary function analysis revealed that %VC and %DLco were significantly reduced in patients with stage 2 disease in comparison with those with stage 1 disease.

There was no difference in the fluid recovery rate of bronchoalveolar lavage (BAL) between the two groups of the subjects. The numbers of total and CD8 cells, and the TIMP-1 concentration in the BAL fluid

were significantly higher in patients with stage 2 sarcoidosis than in those with stage 1 disease. Other BAL indices, i.e. percentage of lymphocytes, the number of lymphocytes and CD4 cells, MMP-9 activity and MMP-9/TIMP-1 ratio) showed a tendency to be higher in patients with stage 2 disease (Table 2).

There were significant associations between %VC and the number of total cells, lymphocytes, macrophages, CD4 cells, CD8 cells and TIMP-1 concentration in the BAL fluid. PaO<sub>2</sub> showed a significant negative correlation with the number of CD4 cells (Table 3).

DISCUSSION

The results of the present study revealed that inflammation and TIMP-1 expression are enhanced in the lungs of patients with lung parenchymal disease, and are associated with reduced pulmonary function in patients with sarcoidosis. Henry et al. [10] conducted a similar study and found that TIMP-1 levels were highest in patients with stage 3 disease, where lung fibrosis is pronounced, followed by those with stage 2 and 1 disease. Furthermore, the MMP-9/TIMP-1 ratio

TABLE 1. Patient Characteristics

	Stage 1	Stage 2	<i>p</i>
Number	23	25	
Age [year]	50.4±15.6	47.0±17.9	0.3
Male	10 (40.0)	6 (26.1)	0.31
Smoker	9 (36.0)	13 (56.5)	0.15
Uveitis	20 (80.0)	16 (69.6)	0.4
Skin lesions	2 (8.0)	2 (8.7)	0.93
%VC [%]	112.4±10.3	100.6±9.7	0.003
%DLco [%]	115.1±18.6	91.3±21.9	0.018
PaO <sub>2</sub> [torr]	86.8±12.6	86.4±15.6	0.98
PaCO <sub>2</sub> [torr]	41.8±4.8	40.1±4.4	0.23

Data are expressed as mean±SD or number (%), depending on the variables.

VC, vital capacity; DLco, diffusing capacity for carbon monoxide

TABLE 2. Characterization of bronchoalveolar lavage fluid

	Stage 1	Stage 2	<i>p</i>
Fluid Recovery, %	55.9±9.1	50.5±10.6	0.07
Total cell, /μl	1.91±0.83	2.81±1.67	0.036
Lymphocyte, %	35.1±16.8	38.8±21.3	0.64
Lymphocyte, /μl	0.67±0.43	1.16±1.06	0.16
CD4 cell, /μl	0.50±0.30	0.90±0.85	0.4
CD8 cell, /μl	0.08±0.06	0.16±0.16	0.027
MMP-9, ng/ml	1.44±1.35	6.03±15.0	0.12
TIMP-1, ng/ml	148.3±72.8	240.3±137.1	0.009
MMP-9/TIMP-1	1.19±1.40	3.47±10.21	0.75

Data are expressed as mean±SD. CD, cluster of designation; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase

TABLE 3. Correlation between lung function and BAL fluid indices

	Total Cell	Lym	Mφ	CD4	CD8	MMP-9	TIMP-1	MMP-9/TIMP-1
%VC	-0.51 (0.002)	-0.50 (0.002)	-0.43 (0.011)	-0.47 (0.006)	-0.61 (0.0001)	NS	-0.37 (0.03)	NS
%DLco	NS	NS	NS	NS	NS	NS	NS	NS
PaO <sub>2</sub>	NS	NS	NS	-0.36 (0.04)	NS	NS	NS	NS

Data were expressed as correlation coefficient (*p* value). Lym; lymphocyte, Mφ; macrophage, NS; not significant

was significantly elevated in patients with stage 3 sarcoidosis [10]. These findings suggest that an altered homeostasis of ECM proteins, which are mediated by relevant mediators such as MMP-9 and TIMP-1, plays a critical role in the development of lung parenchymal disease in patients with sarcoidosis. Importantly, the number of inflammatory cells and TIMP-1 immunoreactivity in the BAL fluid showed a significant negative correlation with %VC in the present study, indicating that these factors play a substantial role in the reduced lung function in affected individuals. In contrast DLco and PaO<sub>2</sub> showed no correlation with BAL indices except that the number of CD4 cells correlated with PaO<sub>2</sub>. These results may indicate that %VC is more closely associated with lung parenchymal inflammation than DLco and PaO<sub>2</sub>.

Although the mechanism for the enhanced expression of TIMP-1 is obscure, inflammation in the lower airways seems to be important. A pathological study on sarcoid granuloma has suggested that multi-nucleated giant cells are the source of MMP-9 and TIMP-1 [11]. Macrophages, which were also increased in patients with parenchymal sarcoidosis in the present study, could be another source of TIMP-1 production in the lower airways [12]. On the other hand, CD4 lymphocytes have been shown as dominant sources for TIMP-1 production *in vitro* [13]. TIMP-1 expression by lung lymphocytes *in vivo* has been demonstrated in the pathological investigation of interstitial pneumonias [14], supporting lymphocytes as one of the sources for TIMP-1 production in the lower airways.

The results of the present study showed a significant correlation between %VC and the number of inflammatory cells in the BAL fluid. Similar findings were reported by Schoenfeld et al. [15], who showed that the number of T lymphocytes was significantly correlated with hypoxia during exercise and %VC in patients with sarcoidosis. Another study has documented a significant correlation between the number of activated lymphocytes and the diffusing capacity [16]. On the other hand, Fireman et al. [17] have shown that TIMP-1 and MMP-9 levels in the BAL fluid were negatively correlated with the diffusion capacity in patients with granulomatous lung diseases. Taken together, enhanced inflammation and associated alterations in the turnover of ECM proteins in the lower airways are likely to mediate functional impairment in lung parenchymal sarcoidosis.

There is a possibility of bias in the present study because of the limited number of patients during the recruitment period. This may be a reason for the in-

consistency of the present findings with those in some previous reports. For instance, Fireman et al. [17] have shown an enhanced expression of MMP-9, but not TIMP-1, in the induced sputum of patients with sarcoidosis, although both parameters were significantly correlated with diffusion capacity. A second limitation of the present study is that it is possible that smoking in a part of the subjects may have influenced the results, since smoking has been documented to affect BAL findings in patients with sarcoidosis [18]. Third, the present study did not clarify whether or not elevated TIMP-1 is directly connected with the inflammation. It is plausible that inflammation induces components other than inflammatory cells, such as fibroblasts, to produce TIMP-1. Despite these limitations, the present study suggested a link between impaired pulmonary function and inflammation in the lower airways in patients with lung parenchymal sarcoidosis. More detailed insight into this link would lead to a better understanding of the pathophysiology of lung parenchymal sarcoidosis.

In conclusion, the present study suggested an association between immunological alterations in the lower airways and impaired pulmonary function in patients with lung parenchymal disease.

## REFERENCES

1. Baughman RP, Teirstein AS, Judson MA, Rossman MD, Yeager H Jr. et al. Clinical characteristics of patients in a case control study of sarcoidosis. *Am J Respir Crit Care Med* 2001; 164:1885-1889.
2. James DG, Neville E, and Siltzbach LE. A worldwide review of sarcoidosis. *Ann N Y Acad Sci* 1976; 278:321-334.
3. American Thoracic Society. Statement on sarcoidosis. Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. *Am J Respir Crit Care Med* 1999; 160:736-755.
4. Agostini C, Cabrelle A, Calabrese F, Bortoli M, Scquizzato E et al. Role for CXCR6 and its ligand CXCL16 in the pathogenesis of T-cell alveolitis in sarcoidosis. *Am J Respir Crit Care Med* 2005; 172:1290-1298.
5. American Thoracic Society. Standardization of Spirometry, 1994 Update. *Am J Respir Crit Care Med* 1995; 152:1107-1136.
6. American Thoracic Society. Single-breath carbon monoxide diffusing capacity (transfer factor). Recommendations for a standard technique-1995 update. *Am J Respir Crit Care Med* 1995; 152:2185-2198.
7. Keogh BA, Hunninghake GW, Line BR, and Crystal RG. The alveolitis of pulmonary sarcoidosis. Evaluation of natural history and alveolitis-dependent changes in lung func-

- tion. *Am Rev Respir Dis* 1983; 128:256-265.
8. Fukuchi Y, Nishimura M, Ichinose M, Adachi M, Nagai A et al. COPD in Japan: the Nippon COPD Epidemiology study. *Respirology* 2004; 9:458-465.
  9. MacIntyre N, Crapo RO, Viegi G, Johnson DC, van der Grinten CPM et al. Standardisation of the single-breath determination of carbon monoxide uptake in the lung. *Eur Respir J* 2005; 26:720-735.
  10. Henry MT, McMahon K, Mackarel AJ, Prikk K, Sorsa T et al. Matrix metalloproteinases and tissue inhibitor of metalloproteinase-1 in sarcoidosis and IPF. *Eur Respir J* 2002; 20:1220-1227.
  11. Gonzalez AA, Segura AM, Horiba K, Qian S, Yu ZX et al. Matrix metalloproteinases and their tissue inhibitors in the lesions of cardiac and pulmonary sarcoidosis: an immunohistochemical study. *Hum Pathol* 2002; 33:1158-1164.
  12. Selman M, Ruiz V, Cabrera S, Segura L, Ramirez R et al. TIMP-1, -2, -3, and -4 in idiopathic pulmonary fibrosis. A prevailing nondegradative lung microenvironment? *Am J Physiol Lung Cell Mol Physiol* 2000; 279:L562-L574.
  13. Johnatty RN, Taub DD, Reeder SP, Turcovski-Corrales SM, Cottam DW et al. Cytokine and chemokine regulation of proMMP-9 and TIMP-1 production by human peripheral blood lymphocytes. *J Immunol* 1997; 158:2327-2333.
  14. Choi KH, Lee HB, Jeong MY, Rhee YK, Chung MJ et al. The role of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in cryptogenic organizing pneumonia. *Chest* 2002; 121:1478-1485.
  15. Schoenfeld N, Schmitt M, Remy N, Ellensohn P, Lodenkemper R et al. Activation of bronchoalveolar lavage T lymphocytes and clinical, functional and radiological features in sarcoidosis. *Sarcoidosis* 1995; 12:135-139.
  16. Xaubet A, Agusti C, Roca J, Picado C, and Rodriguez-Roisin R. BAL lymphocyte activation antigens and diffusing capacity are related in mild to moderate pulmonary sarcoidosis. *Eur Respir J* 1993; 6:715-718.
  17. Fireman E, Kraiem Z, Sade O, Greif J, and Fireman Z. Induced sputum-retrieved matrix metalloproteinase 9 and tissue metalloproteinase inhibitor 1 in granulomatous diseases. *Clin Exp Immunol* 2002; 130:331-337.
  18. Hance AJ, Basset F, Saumon G, Danel C, Valeyre D et al. Smoking and interstitial lung disease. The effect of cigarette smoking on the incidence of pulmonary histiocytosis X and sarcoidosis. *Ann N Y Acad Sci* 1986; 465:643-656.

# Physiological Study of Anal Sphincteric Resection in an Experimental Porcine Model

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*Received 27 October 2006, accepted 17 March 2008*

Edited by MINORU YAGI

**Summary:** This study was conducted to compare and evaluate the extent of anal sphincteric resection and the degree of anal dysfunction in sphincter saving operations for lower rectal cancer using experimental porcine models. Each 10 Clawn miniature pigs underwent transanal intersphincteric resection (ISR), ISR with partial (one-quarter) external sphincteric resection (ESR-25%), and ISR with partial (one-half) external sphincteric resection (ESR-50%). An anorectal physiological study was performed before, one month, and three months after surgery in these three groups. The anal maximum resting pressure (AMRP) decreased from 45.1 cmH<sub>2</sub>O in the control group to 14.8, 14.3 and 11.1 cmH<sub>2</sub>O one month after surgery, and to 15.2, 8.8 and 5.2 cmH<sub>2</sub>O three months after surgery, in the ISR, ESR-25% and ESR-50% groups, respectively. The anal maximum squeezing pressure (AMSP) decreased from 81.7 cmH<sub>2</sub>O in the control group to 42.1, 40.1 and 41.1 cmH<sub>2</sub>O one month after surgery in the ISR, ESR-25% and ESR-50% groups, respectively. Three months after surgery, the MSP increased to 78.1 and 68.1 cmH<sub>2</sub>O in the ISR and ESR-25% groups, respectively, but the ESR-50% group showed a significantly lower MSP of 39.2 cmH<sub>2</sub>O compared with other two groups. The ratio of the potential difference on electromyographic (EMG) was 0.19 in the ESR-50% group, and this value was significantly lower than 0.8 in the ISR and ESR-25% groups, one month after surgery. Three months after surgery, the potential ratio of EMG was increased almost to the pre-operative level both in the ISR and ESR-25% groups, but the ratio of the potential difference in the ESR-50% group with redness, sore and soiling around anus was 0.19 and significantly lower compared with other groups. The results of this study indicate that porcine models with additional resection of less than one quarter of the external anal sphincter have little anal dysfunction. A human clinical trial is needed to determine the ESR for very low rectal cancer.

**Key words** intersphincteric resection (ISR), external sphincteric resection (ESR)

## INTRODUCTION

Surgical treatment for low rectal cancer is shifting from resection with colostomy to sphincter saving operation without colostomy because the latter procedure does not affect postoperative local recurrence adversely compared with abdominoperineal resection

(APR) [1] and also because dyschezia is not severe postoperatively [2]. Since Schiessel et al. [2] reported intersphincteric resection (ISR), with excision of the internal anal sphincter and preservation of the anus, for low rectal cancer close to the anus, oncologic and functional studies of this surgical technique have been performed [3-6]. Recently, a surgical technique

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Abbreviations: AHPZ, anal high pressure zone; AMRP, anal maximum resting pressure; AMSP, anal maximum squeezing pressure; APR, abdominoperineal resection; EMG, electromyographic; ESR, external sphincteric resection; ISR, intersphincteric resection.



involving partial resection of the external anal sphincter also has been reported [7].

However, the evaluation results on the relation between extent of anal sphincter resection and the degree of anal dysfunction differ from one researcher to another. The aim of this study is to compare and evaluate the extent of internal and external sphincteric resection and the degree of anal dysfunction using experimental porcine model.

## MATERIALS AND METHODS

### Preparation of ISR and ESR models

Three-month-old Clawn miniature pigs weighing 25 kg were immobilized with 25 mg/kg ketamine hy-

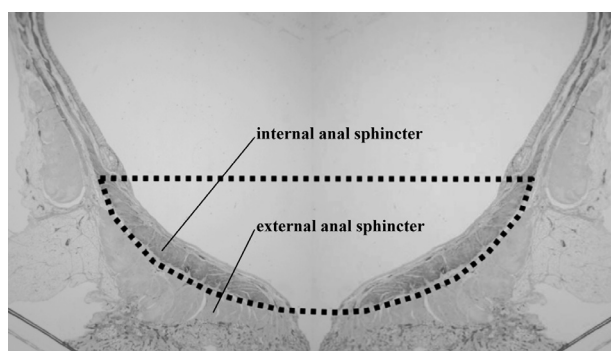


Fig. 1a. ISR model

The anal canal of about 3 cm in length was excised through a circumferential incision into the intersphincter transanally from the intersphincteric groove between the internal and external anal sphincters, with subsequent construction of transanal anorectal anastomosis.

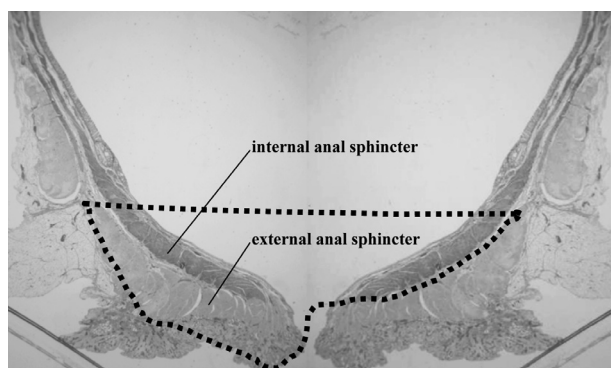


Fig. 1b. ESR model

Animal models which underwent resection of the left anterior quarter of the superficial and deep external sphincters, with complete ISR, served as 25% external sphincteric resection group (ESR-25% group). Animal models which underwent partial resection of the superficial and deep external sphincters, with complete ISR.

drochloride (Ketalar) and generally anesthetized by intravenous injection of 25 mg/kg pentobarbital sodium (Nembutal). The animals were divided into three groups. In the complete intersphincteric resection group (ISR group) (Fig. 1a), about 3 cm of the anal canal was excised through a circumferential incision into the intersphincter transanally from the intersphincteric groove between the internal and external anal sphincters, with subsequent transanal anorectal anastomosis. A second group underwent resection of the left anterior quarter of the superficial and deep external sphincters, with complete ISR, and served as 25% external sphincteric resection group (ESR-25% group). The third group underwent resection of one half of the superficial and deep external sphincters, with complete ISR, served as the 50% external sphincteric resection group (ESR-50% group) (Fig. 1b).

### Anorectal motor functional study

According to St. Mark's technique [8,9], the rectal pressure (RP), anal high pressure zone (AHPZ) and anal maximum resting pressure (AMRP) were measured under general anesthesia preserving spontaneous breathing with a polygraph system LEG-1000 (NIHON KOHDEN CO, Tokyo, Japan). To measure the anal maximum squeezing pressure (AMSP) and electromyographic (EMG) activity of the external anal sphincter, electrical stimuli were given transanally using Stimulator/Isolator. A concentric needle was inserted to the external anal sphincter with a depth of approximately 1 cm, and the EMG waveform was recorded using Neuropack micro MEB-9100 evoked potential/EMG measuring system (NIHON KOHDEN CO, Tokyo, Japan). The finger, containing the stimulating electrode in the tip, was introduced until estab-

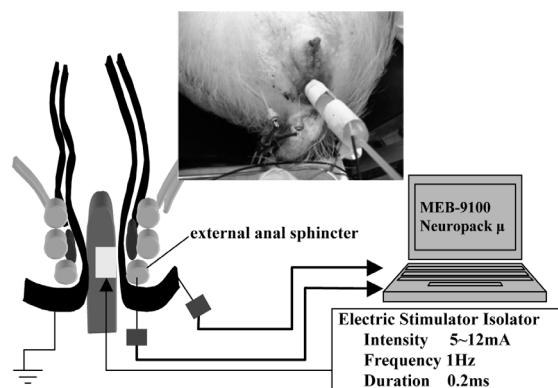


Fig. 2. Electromyographic study

Neuropack micro MEB-9100 evoked potential/EMG measuring system (NIHON KOHDEN Co., Tokyo, Japan).

lishing contact with the ischial spine, while the recording electrode, in the base of the finger remained in contact with the sphincter. A stimulus intensity of 5-12 mA was used for the test. The optimal stimulation potential was applied to induce contractions (Fig. 2). Measured data were evaluated by comparing the optimal stimulation potential one and three months after surgery to the preoperative level.

*Incontinence score*

Pigs have the habit of defecating in one area of the cage, but animals with anal damage show an abnormal behavior of defecating in several areas. Therefore, incontinence score was evaluated by scoring the areas of defecation as an objective symptom and soiling and perineal dermatitis from 0 to 3 (Table 1). All pigs used for this investigation was treated in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

*Statistical analysis*

Data were given as mean (standard deviation). Differences between groups were tested using the un-

paired t-test and the chi-squared test of Fischer's exact test when appropriate. A value of  $P < 0.05$  was considered statistically significant.

RESULTS

The AMRP decreased from 45.1 cmH<sub>2</sub>O in the control group to 14.8, 14.3 and 11.1 cmH<sub>2</sub>O one month after surgery, and to 15.2, 8.8 and 5.2 cmH<sub>2</sub>O three months after surgery, in the ISR, ESR-25% and ESR-50% groups, respectively. The AMSP decreased from 81.7 cmH<sub>2</sub>O in the control group to 42.1, 40.1 and 41.1 cmH<sub>2</sub>O in the ISR, ESR-25% and ESR-50% groups, respectively, one month after surgery. The AMSP increased to 78.1 and 68.1 cmH<sub>2</sub>O in the ISR and ESR-25% groups, respectively, but significantly decreased to 39.2 cmH<sub>2</sub>O in the ESR-50% group three months after surgery (Fig. 3).

The ratio of the potential difference on EMG was 0.19 in the ESR-50% group, and this value was significantly lower than 0.8 in the ISR and ESR-25% groups, one month after surgery. Three months after surgery, the potential ratio of EMG was increased almost to the preoperative level both in the ISR and ESR-25% groups, but the ratio of the potential difference in the ESR-50% group was 0.19 and significantly lower compared with other groups (Fig. 4).

In incontinence score three months after surgery, the ISR and ESR-25% groups had no abnormalities in objective findings of the anus, but the ESR-50% group showed redness, sore and soiling around the anus, and had significantly poorer incontinence score (Fig. 5).

TABLE 1.  
*Incontinence score*

	1	2	3
Perineal dermatitis	none	redness	erosion ulcer
Soiling	none	sometimes	always
Stool in the rectum	none	sometimes	always
Frequency of defecation / day	1	2-3	3<

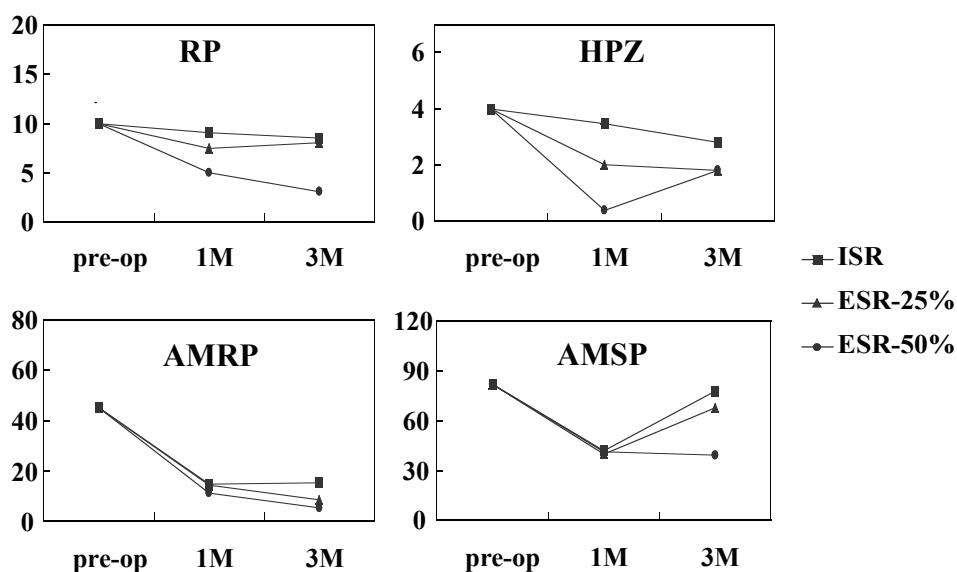
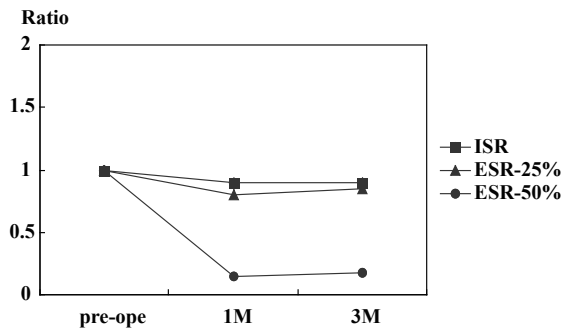


Fig. 3. Profile of pre-and post-operative manometric study.



Ratio : postoperative voltage ( $\mu$ V) / preoperative voltage ( $\mu$ V)

Fig. 4. Profile of pre-and post-operative electromyographic study.

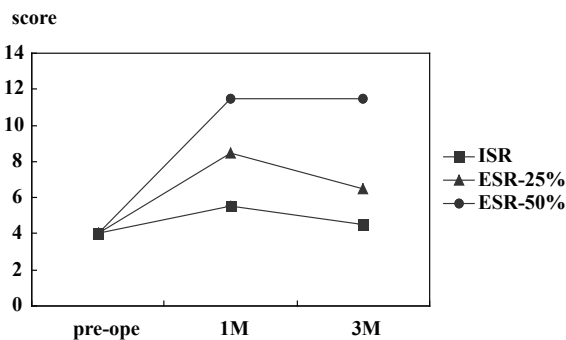


Fig. 5. Profile of pre-and post-operative incontinence score.

## DISCUSSION

Parks [10], retrospectively studied the patients with APR and reported that, owing to mechanical anastomosis and transanal anastomosis for lower rectum, a sphincter saving operation was oncologically possible in 20% of patients. Since Schiessel et al. [2], reported ISR, which consists of resection of the internal anal sphincter and preservation of the anus, for low rectal cancer close to the anus, oncologic and functional studies of this surgical procedure have been performed [3-6,11].

Some authors recommended ISR only for early staged tumors [12], whereas others included more advanced disease by use of neoadjuvant treatment [6,13,14]. Oncologic results showed local recurrence rates of 0 to 13% [2,6,13,15-18]. Contraindications are infiltration of the external anal sphincter and fixed tumors (T4), although ISR has been proposed in very selected cases with fixation on levator ani muscles [19].

There was no difference in stool frequency, fragmentation, urgency, dyschezia or alimentary restriction between patients with ISR and those with conventional coloanal anastomosis for very low rectal cancer [11].

Clinical studies demonstrated that the AMRP decreased by 38% after total ISR [2,4]. The AMRP decreased similarly by 34% in the present study using experimental models. If partial resection of the external anal sphincter is possible, ISR can be oncologically indicated for more patients. We therefore planned this experimental study to investigate the postoperative anal dysfunction after partial resection of the external anal sphincter.

Pigs have the habit of defecating in one area of the cage, but animals with anal damage show an abnormal behavior of defecating in several areas. Animals in the ISR and ESR-25% groups defecated normally in one area, but those in the ESR-50% group defecated in several areas and had severe anal dysfunction with persistent soiling and soreness around the anus. The degree of anal dysfunction after ISR with resection of one quarter of the superficial external sphincter is similar to that of the postoperative dysfunction after ISR.

The data on anal function in porcine subjects receiving ISR with 25% ESR were similar to those in animal models receiving ISR. As anal function as well as the remaining colon function and fecal quality are involved in the postoperative dyschezia, the results of this experimental study may not be immediate clinical implications, but our findings suggested a possibility of partially resecting the external anal sphincter in respect of preserving anal function.

## CONCLUSION

Our study suggested that subjects with one quarter or less of the external sphincter resected have little anal dysfunction. A human clinical trial will be needed to determine the usefulness of ESR in very low rectal cancer.

## REFERENCES

1. Pollett WG, and Nicholls RJ. The relationship between the extent of distal clearance and survival and local recurrence rates after curative anterior resection for carcinoma of the rectum. *Ann Surg* 1983; 198:159-163.
2. Schiessel R, Karner-Hanusch J, Herbst F, Teleky B, and Wunderrlich M. Intersphincteric resection for low rectal tumors. *Br J surg* 1994; 81:1376-1378.
3. Renner K, Rosen HR, Novi G, Holing N, and Schiessel R. Quality of life after surgery for rectal cancer: do we still need a permanent colostomy? *Dis Colon Rectum* 1999;

- 42:1160-1167.
4. Rullier E, Zerbib F, Laurent C, Bonnel C, Caudry M et al. Intersphincteric resection with excision of internal anal sphincter for conservative treatment of very low rectal cancer. *Dis Colon Rectum* 1999; 42:1168-1175.
  5. Bretagnol F, Rullier E, Laurent C, Zerbib F, Gontier R et al. Comparison of functional results and quality of life between intersphincteric resection and conventional coloanal anastomosis for low rectal cancer. *Dis Colon Rectum* 2004; 47:832-838.
  6. Rullier E, Goffre B, Bonnel C, Zerbib F, Caudry M et al. Preoperative radiotherapy and sphincter-saving resection for T3 carcinomas of the lower third of the rectum. *Ann Surg* 2001; 234:633-640.
  7. Shirouzu K, Ogata Y, Araki Y, and Sato Y. A new ultimate anus-preserving operation for extremely low rectal cancer and for anal canal cancer. *Tech Coloproctol* 2003; 7:203-206.
  8. Kiff E, and Swash M. Slowed conduction in the pudendal nerves in idiopathic (neurogenic) fecal incontinence. *Br J Surg* 1984; 71:615-616.
  9. Gloria L, Miguel P, Josep V, Xavier G, Montserrat P et al. Electrophysiologic studies and clinical findings in females with combined fecal and urinary incontinence: a prospective study. *Dis Colon Rectum* 2006; 49:353-359.
  10. Parks AG. Endoanal technique of low colonic anastomosis. *Surgical Technique (Illinois)* 1977; 2:63-70.
  11. Frederic B, Eric R, Christophe L, Franc Z, Renaud G et al. Comparison of functional results and quality of life between intersphincteric resection and conventional coloanal anastomosis for low rectal cancer. *Dis Colon Rectum* 2004; 47:832-838.
  12. Teramoto T, Watanabe M, and Kitajima M. Per anum intersphincteric rectal dissection with direct coloanal anastomosis for lower rectal cancer: the ultimate sphincter-preserving operation. *Dis Colon Rectum* 1997; 40:S43-47.
  13. Marks G, Mohiuddin M, and Masoni A. High-dose preoperative radiation therapy as the key to extending sphincter-preservation surgery for cancer of the distal rectum. *Surg Oncol Clin North Am* 1992; 1:71-86.
  14. Rouanet P, Fabre JM, Dubois JB, Dravet F, and Saint Aubert B. Conservative surgery for low rectal carcinoma after high-dose radiation. Functional and oncologic results. *Ann Surg* 1995; 221:67-73.
  15. Braun J, Trreutner KH, Winkeltau G, Heidenreich U, Lerch MM et al. Results of intersphincteric resection of the rectum with direct coloanal anastomosis for rectal carcinoma. *Am J Surg* 1992; 163:407-412.
  16. Rullier E, Zerbib F, Laurent C, Bonnel C, and Caudry M. Intersphincteric resection with excision of internal anal sphincter for conservative treatment of very low rectal cancer. *Dis Colon Rectum* 1999; 42:1168-1175.
  17. Köhler A, Athanasiadis S, Ommer A, and Psarakis E. Long term results of low anterior resection with intersphincteric anastomosis in carcinoma of the lower one-third of the rectum: analysis of 31 patients. *Dis Colon Rectum* 2000; 43:843-850.
  18. Rouanet P, Saint-Aubert B, Lemanski C, Senesse P, and Goutgou S. Restorative and nonrestorative surgery for low rectal cancer after high-dose radiation: long-term oncologic and functional results. *Dis Colon Rectum* 2002; 45:305-315.
  19. Fucini C, Elbetti C, Petrolo A, and Casella D. Excision of the levator muscles with external sphincter preservation in the treatment of selected low T4 rectal cancers. *Dis Colon Rectum* 2002; 45:1697-1705.

# Increased Expression of Heparan Sulfate Proteoglycan on the Cultured Renal Epithelial Cells during Oxalate Exposure

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*Received 18 December 2007, accepted 2 July 2008*

Edited by KEI MATSUOKA

**Summary:** We have previously reported that heparan sulfate (HS) / heparan sulfate proteoglycan (HSPG, syndecan-1) expression significantly increased in the rat kidney during calcium oxalate (CaOx) nephrolithiasis. Although the exact mechanism of the increased syndecan expression still remains unclear, HS/syndecan is thought to have some important roles in CaOx crystal formation. The present study examined the role of HS during oxalate exposure by using a newly developed cell line (KIC-synd-1) that expresses human heparan sulfate proteoglycan (syndecan-1). Quantitative competitive (QC)-RT-PCR was used to examine change of syndecan-1 mRNA expression in KIC-synd-1 cells. Production of syndecan-1 core protein and glycosaminoglycans (GAGs) were also confirmed by Western blot, immunohistochemistry and HPLC, respectively. Wild type Mardin-Darby canine kidney (MDCK) cells were also examined in the same manner. The stable expression of syndecan-1 gene and production of both core protein and HS chains were confirmed in the newly developed KIC-synd-1 cell line. Increased syndecan-1 mRNA expression and production of core proteins were confirmed in KIC-synd-1 cells during oxalate exposure. MTT assay revealed that the cell viability decreased significantly in the MDCK cells after 1 mM oxalate exposure ( $p < 0.05$ ). On the other hand, there was no significant difference in the oxalate exposed KIC-synd-1 cells. However, the cell viability in KIC-synd-1 cells pretreated with heparitinase digestion decreased significantly before oxalate exposure ( $p < 0.05$ ). The present data suggests that both exogenous and endogenous HS exerts protective effect against oxalate-induced cell injuries. Previous studies in our laboratory have indicated that hyperoxaluria and deposition of CaOx crystals resulted in renal tubular cellular injury inducing the synthesis of HSPG to protect and repair the damaged epithelial cell surface. The present data offers strong support for this hypothesis. Finally, HS could be potent inhibitor of CaOx nephrolithiasis and the absence of this substance on the tubular surface may increase the risk of CaOx crystal formation and retention.

**Key words** calcium oxalate, nephrolithiasis, heparan sulfate proteoglycan, syndecan-1, cellular injury

## INTRODUCTION

Calcium oxalate (CaOx) is a major constituent of calcium-containing stones in the kidney. However, the mechanism underlying the formation of CaOx stones remains unclear. Recent studies indicate that heparan sulfate (HS) is a major constituent of matrix

glycosaminoglycans (GAGs) in CaOx stones and is a potent inhibitor of CaOx crystallization [1-3]. In general, GAGs are present in tissues as a proteoglycan. Free GAGs are metabolic turnover products of tissue proteoglycan, and appear in urine by glomerular filtration. In our previous reports we suggest that the increased expression of heparan sulfate proteoglycan

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Abbreviations: CaOx, calcium oxalate; GAGs, glycosaminoglycans; HS, heparan sulfate; HSPG (syndecan-1), heparan sulfate proteoglycan; MDCK, Mardin-Darby canine kidney.

(HSPG) mRNA in rat kidney may correlate with CaOx crystal formation and deposition in the renal tubules [4,5]. Oxalate, which is an end product and secreted in urine, can induce renal cell injury [6-8]. On the other hand, several researchers have reported that heparin or HS inhibit calcium oxalate crystal aggregation, as well as crystal adhesion on the renal epithelial cells via the formation of a charge barrier [1-3]. Previously, we reported that HSPG (syndecan) mRNA significantly increased in the rat kidney during CaOx nephrolithiasis [4]. However, exact mechanism of increased expression of HSPG remains unclear. Various types of cultured epithelial cell lines have been used for experiments on kidney stone research. Mardin-Darby canine kidney (MDCK) cells also have been used commonly. Recently, several investigators have reported that presence of GAGs, such as HS and chondroitin sulfate (ChS) prevent crystal attachment to the tubular epithelial surface [3,9,10]. Unfortunately, previous reports failed to confirm the exact role of cell surface HS, because the wild type MDCK cells do not express any type of cell surface heparan sulfate proteoglycan (HSPG, syndecan) [11]. Recently, we established novel MDCK cell lines (KIC-synd-1) that expressed the human syndecan-1 gene, and we confirmed that cell surface HS inhibited the COM crystal cell attachment [12]. In the present study, we examined the change of heparan sulfate proteoglycan (syndecan-1) expression on the KIC-synd-1 cells during oxalate exposure.

## MATERIALS AND METHODS

### *Cell cultures*

MDCK cells (ATCC CCL 34) were obtained from the Laboratory Products Division of Dainippon Pharmaceutical Co. (Osaka, Japan) and sub-cultured in minimum essential medium (MEM; Gibco BRL, Life Technologies, Inc, Gaithersburg, MD) containing 10% fetal calf serum (FCS; Gibco BRL) and 1% antibiotics (penicillin and streptomycin) / anti-micotic solution (Gibco BRL) at 37°C in a 5% CO<sub>2</sub> and 95% air atmosphere. Newly established MDCK transfectant that expressed human-syndecan-1 (KIC-synd-1) were grown in similar growth media containing neomycin (G418, 20 µg/ml). This cell line was transformed with human syndecan-1 cDNA derived from the human prostate cancer cell line (LNCaP; ATCC CRL 1740, Dainippon Pharmaceutical Co., Osaka, Japan) [12]. KIC-synd-1 cells that we established from MDCK transfectant has already been recognized at our ethical

committee.

### *Reverse Transcription (RT)-PCR*

To evaluate the expression of human-syndecan-1 mRNA in both MDCK cells and KIC-synd-1 cells, the following experiments were performed. In brief, 1×10<sup>6</sup> cells were harvested and their poly-(A<sup>+</sup>)-tailed RNA was isolated, then first strand cDNA was synthesized by manufacturer's protocol. RT-PCR was performed according to protocols detailed elsewhere. Amplifications were performed as 25 µl reactions consisting of 1 µg of cDNA, 0.65 U of recombinant Taq DNA polymerase (Qiagen GmbH, Hilden, Germany), 2.5 µl of 10× reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>), 200 µM of each deoxyribonucleotide triphosphate (dNTPs), and 1.25 µl each of the 5' and 3' primers (20 µM). After 3 min denaturation step, PCR consisted of 35 cycles, where each cycle consisted of 45 sec of denaturation at 94°C, 60 sec of annealing at 60°C, and 90 sec of extension at 72°C. The primer sequences for syndecan-1 were as follows: sense primer, 5'-ATGAG-GCGCGCGGCTCTGG-3' specific for human syndecan-1 (206-226); antisense primer: 5'-TGCAG-GGGTTGAGGTCTCATG-3' (689-709) [13]. To control for the presence of different amounts of cDNA in different mRNA preparations, human cyclophilin mRNA was amplified from each cDNA. Primer sequences for cyclophilin were as follows: sense primer, 5'-TTTATGTGTCAGGGTGGTGA CTTCA-3', corresponding to nucleotides 192 to 216, antisense primer, 5'-TATTCATGCCTTTCACTTTGC-3', corresponding to nucleotides 403 to 426 [14].

### *Application of oxalate containing solutions*

To evaluate the effect of oxalate on the cultured renal epithelial cells, we added the either 0.5 mM or 1 mM sodium oxalate in calcium nominally free solution which composition is the same with NT solution without calcium chloride (pH 7.4). Each test solution was applied to a 25 cm<sup>2</sup> culture dish (controlled to a total volume of 5 ml) and incubated at 37°C in the CO<sub>2</sub> incubator for 1 hr. In each experiment, a minimum of 5 replications was performed for each treatment.

### *Quantitative-competitive RT-PCR*

QC-RT-PCR was performed using an internal competitive template, as described elsewhere [15]. In brief, this QC-PCR is based on the assumption that the cDNA template and the competitive template compete equally for the primers and that amplification is internal control, a 318 bp fragment of the syndecan-1 gene

flanked by sequences homologous for the 5' and 3' ends and containing the gene specific probe sites was synthesized. To accomplish this, a PCR using the 5'-primer plus a modification of the 3'-primer were used. The modified 3'-primer as follows: 5'-TGC AGG GGT TGA GGT CTC ATG GAC TAC AGC CTC TCC CTC CTT-3' corresponding to nucleotides 689-709 and 503-523 of human syndecan-1 mRNA consists of two portions: a 5'-end which annealed to the normal binding site of the 3'-end of the syndecan-1 gene leaving the 3'-end of the primer un-annealed, plus a 3'-end which annealed at an internal site leaving the primer un-annealed. The PCR using this primer pair preferentially amplified the shorter 318 bp segment and synthesized the 3'-end primer site resulting in a 339 bp product. This PCR fragment was size-fractionated by electrophoresis through 1.3% agarose gel, the 339 bp band extracted using the QIAquick DNA extraction protocol (Qiagen Inc.), and ligated into the TA cloning vector, PCR 2.1 plasmids were selected following transformation of and growth in INV $\alpha$ F competent cells. Positive colonies were confirmed by identification and sequencing of a proper insert fragment following digestion with *Eco*RI. The plasmid DNA was purified by alkaline lysis and polyethylene glycol (PEG) precipitation, then quantified. QC-PCR was performed using 1  $\mu$ l of a dilution (from 10<sup>1</sup>-10<sup>9</sup> copies) of the synthetic internal competitive DNA template.

PCR products were resolved by electrophoresis through 1.3% agarose gel containing ethidium bromide, and then visualized in UV light. The gels were scanned and intensity of each PCR fragment was measured using image analyzer (Gel Doc 1000, Bio Rad, Hercules, CA). Band intensities were normalized for differences in molecular weights. We also sequenced the PCR products and compared them with those of cDNA clone.

#### *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of culture medium*

Culture supernatant (10 ml) was collected and concentrated to a volume of 500  $\mu$ l using a centricon 10 centrifuge tube assembly (Amicon, Inc, Beverly, MA). The GAG chains were digested by 0.01 U/ml heparitinase and 0.2 U/ml chondroitinase ABC protease free (Seikagaku Kogyo, Tokyo, Japan) for 1 hr at 37°C. For each supernatant, 5  $\mu$ l were applied through 10-20% gradient gels using the Laemmli buffer system and a Bio Rad Mini-Protean II apparatus (Bio Rad Laboratories, Hercules, CA). Proteins were visualized

by staining the gels with CBB-250R. Following SDS-PAGE, unstained gels were transferred (100 V for 2 hrs) to BioBlot nitrocellulose membranes (Coster Scientific Co., Cambridge, MA) by using a Mini Transblot apparatus (Bio Rad Laboratories). The membranes were blocked with bovine serum albumin (1%) in TBST (6.38 mM Tris-HCl, 25 mM NaCl, 0.05% Tween 20 [pH 8.0]). Membranes were treated for 16 hrs with mouse anti-human syndecan-1 monoclonal antibody (clone MCA681; Serotec Ltd, Oxford, U.K.) diluted 1: 500 in 1% BSA-TBST. Membranes were then treated for 2 hrs with peroxidase-conjugated IgG (Jackson ImmnoResearch, Pennsylvania, U.S.A.) for 30 min. Bound syndecan-1 was detected by ECL detection system (NEN Life Science Products, Boston, MA) and autoradiographed on rx FILM (Polaroid).

#### *Analysis of syndecan-1 production by immunohistochemistry*

The localization of syndecan-1 on KIC-synd-1 cells and on wild type MDCK cells were confirmed by immunohistochemistry with mouse anti human syndecan-1 core protein monoclonal antibody (clone MCA681). In brief, cells were grown by confluent in the 35 mm  $\phi$  glass bottom dish. Cells were rinsed with PBS twice and then fixed with 10% formalin for 40 min at room temperature. Formalin was removed and cells were rinsed with PBS twice. Add 2 ml of blocking buffer and incubate at room temperature. The first antibody (1:200 dilution) was added and incubated at room temperature for 30 min. The cells were rinsed with PBS and biotinylated second antibody was added and incubated for 30 min. After rinsed the cells with PBS and ABC buffer was added and incubated for 30 min. Immunohistochemical reaction was detected with DAB stain kit (Dako).

#### *In situ hybridization*

To evaluate the localization of syndecan-1 gene, in situ hybridization was performed. In brief, the syndecan-1 antisense oligo-DNA using thymine-thymine (T-T) dimerized DNA as a haptenic probe was selected complimentary to the mRNA sequence coding for amino acids of the human syndecan-1 core protein and the syndecan-1 sense oligo-DNA corresponded to the mRNA sequence (Hishikawa Y et al., Molecular Histochemical Techniques, Koji T. Ed. Springer-Verlag Tokyo). These two oligo-DNAs were added with two and three repeats of adenine-thymine-thymine (ATT) at the 5' and 3' ends for the T-T dimmers respectively. The oligo-DNAs were haptenized by UV (10,000 Jm<sup>-2</sup>) irradiation [16]. The oligo-DNA probe sequenc-

es of human syndecan-1 for *in situ* hybridization were as follows:

sense:

5'-TTATTACGACCCAGGGAGACCACACAGCTC-  
CCGACCACTCATCAGGCCTCAATTATTATT-3'  
(613-658, GenBank accession # BC008765)

anti-sense:

5'-TTATTATGAGGCCTGATGAGTGGTCG-  
GGAGCTGTGTGGTCTCCCTGGGTCGATTAT-  
TATT-3' (658-613, GenBank accession # BC008765)

MDCK cells and KIC-synd-1 cells, both subconfluent, were cultured on 4 well glass slides (Lab-Tek II chamber; Nalge Nunc, Naperville, IL) in a water-jacketed incubator according to the standard procedures. These cells were treated with 0.2 N hydrochloric acid (RT, 20 min) and digested with 2  $\mu$ g/ml of proteinase K (37°C, 15 min). After post-fixation with 4% paraformaldehyde in PBS (5 min), the cells were immersed in 2 mg/ml glycine in PBS (15 min) twice and kept in 40% de-ionized formamide in 4 X SCC until used for hybridization. Hybridization was carried out at 42°C for overnight with 2  $\mu$ g/ml T-T labeled oligo-DNA probes dissolved in the hybridization medium. After repeated washings, the signals were detected by enzyme immunohistochemistry as described above, without any counterstaining. To confirm the specificity of syndecan-1 mRNA signals, we conducted various types of control experiments concurrently with the test experiments. First, the sense probe was used as a negative control in every run. The cells were used to evaluate the level of hybridizable RNAs for 28S rRNA probe as a positive control in every case [17]. Furthermore, these cells were hybridized with T-T complementary oligo-DNA for 28S in the presence of an excess amount of 50-fold non-haptenized complementary oligo-DNA for 28S rRNA to provide definitive evidence for the sequence specificity.

#### *Analysis of GAGs chain by high performance liquid chromatography (HPLC)*

To evaluate the difference of GAGs composition between KIC-synd-1 cells and wild type MDCK cells, we performed HPLC. Both types of cells ( $1.0 \times 10^6$  cells) were seed on 25 cm<sup>2</sup> Falcon Flasks and grown by confluent. The cells were rinsed twice by PBS solution, then the cells were treated with 0.05 units of heparitinase I and II (from Flavobacterium heparinum, Seikagaku Kogyo, Tokyo, Japan) in 1 ml of 10 mM calcium acetate buffer (pH 7.0) at 37°C for 2 hrs. After each digestion, collect the supernatant and four volumes of ethanol (99.5%) were added to each reaction and mixture allowed to stand overnight at 4°C. Subse-

quently, these mixtures were centrifuged at 3,000 rpm for 20 min to remove any residual proteins. The supernatant was then dried under nitrogen gas and the residue dissolved in 100  $\mu$ l of mobile phase solution (0.05-2.3 M LiCl buffer). The HPLC system consisted of a Waters chromatography Model 626bLC system (Waters Co, Milford, MA) fitted with a Dionex CarboPac PA1 column ( $\phi$  4 $\times$ 250 mm). Using a sample of 100  $\mu$ l, chromatography was carried out at 40°C at a flow rate of 1.0 ml/min. Each component was detected at 230 nm with a UV detector (Waters 486 Tunable absorbance detector) and the elution response was monitored by an 805 data station computer system (Waters Ltd.) The concentration of HS was estimated from a calibration curve using by 805 data station computer system.

#### *Measurement of cell viability*

To assess cell viability under the presence or absence of oxalate, an MTT assay was performed [18]. In brief, both MDCK cells ( $4 \times 10^3$  cells/well) and KIC-synd-1 cells were seeded on a 96-well-plate (Falcon) and cultured for 24 hrs, and then used in the following experiment. Cells were incubated in 100  $\mu$ l of Ca<sup>2+</sup> free solution with or without NaOx (1 mM) for 30 min and one hr. For each plate, absorbance was monitored using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell growth assay kits (Chemicon, Temecula, CA, USA), according to the manufacturer's instructions. The number of viable cells was estimated by measuring the absorbance of each well with Easy Reader EAR 400 (SLT Lab Instruments, Salzburg, Austria). The rates of viable cells were obtained as the ratio of experimental absorbance (viable cell number in the experimental wells) to control absorbance (viable cell number in the control wells).

#### *Statistical analysis*

Data are expressed as mean  $\pm$  S.E.. Differences between data sets were analyzed by either the unpaired t or Mann-Whitney tests. A P < 0.05 was considered to be statistically significant.

## RESULTS

#### *Expression of syndecan-1 in MDCK cells*

As shown in Fig. 1, KIC-synd-1 cells and LNCaP cells show high expression of transfected human syndecan-1 gene, which appears as a band at 504 bp. In contrast, however, wild type MDCK cells do not ex-



press syndecan-1 gene. The PCR bands for cyclophilin (Fig. 1, bottom panel) showed similar band intensities for the different cDNA preparations, indicating that similar amounts of cDNA were available. Figure 2 shows Western blot analysis in each cell lines. Both KIC-synd-1 cells and LNCaP cells expressed syndecan-1 core protein (Fig. 2). However, wild type MDCK cells do not produce syndecan-1 core protein (Fig. 2). The localization of syndecan-1 on KIC-synd-1 cells and on wild type MDCK cells were confirmed by immunohistochemistry with anti-human syndecan-1 core protein monoclonal antibody. Syndecan-1 was significantly stained on basolateral surface and cytosol in KIC-synd-1 cells (Fig. 3C). However, both wild type MDCK cells and negative control (without antibody) did not show any stain against syndecan-1 monoclonal antibody (Figs 3A, B, D).

*Localization of syndecan-1 mRNA*

To examine the localization of syndecan-1 mRNAs in both MDCK and KIC-synd-1 cells, we performed in situ hybridization. The antisense probe for syndecan-1 mRNA was confirmed after staining KIC-synd-1 cells (Fig. 4C). Yet, the antisense probe for syndecan-1 mRNA gave negative results after MDCK cells (Fig. 4A) were stained. As a negative control, when cells were hybridized with T-T dimerized syn-

decn-1 sense probe, no signal was observed (Figs 4B, D).

*Increased production of HS in KIC-synd-1 cells*

To assess the level of cell surface HS concentration in both MDCK and KIC-synd-1 cell lines, HPLC was performed. Figure 5 shows the total amount of cell surface HS in both cell lines. Interestingly, the concentration of total HS was significantly higher in the KIC-synd-1 cells than that of MDCK cells ( $p < 0.05$ ).

*Increased expression of syndecan-1 mRNA in the KIC-synd-1 cells following exposure to oxalate*

A level of syndecan-1 mRNA expression in KIC-synd-1 cells prior to and following exposure to oxalate was determined by QC-RT-PCR (Fig. 6). Exposure of the KIC-synd-1 cells to oxalate resulted in nearly 20 times increase in the expression of syndecan-1 mRNA (Fig. 6A). Figure 6B shows the relative gene expression of syndecan-1 in each group. The expression of syndecan-1 gene was significantly increased in the oxalate-exposed cells ( $p < 0.01$ ).

*Increased production of syndecan-1 core proteins in KIC-synd-1 cells following oxalate exposure*

Culture medium collected from KIC-synd-1 cell

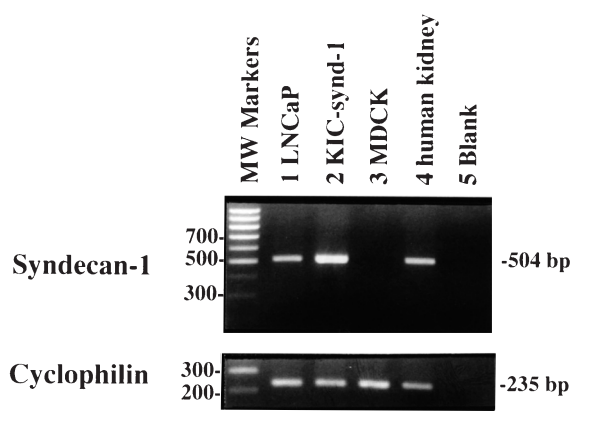


Figure 1 shows RT-PCR for human syndecan-1 mRNA in the cells and kidney tissue. KIC-synd-1 cells and LNCaP cells show high expression of transfected human syndecan-1 gene, which appears as a band at 504 bp. In contrast, however, wild type MDCK cells do not express syndecan-1 gene. The PCR bands for cyclophilin showed similar band intensities for the different cDNA preparations, indicating that similar amounts of cDNA were available. Lane 1; LNCaP, Lane 2; KIC-synd-1 cells, Lane 3; MDCK cells, Lane 4; human kidney, Lane 5; Blank

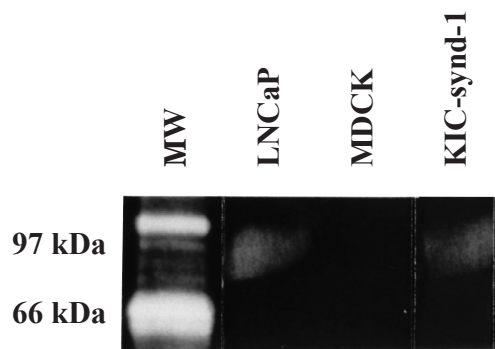
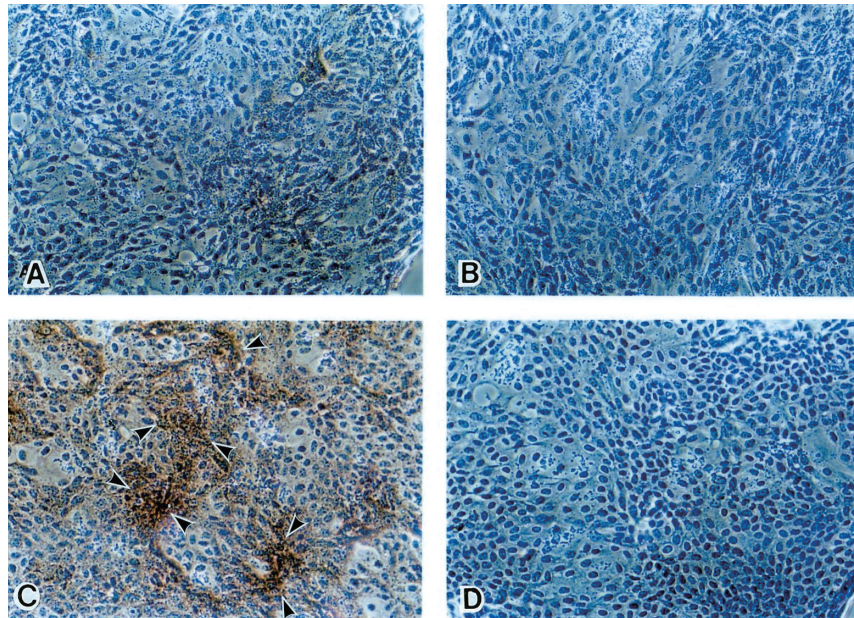
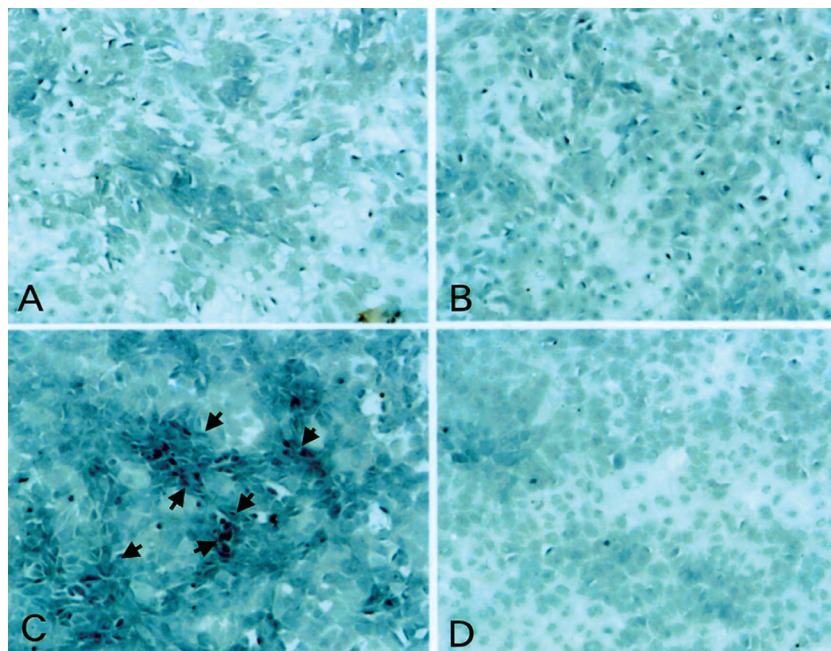


Figure 2 shows Western blotting for human syndecan-1 core protein in each cell line. Culture medium collected from each cell culture and subjected to SDS-PAGE. Each protein gel was transblotted onto PVDF membranes. The membranes treated with mouse anti-human syndecan-1 cells produced syndecan-1 core proteins. MDCK cells did not express syndecan-1 core protein. Lane 1; MW markers, Lane 2; LNCaP, Lane 3; MDCK cells, Lane 4; KIC-synd-1 cells



*Fig. 3.* The localization of syndecan-1 on KIC-synd-1 cells and on wild type MDCK cells were confirmed by immunohistochemistry with anti-human syndecan-1 core protein monoclonal antibody. Syndecan-1 was significantly stained on basolateral surface and cytosol in KIC-synd-1 cells (C). However, both wild type MDCK cells (A, B) and negative control (D, w/o antibody) did not show any stain against syndecan-1 monoclonal antibody. A; MDCK cells (negative control), B; MDCK cells (+MAB), C; KIC-synd-1 cells (+MAB), D; KIC-synd-1 cells (negative control)



*Fig. 4.* In situ hybridization in culture cells of MDCK and KIC-synd-1 cells is shown. Although syndecan-1 antisense in KIC-synd-1 cells (C) shows positive in cytoplasm through staining, negative indications were observed through MDCK cells after staining (A). Syndecan-1 sense in both MDCK (B) and KIC-synd-1 cells (D) gave negative findings after staining. Arrows indicate positive findings (C).

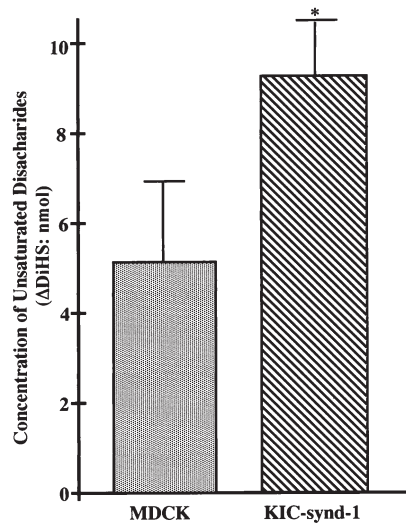


Fig. 5. Figure 5 shows the total amount of cell surface HS in both cell lines. The concentration of total HS was significantly higher in the KIC-synd-1 cells than that of wild type MDCK cells ( $P < 0.05$ ). (N=5 dishes in each group)

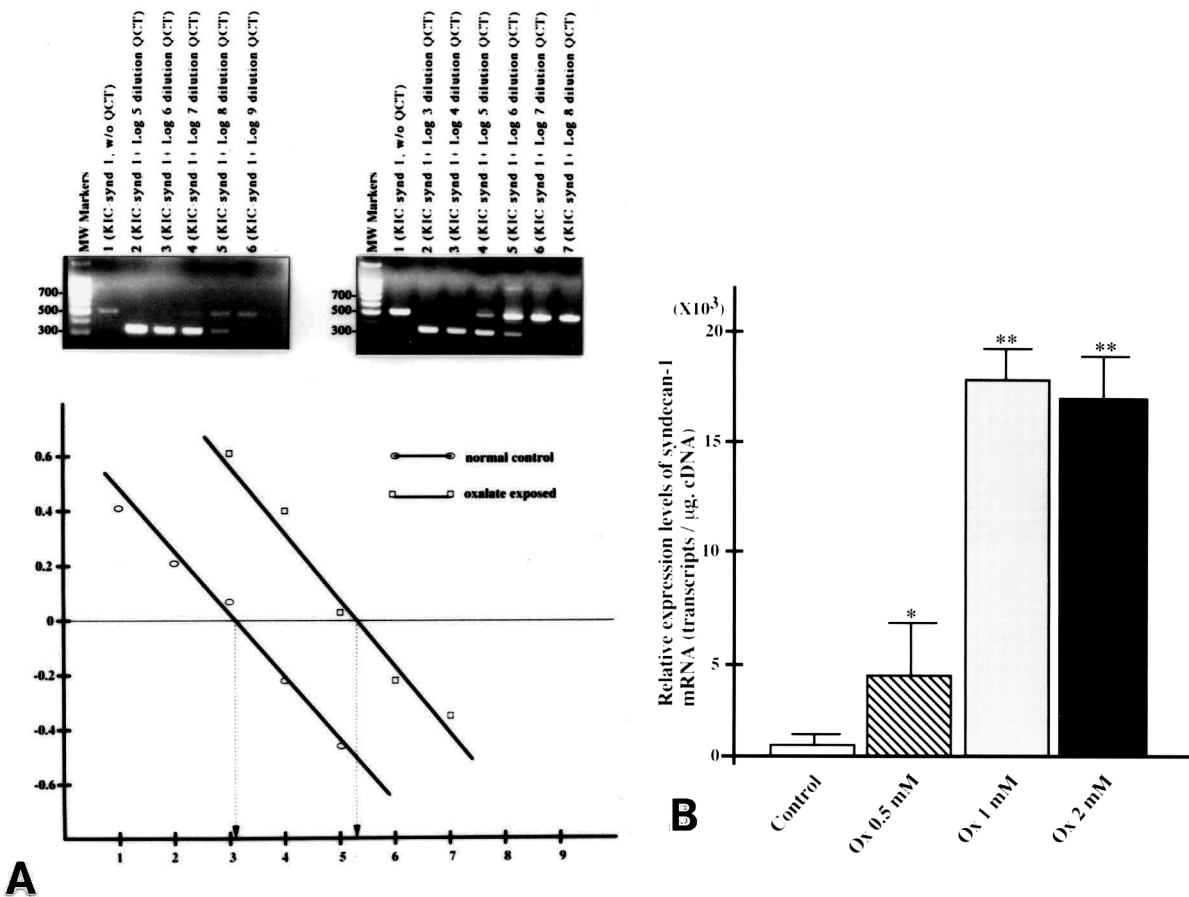
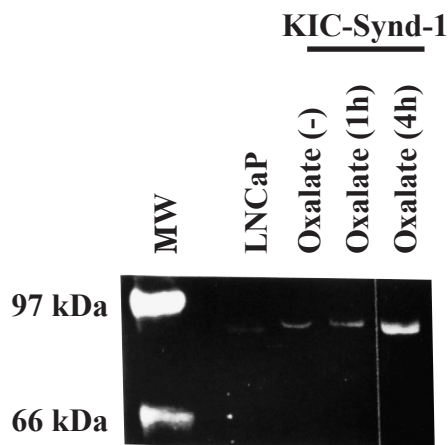
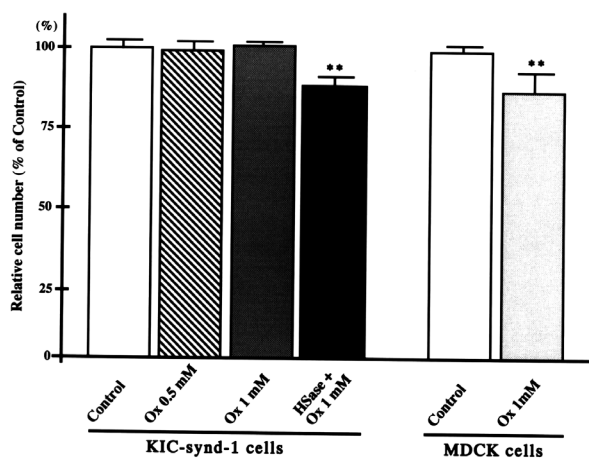


Fig. 6. Figure 6 shows the relative expression level of syndecan-1 mRNA in each group. Levels of syndecan-1 mRNA expression in KIC-synd-1 cells prior to and following exposure to oxalate was determined by QC-RT-PCR (Fig. 6A, top panel). Exposure of the KIC-synd-1 cells to oxalate resulted in nearly 20 times increase in the expression of syndecan-1 mRNA (Fig. 6A, bottom panel). Figure 6B shows the relative gene expression of syndecan-1 in each group. The expression of syndecan-1 gene was significantly increased in the oxalate-exposed cells (\* $p < 0.05$ , \*\* $p < 0.01$ ).



**Fig. 7.** Figure 7 shows that oxalate (1 mM) exposure for 1 hr increased production of syndecan-1 core protein (72 kDa). Syndecan-1 core protein slightly increased just after termination of oxalate exposure. Following 3 hrs incubation in oxalate-free medium developed farther production of protein. Lane 1; MW markers, Lane 2; LNCaP, Lane 3; KIC-synd-1, (control), Lane 4; KIC-synd-1, (1 mM oxalate exposed for 1hr and 4 hrs)



**Fig. 8.** Figure 8 shows the change of cell viability either presence nor absence of oxalate in each cells. The cell viability decreased significantly in wild type MDCK cells after 1 mM oxalate exposure ( $P < 0.05$ ). On the other hand, there was no significantly difference in the oxalate exposed KIC-synd-1 cells. However, cell viability decreased significantly in the heparitinase-treated cells before oxalate exposure. (N=5 dishes each)

cultures that were either untreated or treated with oxalate were concentrated 10-fold, and then subjected to SDS-PAGE. Each protein gel was transblotted onto PVDF membranes, the membranes treated with mouse anti-human syndecan-1 monoclonal antibody (MCA681) as described above. Figure 7 shows increased production of syndecan-1 core protein after oxalate exposure. Syndecan-1 core protein slightly increased just after termination of oxalate exposure. Moreover, the production of core protein tended to increase further 3 hrs incubation without oxalate exposure.

#### MTT assay

To assess the viability of the cells after oxalate exposure, MTT assay was performed. The cell viability decreased significantly in the MDCK cells after 1 mM oxalate exposure (Fig. 8). On the other hand, there was significantly difference in the oxalate exposed KIC-synd-1 cells. However, cell viability decreased significantly in the heparitinase-treated cells before oxalate exposure (Fig. 8).

## DISCUSSION

In this report, we provide the first direct evidence that a high concentration of extracellular free oxalate can induce an increased production of cell surface HSPG syndecan-1. In the kidney, HS is the major acid glycosaminoglycans (GAGs) constituent of both the glomerular and tubular basement membranes (GBM and TBM) [19]. Basement membrane type HSPG in GBM, so called perlecan is important for maintaining the integrity of glomerular size and the charge barrier [20,21]. A decrease in negatively charged molecules within the GBM is thought to be responsible for the albuminuria that arises under various nephropathic conditions [19-22]. On the other hand, the function of other type of HSPG, such as syndecan [23,24], remains unclear in the kidney. In general, syndecan is localized on the cell surface of a wide variety of mammalian epithelial cells, where it is involved in maintaining epithelial morphology and stabilization [23,24].

In the previous reports indicated that GAGs play an important role in crystal-cell interactions [3,9,10]. Removal of GAGs from renal epithelial cell surfaces promotes crystal adhesion and the process is recovered by treatment with sulfated polysaccharides, such as heparin, HS and chondroitin sulfate [9,10]. Pretreatment of CaOx crystals with sulfated GAGs prevents their adherence to LLC-PK1 and MDCK cells in cul-

ture [10]. Heparin also inhibits endocytosis of CaOx crystals on BSC-1 monkey kidney epithelial cells via an interaction with cells and not with crystals [25]. These observations support the hypothesis that cell-surface GAGs provide a protective barrier against crystals and oxalate ions. Recently, we have reported that oxalate induced the decline in cytosolic  $Ca^{2+}$  levels in the renal epithelial cells [26]. Moreover, the findings of both transmission electron microscopy and MTT assay revealed higher concentration of oxalate might injure the cells within short duration. Although the exact mechanism of the oxalate-induced change of cytosolic  $Ca^{2+}$  levels remains unclear, this phenomenon was strongly inhibited by presence of HS [26]. Ichimiya et al. [27] reported that decline of intracellular  $[Ca^{2+}]_i$  level reduced  $H_2O_2$ -induced cytotoxicity in rat renal kidney epithelial cells (NRK cells). They concluded that increasing of intracellular  $Ca^{2+}$  initiate cell death and Bcl-2 prevent oxidant-induced cell death by accelerate restoring  $Ca^{2+}$  into the mitochondria [27]. Our previous study suggest that the decline in  $[Ca^{2+}]_i$  is dramatically inhibited by the presence of either heparin or HS in a dose dependent manner [26]. Although the exact mechanism still remains unclear, these phenomena imply that cell surface HS prevent oxalate induced-cell injury.

Interactions between renal epithelial cells and CaOx crystals (or oxalate ions) no doubt play a crucial role in the formation of urinary stones [28,29]. Renal epithelial cells respond to hyperoxaluria and the presence of CaOx crystals both in vivo [15,30] and in vitro [31,32]. It is well known that calcium-binding proteins as well as GAGs chelate calcium and coat the surfaces of calcium containing crystals, which are then either excreted as crystalluria particles or endocytosed by the epithelial cells. Crystals produced in such an environment have protein coats and are apparently less reactive with renal epithelial cells [29]. Our previous study implied that CaOx nephrolithic conditions might induce increased expression of HSPG in the tubular epithelial cells and thereby protect and repair the damaged epithelial surface [4,5]. Moreover, Barsotti and colleagues reported on the effect of administered HS in rat remnant kidney model [33]. In their study, HS-treated rat kidney showed a lower prevalence of glomerular sclerosis, mesangial proliferation, and much less evident tubulointerstitial damage than controls. They concluded that HS might act as a renoprotective substance via anti-proliferate and anti-inflammatory effects [33].

Oxalate, which is a common constituent of kidney stones, induces renal epithelial toxicity [6,7,34]. More-

over, a high concentration of both oxalate and COM crystals also induces expression of various genes in renal epithelial cells as well as nephrolithic rat kidneys [15,35-38].

In this study, we explored the acute effect of oxalate on the expression of syndecan-1 in MDCK cells. Based on our data, the increased expression of syndecan-1 strongly correlated with oxalate induced cell injury. These facts mean the HS/HSPG have some important role to prevent CaOx nephrolithiasis. To extend present study, a prolonged period of lower concentration of oxalate exposure on the cells should be also explored to estimate an influence of chronic mild hyperoxalic condition. Further study is warranted to investigate these mechanisms.

**ACKNOWLEDGMENTS:** This work was supported in part by a Grant-in-Aid (#12307072) from the Ministry of Education, Science, Sports, and Culture in Japan. We thank Ms. Satoko Yamada for her help in preparing the cells for the transmission electron microscopic study.

## REFERENCES

1. Suzuki K, and Ryall RL. The effect of heparan sulfate on the crystallization of calcium oxalate in undiluted, ultrafiltered human urine. *Br J Urol* 1996; 78:15-21.
2. Yamaguchi S, Yoshioka T, Utsunomiya M, Koide T, Osafune M et al. Heparan sulfate in the stone matrix and its inhibitory effect on calcium oxalate crystallization. *Urol Res* 1993; 21:187-192.
3. Ebisuno S, Kohjimoto Y, Tamura M, and Ohkawa T. Adhesion of calcium oxalate crystal to Madin-Darby canine kidney cells and some effects of glycosaminoglycans or cell injuries. *Eur Urol* 1995; 28:68-73.
4. Iida S, Miyajima J, Suzuki K, Matsuoka K, Inoue M et al. Expression of heparan sulfate proteoglycan mRNA in rat kidneys during calcium oxalate nephrolithiasis. *Urol Res* 1997; 25:361-364.
5. Iida S, Inoue M, Yoshii S, Yanagaki T, Chikama S et al. Molecular detection of heparan sulfate proteoglycan mRNA in rat kidney during calcium oxalate nephrolithiasis. *J Am Soc Nephrol* 1999; 10:S412-416.
6. Scheid C, Koul H, Hill WA, Luber-Narod J, Jonassen J et al. Oxalate toxicity in LLC-PK1 cells, a line of renal epithelial cells. *J Urol* 1996; 155:1112-1116.
7. Koul HK, Koul S, Fu S, Santosham V, Seikhon A et al. Oxalate: From crystal formation to crystal retention. *J Am Soc Nephrol* 1999; 10:S417-421.
8. Wiessner JH, Hasegawa AT, Hung LY, Mandel GS and Mandel NS. Mechanisms of calcium oxalate crystal attachment to injured renal collecting duct cells. *Kidney International* 2001; 59:637-644.
9. Verkoelen CF, Romijn JC, Cao LC, Boeve' ER, Bruijn WC et al. Crystal-cell interaction inhibition by polysaccharides. *J Urol* 1996; 155:749-752.
10. Gill WB, Jones KW, and Ruggiero KJ. Protective effects of

- heparin and other sulfated glycosaminoglycans on crystal adhesion to injured epithelium. *J Urol* 1982; 127:152-154.
11. Svennevig K, Prydz K, and Kolset SO. Proteoglycans in polarized epithelial Mardin-Darby canine kidney cells. *Biochem J* 1995; 311:881-888.
  12. Chikama S, Iida S, Inoue M, Kawagoe N, Tomiyasu K et al. Role of heparan sulfate proteoglycan (syndecan-1) on the renal epithelial cells during calcium oxalate monohydrate crystal attachment. *Kurume Med J* 2002; 49:201-210.
  13. Mali M, Jaakkola P, Arvilommi A-M, and Jalkanen M. Sequence of Human Syndecan Indicates a Novel Gene Family of Integral Membrane Proteoglycans. *J Biol Chem* 1990; 265:6884-6889.
  14. Haendler B, Hofer-Warbinek R, and Hofer E. Complementary DNA for human T-cell cyclophilin. *EMBO J* 1987; 4:947-950.
  15. Iida S, Peck AB, Johnson-Tardieu J, Moriyama M, Glenton PA et al. Temporal changes in mRNA expression for bikunin in the kidneys of rats during calcium oxalate nephrolithiasis. *J Am Soc Nephrol* 1999; 10:986-996.
  16. Koji T, and Nakane PK. Recent advances in molecular histochemical techniques : in situ hybridization and southwestern histochemistry. *J Electron Microscopy* 1996; 45:119-127.
  17. Yoshii A, Koji T, Ohsawa N, and Nakane PK. In situ localization of ribosomal RNAs is a reliable reference for hybridizable RNA in tissue sections. *J Histochem Cytochem* 1995; 43:321-327.
  18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Imm Met* 1983; 65:55-63.
  19. Heuvel LPWJ, Born J, Veerkamp JH, Velden TJAM, Schenkels L et al. Heparan sulfate proteoglycan from human tubular basement membrane. Comparison with this component from the glomerular basement membrane. *Biochim Biophys Acta* 1990; 1025:67-76.
  20. Farquhar MG. The glomerular basement membrane: a selective macromolecular filter. In: *Cell biology of extracellular matrix*, ed. Hay ED. Plenum Press, New York, pp 335-378, 1981.
  21. Farquhar MG, Lemkin MC, and Stow JL. Role of proteoglycans in glomerular function and pathology. In: *Nephrology*, ed. Robinson RR. Springer-Verlag, New York pp 580-600, 1985.
  22. Tamsma JT, Van Den Born J, Bruijin JA, Assmann KJM, Weening JJ et al. Expression of glomerular extracellular matrix components in human diabetic nephropathy: decrease of heparan sulphate in the glomerular basement membrane. *Diabetologia* 1994; 37:313-320.
  23. Bernfield M, Kokenyesi R, Kato M, Hinkes MT, Spring J et al. Biology of the syndecans: a family of transmembrane heparan sulfate proteoglycans. *Annu Rev Cell Biol* 1992; 8:365-393.
  24. Jalkanen M. Biology of cell surface heparan sulfate proteoglycans. *Med Biol* 1987; 65:41-47.
  25. Lieske JC, and Toback FG. Regulation of renal epithelial cell endocytosis of calcium oxalate monohydrate crystals. *Am J Physiol* 1993; 264:F800-807.
  26. Iida S, Ishimatsu M, Chikama S, Inoue M, Matsuoka K et al. Protective role of heparin/heparan sulfate on oxalate induced changes in cell morphology and intracellular  $Ca^{2+}$ . *Urol Res* 2003; 31:198-206.
  27. Ichimiya M, Chang SH, Liu H, Berezsky IK, Trump BF et al. Effect of Bcl-2 on oxidant-induced cell death and intracellular  $Ca^{2+}$  mobilization. *Am J Physiol* 1998; 275:C832-839.
  28. Khan SR. Calcium oxalate crystal interaction with renal tubular epithelium, mechanism of crystal adhesion and its impact on stone development. *Urol Res* 1995; 23:71-79.
  29. Khan SR. Tubular cell surface events during nephrolithiasis. *Curr Opin Urol* 1997; 7:240-247.
  30. Gokhale JA, Glenton PA, and Khan SR. Localization of tamm-horsfall protein and osteopontin in a rat nephrolithiasis model. *Nephron* 1996; 73:456-461.
  31. Hammes MS, Lieske JC, Spargo BH, and Toback FG. Calcium oxalate monohydrate crystals stimulate gene expression in renal epithelial cells. *Kidney International* 1995; 48:501-509.
  32. Lieske JC, Hammes MS, Hoyer JR, and Toback FG. Renal cell osteopontin production is stimulated by calcium oxalate monohydrate crystals. *Kidney International* 1997; 51:679-686.
  33. Barsotti G, Cupisti A, Gervasi GB, Bartoli C, Barsotti M et al. Effects of oral administration of heparan sulphate in the rat remnant kidney model. *Nephron* 1999; 81:310-316.
  34. Kohjimoto Y, Kennington L, Scheid CR, and Honeyman TW. Role of phospholipase A2 in the cytotoxic effects of oxalate in cultured renal epithelial cells. *Kidney International* 1999; 56:1432-1441.
  35. Koul H, Kennington L, Nair G, Honeyman T, Menon M et al. Oxalate-induced initiation of DNA synthesis in LLC-PK1 cells, a line of renal epithelial cells. *Biochem Biophys Res Commun* 1994; 205:1632-1637.
  36. Iida S, Peck AB, Byer KJ, and Khan SR. Expression of bikunin mRNA in renal epithelial cells after oxalate exposure. *J Urol* 1999; 162:1480-1486.
  37. Umekawa T, Kohri K, Kurita T, Hirota S, Nomura S et al. Expression of osteopontin messenger RNA in the rat kidney on experimental model of renal stone. *Biochem Mol Biol Int* 1995; 35:223-230.
  38. Grover PK, Miyazawa K, and Ryall RL. Renal prothrombin: quantification of mRNA using reverse transcription-polymerase chain reaction in a rat model. *Electrophoresis* 2004; 25:797-803.

# Longitudinal Psychological Effects of the Garuda Indonesia Air Disaster in Japan

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*Received 4 December 2007, accepted 31 January 2008*

**Summary:** We examined the general health and psychological symptoms among survivors of the 1996 Garuda Indonesia air disaster in Japan. We conducted a prospective study 6 months and 1 year (Study 1) after the disaster. A retrospective follow-up study was performed ten years after the disaster (Study 2). The mean score on the 28-Item General Health Questionnaire was 6.5 (SD=6.9) 1 year after the disaster. Those who witnessed the death of an acquaintance in the disaster were classified into the high risk group. In Study 2, more than one-third of respondents complained of a flying phobia. These findings indicate that the psychological burdens of air disasters may last as long as 10 years.

**Key words** air disasters, coping behavior, flying phobia, general health questionnaire, longitudinal study

## INTRODUCTION

Transportation accidents on land, in the air, or at sea form an important class of technological disasters [1,2]. Air disasters in particular can produce a greater number of victims at one time than other forms of transportation. Because air disasters often have a high mortality rate, there are few systemic studies on the psychosocial consequences of air disaster survival on passengers, in contrast to rescue workers [3-5] or community residents [6,7]. One of the most systematic surveys of surviving passengers was conducted after the Kegworth air disaster, in which 47 people died. Gregg et al. [8] assessed 68 of the 79 survivors at a clinical interview within one year after the disaster. Twenty-seven survivors had posttraumatic stress disorder (PTSD) in the first year, and 9 of this group also met DSM-III-R criteria for major depression. Those who saw injured or dead people at the scene, sustained less severe injuries, or were under 35 years of age were significantly more likely to develop PTSD. Sloan [9] followed-up 30 survivors of a non-fatal airplane crash and found high levels of stress in the following months.

The Garuda Indonesia air disaster in Japan occurred in 1996. Three passengers died and 108 were injured. A mental health care service team was organized after the disaster in cooperation with mental health experts (i.e., psychiatrists, clinical psychologist, nurses, and social workers) of Fukuoka Prefecture and the Department of Psychiatry at Kurume University. We planned a prospective study of general health and psychological symptoms related to the disaster among the survivors. This study was carried out at six months (first examination) and one year (second examination) after the disaster. Members of the study team visited the homes or offices of survivors, and interviews were conducted in an outreach setting. This was the first psychological intervention study of the survivors of an air accident in Japan. Initially we assumed that psychological effects would decrease at one year after the disaster. However, there was no improvement in symptoms, such as vehicle phobias or mental health, at the time of the second examination. A retrospective follow-up study was conducted ten years after the disaster, consisting of a mail survey on general health and psychological symptoms that was almost identical to the one used in the first examina-

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Abbreviations: GHQ-28, 28-Item General Health Questionnaire; PTSD, posttraumatic stress disorder.

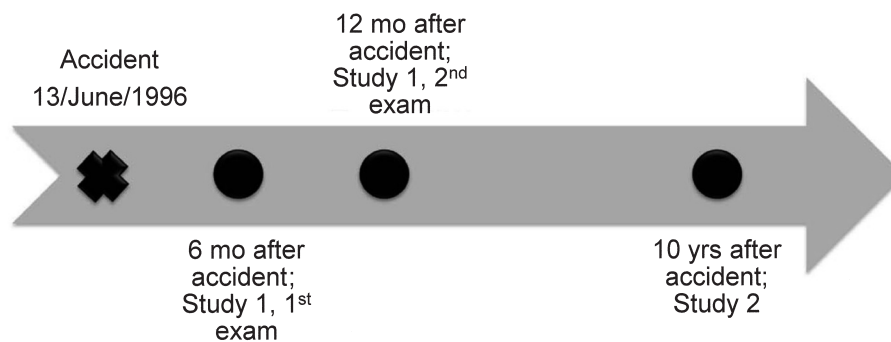


Fig. 1. Schedule of Study 1 and Study 2. Study 1, 1st exam was held at 6 months after the accident. Study 1, 2nd exam was held at 1 year after the accident. Study 2 was held 10 years after the accident.

tion, as well as an interview session to further elucidate psychological effects. In this article, we report the initial two-part prospective study as Study 1, and the retrospective follow-up as Study 2.

This study had two aims. One was to elucidate the psychological effects of the Garuda Indonesia on the survivors. The other was to consider the necessity of a long-term mental health care system for victims of transportation disasters.

#### *The incident and the interventions*

On 13 June 1996, Garuda Indonesia Airways Flight 865 (260 passengers, 15 crew members) failed to take off and crashed at the Fukuoka Airport in Japan. The entire fuselage of the plane went up in flames. Despite the great efforts of rescue teams, three passengers died and 108 were injured. All of the dead passengers were seated in the rear of the plane, on the right side. Most of the passengers lived in Fukuoka and were going on a trip to Bali Island with their colleagues from work. One survivor described the incident thus: "I felt frightened when the airplane started slipping on the runway. Fire came from the floor, and I could not see through the clouds of smoke. I desperately tried to remove my seat belt, but it would not move. I thought it was the end. I wanted to cry out for help; but I don't know if I did. I don't remember anything after that." (author's translation)

Fukuoka Prefecture's mental health team planned an outreach program and prospective mental health survey (Study 1) in September 1996, three months after the disaster. The first outreach and the first examinations were conducted by 50 experts, in pairs. Some survivors received continuous outreach service due to

health problems (physical or mental) or on request. After the second examination, conducted one year after the disaster, a single session of group psychoeducation was provided at one company where more than ten survivors worked. After the second examination, survivors received only individual care or treatment until the follow-up study. Schedule of the studies was shown in Fig. 1.

## METHODS

### *Study 1*

Study 1 was a prospective survey. Of the 87 survivors (all Japanese), excluding children under 11 years of age, living in Fukuoka Prefecture, study respondents comprised 84 at the first examination and 83 at the second. The response rate reached approximately 95%. Unfortunately, due to our own methodological errors, only 57 survivors responded to the self-rating questionnaire at the second examination. Table 1 summarizes the number of respondents.

Assessments were conducted in December 1996 for the first examination and in June 1997 for the second. Subjects were evaluated using the 28-item General Health Questionnaire (GHQ-28 [10]; the cut-off score of the Japanese version is 6/7) and a self-rating questionnaire on psychological symptoms. The latter questionnaire consisted of 6 items (flying phobia, phobia of other vehicles, difficulty in concentrating, hypersensitivity to noise or vibration, irritability and insomnia). Each item was rated in terms of its severity (rated on a 0-3 scale). We considered scores over 2 to confirm the existence of a symptom.



### Study 2

Study 2 was constructed as a retrospective follow-up survey 10 years after the disaster. The self-rating questionnaire and the interview by a psychiatrist or clinical psychologist were administered separately. Only 21 survivors (16 males, 5 females) responded to the self-rating questionnaire. The response rate of 24% was partially due to the fact that 22 survivors could not be contacted. We interviewed nine survivors among the respondents to the self-rating questionnaire, after obtaining written informed consent.

TABLE 1.  
*Study 1 variables*

Variables	Number of Respondents	
	First	Second
Exam		
Demographic characteristics	83	83
GHQ-28	83	82
Self-rating questionnaire	75	57

(Data missing for some subjects)

TABLE 2.  
*General Health Questionnaire (28 items) scores for Study 1*

Variables	First exam	S.D.	Second exam	S.D.
	Average		Average	
GHQ-28 total score	5.7	6.1	6.5	6.9
Subscales of GHQ-28				
Somatic concern	2.1	2.2	2.2	2.3
Anxiety/insomnia	2.2	2.3	2.5	2.4
Social dysfunction	1.0	1.5	1.3	2.0
Depression	0.5	1.4	0.5	1.5

TABLE 3.  
*Characteristics at the first examination*

Variables	GHQ high risk	GHQ low risk
	at Wave 1	at Wave 1
Sex		
Male	20	38
Female	8	17
Age at the accident		
12-40	10	16
Over 40	17	39
Seat		
Near victims	22	38
Distant to victims	6	17
Physically injured		
Underwent treatment	22	33
No treatment	6	21
Witnessed death of acquaintance*		
Yes	11	7
No	17	45

\*  $p=0.012$  by Fisher's exact test

For Study 2, Questions on Useful Coping Behavior [11] were added to the GHQ-28 and self-questionnaire on psychological symptoms to assess the coping behaviors which survivors considered useful for their recovery. There were 6 categories in this questionnaire: 1) talking and gathering with others, 2) obtaining information on health problems from public organizations, 3) leisure activities, 4) work, 5) avoidance (including sleeping) and 6) humor. For each category, we asked survivors whether or not these behaviors had been useful for their recovery.

In interview sessions, we focused on 4 themes: 1) psychological symptoms, 2) duration of recovery, 3) useful coping behaviors and 4) views on post-disaster mental health services. The main purpose of the interview sessions was to obtain survivors' narratives after ten years.

### Statistical analysis

Statistical analysis employed SPSS, version 14.0 for Windows. Fisher's exact tests were used for comparing the different groups and a Wilcoxon signed-rank test were used for comparing GHQ-28 scores. The significance level was less 5%.

### Ethical issues

This research received approval from the ethics review board at Kurume University. Written informed consent was obtained from all the respondents and confidentiality of ratings was assured. Respondents' anonymity is preserved. Authors followed the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1984 and Declaration of Tokyo, 1975).

## RESULTS

### Study 1

The mean GHQ-28 score was 5.7 (SD=6.1) at the first examination and 6.5 (SD=6.9) at the second examination. There was no significant difference between the results of the first and second examinations (Table 2). Results for subscales of GHQ-28, of which the anxiety/insomnia subscale was the highest, followed by somatic concerns, social dysfunction and depression, in that order, did not change throughout the examinations. The high-risk ratio (over the cut-off score) remained over 30% (33.7% at the first examination, 34.1% at the second examination).

To assess the characteristics of GHQ-28 for the high risk group, Fisher's exact tests were conducted. Table 3 showed that those who witnessed the death of

an acquaintance in the disaster were more often classified into the high risk group at the first examination (P=0.012). A similar pattern was observed at the second examination (Table 4; p=0.001). Other variables, such as sex, age, physical injury and seat location, were not significantly different.

Psychological symptoms reported by survivors are shown in Fig. 2. At the first examination, flying phobia was reported by 89% of respondents, followed by hypersensibility (60.8%) and phobia of other vehicles

(47.9%). At the second examination, although the ratio of flying phobia and phobia for other vehicles decreased, irritability and insomnia got worse. Hypersensibility showed some improvement but was still over 40% at the 2nd exam.

*Study 2 (self-rating questionnaire)*

The mean GHQ-28 score of 21 respondents was 6.6 (SD=5.9). The order of subscales in GHQ-28 did not differ from Study 1, with anxiety/insomnia the

TABLE 4.  
*Characteristics at the second examination*

Variables	GHQ high risk at Wave 2	GHQ low risk at Wave 2
Sex		
Male	20	37
Female	8	17
Age at the accident		
12-40	6	20
Over 40	21	34
Seat		
Near victims	22	37
Distant to victims	6	17
Physically injured		
Underwent treatment	19	35
No treatment	9	18
Witnessed death of acquaintance**		
Yes	12	5
No	16	46

TABLE 5.  
*Characteristics at Study 2*

Variables	GHQ high risk at Study 2	GHQ low risk at Study 2
Sex		
Male	5	9
Female	4	2
Age at the accident		
12-40	3	2
Over 40	6	9
Seat		
Near the victims	5	7
Distant to victims	4	4
Physically injured		
Underwent treatment	9	7
No treatment	0	4
Witnessed death of acquaintance		
Yes	3	2
No	5	8

\*\* p=0.001 by Fisher's exact test

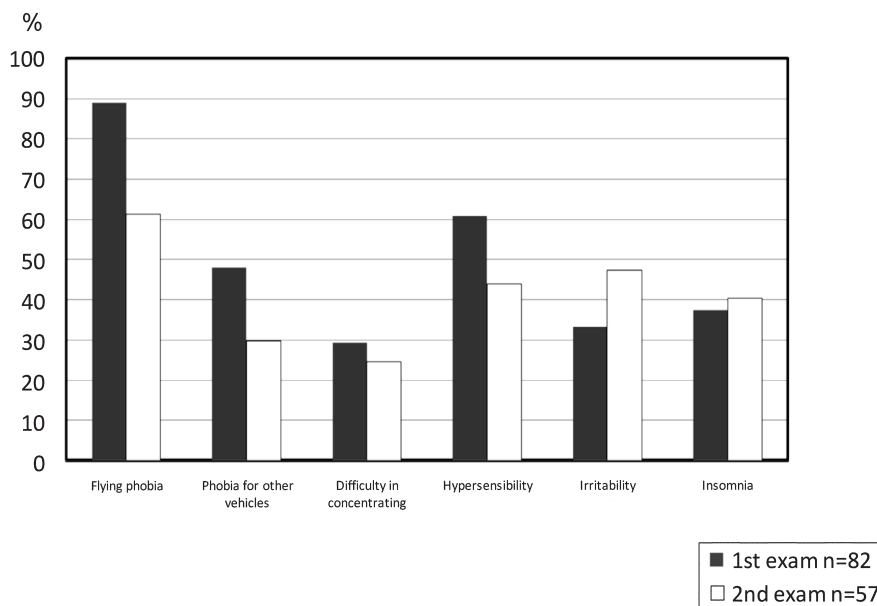


Fig. 2. The ratio of subjects with psychological symptoms at Study 1. Left (Black bar) indicates the 1st exam and Right (White bar) the 2nd exam.

highest, followed by somatic concerns, social dysfunction and depression. The high risk ratio reached 45%. Fisher's exact tests revealed no significant differences among variables.

More than one-third of respondents complained of flying phobia or hypersensitivity in the questionnaire on psychological symptoms, and one-fourth reported insomnia. No respondent in Study 2 reported phobias of other vehicles.

Results from Questions on Useful Coping Behaviors (multiple answers), showed that the most commonly reported coping behaviors were talking and gathering with others (76.2%), work (71.4%), avoidance (70%) leisure activities (63.2%), humor (55.0%), and obtaining information on health problems from public organizations (52.4%).

#### *Study 2 interviews*

As respondents included only nine survivors, we were unable to conduct a statistical analysis of the interview sessions. Instead, the interviews indicated overall points of view. Individual statements were omitted for the protection of privacy.

Most survivors stated that while they could board an airplane, flying phobias remained even after 10 years. Some reported that they felt great fear at take-off; one survivor had made a habit of checking the exit when he/she entered a building because he/she felt anxious if there was no place to escape. None of the respondents reported use of professional mental health care services.

The majority of respondents said it had taken about 5 years to recover. They considered one or two years to be too short to achieve full recovery. In a free discussion on coping behavior, although there were few concrete answers, several respondents mentioned spiritual aspects of recovery, e.g., "I believe in the existence of God, so I will make the most of my opportunities". Another respondent replied, in reference to his/her way of thinking, "I changed my mind to think positively".

Survivors were asked about the types of mental health services that are required for the victims of disasters. Most respondents preferred to receive outreach services at home instead of at their offices. They also agreed that local governments, not residents, should take the initiative in providing information on mental health.

## DISCUSSION

Survivors of the Garuda Indonesia air disaster suf-

fered significant losses in mental health for at least one year. Further, our study of these survivors indicates that psychological consequences may remain after 10 years. Our results in Study 1 were nearly the same as those of the Kegworth air disaster [8], but provided a striking contrast to Sloan's result [9], in which most passengers recovered in only a few months. However, the disaster reported by Sloan was a non-fatal accident, and all passengers belonged to the same college sport club. Thus, they were able to derive additional psychological support from their friends and teammates.

Although we must take into consideration that the response rate was relatively low in Study 2, the risk rate on the general health questionnaire was unexpectedly higher in Study 2 than in Study 1. A prospective, longitudinal epidemiologic study in a community sample of 2,548 adolescents and young adults (aged 14-24 years) showed that PTSD symptoms in 52% of cases remitted after 34-50 months [11]. If it is true that the psychological burdens of traumatic experiences gradually decrease in the natural course of recovery, some biases may have affected the results of Study 2. We must then identify these biases. One hypothesis is that respondents of Study 2 might have more severe psychopathology than non-respondents. The high risk ratio of Study 2 respondents was 52.4% at 6 months and 33.7% at 1 year after the accidents. Moreover, a review article by Gavrilovic et al. [12] considered that the most important factors associated with treatment-seeking behavior for mental health services appears to be a higher level of current psychopathology. Based on their findings, it is possible that those who had more severe mental health problems were more likely to have responded to our surveys in Study 2.

Our results showed the importance of research studies with a longitudinal view. Recently, ethical issues related to trauma research have become an issue for discussion, because the risks and benefits of participation in disaster-focused research are not fully understood [13]. For example, whether or not survivors are harmed when they participate in surveys is a point of controversy. If the risks of longitudinal studies are high, ethical considerations may preclude this type of research. Griffin et al. [14] examined participant reactions to different trauma assessment procedures for domestic violence, rape and physical assault and found that trauma survivors in fact viewed research studies as interesting and valuable experiences. Survivors might also recognize studies as opportunities to communicate their experiences and attitudes to others. While researchers should consider the potential risk or

harm of a study, overly cautious inquiry can also render data useless. In some cases, therapeutic significance may be achieved if researchers are able to provide longitudinal intervention for survivors. Survivors' statements from our interviews support our view that outreach or information from public offices is important. These results indicate that survivors may desire the active involvement of mental health service providers or researchers.

This study had numerous limitations. Subjects comprised residents of Fukuoka Prefecture only, and we could not consider a causal relationship between the studies, as Study 1 was prospective and Study 2 was retrospective. In Study 2, only 24% of respondents answered the self-rating questionnaire, and we were able to interview only nine survivors. Thus, the results of Study 2 were used to create hypotheses on longitudinal psychological consequences, instead of as supporting data. A well-designed, longitudinal prospective research study is still required. Although we have insufficient evidence to support our conclusion that the psychological burden of air disasters might be long lasting, we believe our data may have great value in considering longitudinal psychological effects on air disaster survivors and the usefulness of mental health care systems.

**ACKNOWLEDGMENTS:** This work was supported in part by a grant from the Japanese Ministry of Health, Labour and Welfare (Emergency medicine and public mental health services for transportation disasters).

## REFERENCES

- Norris FH. Psychosocial consequences of disasters. *PTSD Research Quarterly* 2002; 2:1-7.
- Norris FH, Friedman MJ, Watson PJ, Byrne CM, Diaz E et al. 60,000 disaster victims speak: Part I. An empirical review of the empirical literature, 1981-2001. *Psychiatry* 2002; 65:207-239.
- Epstein RS, Fullerton CS, and Ursano RJ. Posttraumatic stress disorder following an air disaster: a prospective study. *Am J Psychiatry* 1998; 155:934-938.
- Fullerton CS, Ursano RJ, and Wang L. Acute stress disorder, posttraumatic stress disorder, and depression in disaster or rescue workers. *Am J Psychiatry* 2004; 161: 1370-1376.
- Witteveen AB, Bramsen I, Twisk JW, Huizink AC, Slottje P et al. Psychological distress of rescue workers eight and one-half years after professional involvement in the Amsterdam air disaster. *J Nerv Ment Dis* 2007; 195:31-40.
- Brooks N, and McKinley W. Mental health consequences of the Lockerbie disaster. *J Trauma Stress* 1992; 5:527-543.
- Chung MC, Chung C, and Easthope Y. Traumatic stress and death anxiety among community residents exposed to an aircraft crash. *Death Stud* 2000; 24:689-704.
- Gregg W, Medley I, Fowler-Dixon R, Curran P, Loughrey G et al. Psychological consequences of the Kegworth air disaster. *Br J Psychiatry* 1995; 167:812-817.
- Sloan P. Post-traumatic stress in survivors of an airplane crash-landing: a clinical and exploratory research intervention. *J Trauma Stress* 1988; 1:211-229.
- Goldberg D. *Manual of the General Health Questionnaire*. NFER-Nelson, London, 1978.
- Perkonig A, Pfister H, Stein MB, Hofler M, Lieb R et al. Longitudinal course of posttraumatic stress disorder and posttraumatic stress disorder symptoms in a community sample of adolescents and young adults. *Am J Psychiatry* 2005; 162:1320-1327.
- Gavrilovic JJ, Schutzwahl M, Fazel M, and Priebe S. Who seeks treatment after a traumatic event and who does not? A review of findings on mental health service utilization. *J Trauma Stress* 2005; 18:595-605.
- Collogan LK, Tuma F, Dolan-Sewell R, Borja S, and Fleischman AR. Ethical issues pertaining to research in the aftermath of disaster. *J Trauma Stress* 2004; 17:363-372.
- Griffin MG, Resick PA, Waldrop AE, and Mechanic MB. Participation in trauma research: is there evidence of harm? *J Trauma Stress* 2003; 16:221-227.

## The Oldest Patient with Gallstone Ileus: Report of a Case and Review of 176 Cases in Japan

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*Received 29 February 2008, accepted 10 June 2008*

Edited by HISAFUMI KINOSHITA

**Summary:** We report a 91-year-old woman presenting bowel obstruction due to impacted gallstone, who was the oldest patient which has been reported in Japanese scientific literature. The patient was referred to our hospital due to vomiting and abdominal pain. Computed tomography and abdominal X-ray showed dilated loops of small intestine associated with air-fluid levels, pneumobilia, and a calcified mass in the left iliac fossa. After the diagnosis of bowel obstruction due to gallstone was made, an enterotomy and lithotomy was performed under spinal anesthesia. The postoperative recovery was uneventful. We also reviewed 176 cases of gallstone ileus which were reported in the Japanese literature in the past 20 years. The retrospective analysis demonstrated that one-stage enterolithotomy alone may be acceptable as the first choice of operative treatment. The gallstone ileus is a rare, but important disease because urgent and appropriate surgical therapy is required.

**Key words** gallstone ileus, bowel obstruction, pneumobilia, cholecystoduodenal fistula, surgery

### INTRODUCTION

Gallstone ileus is a rare mechanical intestinal obstruction caused by the passage of gallstones into the gastrointestinal lumen. Gallstone ileus accounts for only about 1-3% of cases of mechanical obstruction of the small bowel, but it is a quite important disease especially in elderly patients, as this disease shares 25% of all small bowel obstructions in patients older than 65 years [1]. As the majority of the elderly patients frequently have concomitant diseases and show relatively poor clinical manifestations, it is important to recognize this disease to avoid delayed diagnosis. Furthermore, its high incidence among elderly people may explain its association with chronic and degenerative factors, which may increase the complexity of the treatment choice.

Herein, we present a case of gallstone ileus in a 91-year-old woman with small bowel obstruction, who was successfully treated by surgery. Also, we reviewed the literature written in Japanese for Japanese Medical Journals or abstracts presented at Japanese conferences with available information between 1985 and 2005 using the website of IGAKUCHUO ZASSHI, and analyzing the clinical data for 176 Japanese patients of gallstone ileus. This article may assist the physician and surgeons to further understand this disease, as well as to illustrate diagnostic and therapeutic pitfalls, and determine the current therapeutic strategy based on a detailed analysis of a number of Japanese cases. Notably, our patient was the oldest patient which has been reported in Japanese scientific sources.

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Abbreviation: CT, computed tomography.

## CASE REPORT

A 91-year-old Japanese woman was referred to our hospital due to vomiting and abdominal pain. She appeared dehydrated with a fever of 38.3 degrees. Past medical history was unremarkable with no episodes of cholecystitis. No other concomitant disease was determined to be present. Blood pressure was 116/65 mmHg and pulse rate was 74 beats/min. Her abdomen was slightly distended but soft with central and left-sided tenderness without muscle guarding. Bowel sounds were weak but audible. Neither jaundice nor anemia was recognized. Significant laboratory results were: white blood cell count, 10400/ $\mu$ L, C-reactive protein, 1.26 mg/mL; total bilirubin, 2.8 mg/dL; lactate dehydrogenase (LDH), 486 IU/L. Plain abdominal X-ray showed marked small bowel distention with air-fluid level, and the presence of a round calcified component in the pelvic cavity (Fig. 1). Computed tomography (CT) of the abdomen revealed pneumobilia and distention of the jejunum, with a high density mass of 2.5 cm in diameter in the alimentary tract (Fig. 2). Air in the gallbladder and an adhesion between the thickened gallbladder wall and duodenal wall was visualized. The targeted lesion was located inside the ileum and consisted of two layers; rim calcification and an internal area of isodensity. There was fluid pooling in the proximal intestine of the high density mass, suggesting the cause of the mass to be bowel obstruction. Based on these radiological and clinical assessments, the diagnosis of bowel obstruction due to gallstone was made. Since transnasal drainage of gastrointestinal tract for decompression was not efficient, the general status of the patient prompted us to undertake a surgical intervention. After obtaining an adequate informed consent, enterotomy and surgical lithotripsy were performed under spinal anesthesia 48 hrs after disease onset. The location of stone was detected by palpation and found to be impacted in the ileum at 50 cm proximal to the terminal ileum. After the stone was removed, the ileum was primarily closed with double-layer running stiches of a bioabsorbable material. The stone was of a combined nature 3 $\times$ 2 cm in size (Fig. 3). The patient's postoperative recovery was uneventful.

## DISCUSSION

Since Kasahara et al. [2] reported the retrospective analysis of the clinical features of gallstone ileus in 112 Japanese patients between 1903 and 1978, a single or a few case reports restricted to the Japanese language have been available in Japan. Decades ago,

gallstone ileus was known to be a disease with relatively high mortality rates, however current progress in surgical technique, diagnostic imaging, and innovation of drugs might have improved the outcome of this disease. Therefore, it is important to reevaluate the current feature of the gallstone ileus aiming to clarify the diagnosis and management, and to summarize clinical data from multi-Japanese centers in the English literature.

As far as we know, there have been 176 reported cases of gallstone ileus written in the Japanese literature in these 20 years between 1985 and 2005. The number of male and female patients was 49 and 127, respectively. The age of the patients ranged from 24 to 91 years old. (Fig. 4) Thus elderly people are an important group of patients with gallstone ileus. Typically, the pathogenesis of gallstone ileus is based on acute or chronic cholecystitis associated with cholelithiasis, spreading inflammations and adhesion to alimentary tracts, leading to biliary-enteric fistula. In the United States, about 50% of the patients have a history of gallbladder disease [3]. In our series of Japanese patients, 124 of 176 reported cases (66%) to have the presence of stones in the biliary tracts. Our review of the Japanese literature revealed that cholecystoduodenal fistula is most frequently found in 96.5% of Japanese patients with gallstone ileus. Similarly, cholecysto-duodenal fistulas are those which are most frequently described in the worldwide-reports, followed by cholecysto-colonic, cholecysto-gastric and duodeno-left hepatic duct fistulas [4-6]. In one rare case, the bile duct stone disrupted through the Papilla Vater into the duodenal lumen [7]. Potentially reactive substances in bile juice may react with the intestinal epithelial cells which may induce subsequent impacted stones which are associated with mucosal injuries [8].

Impacted gallstones ranged in size from 2 cm to 10 cm, with a mean of 4.3 cm in the series of Japanese patients. A gallstone is usually more than 2.5 cm in diameter to cause an intestinal obstruction in the normal small intestine [9]. As the smaller stones may be easily evacuated, the impaction of the gallstone smaller than 2 cm is unusual, but, may occur when some pathologic state of the intestine including spasms, angulation or adhesion causes narrowing of the intestinal lumen [10]. The review of Japanese patients revealed that intestinal obstruction by gallstones is seen more frequently at the ileus, followed by jejunum and duodenum (Fig. 5). The impacted stone at the colon is uncommon and occurred in only 2.9% of Japanese patients, which was comparable to that of US reports



Fig. 1. Plain abdominal X-ray showed marked small bowel distention with air-fluid level, and demonstrated a round calcified component in the pelvic cavity.

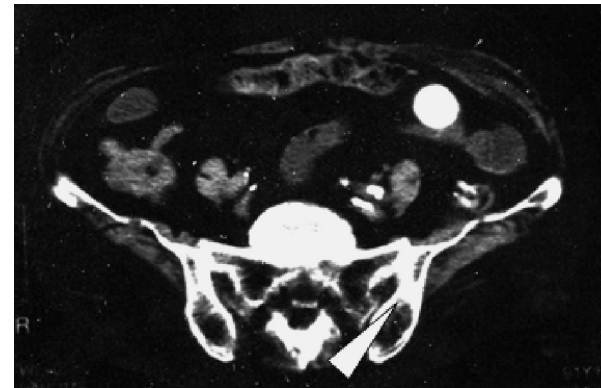
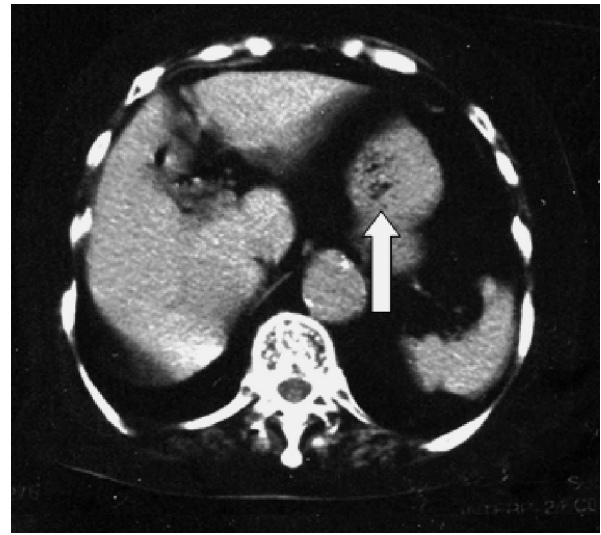


Fig. 2. Pneumobilia (arrow) and air in the gallbladder are seen (upper panel). CT demonstrated the distention of the jejunum with a high density mass of 2.5 cm in the diameter in the alimentary tract (lower panel).



Fig. 3. The impacted stone was a combined stone 3x2 cm in size.

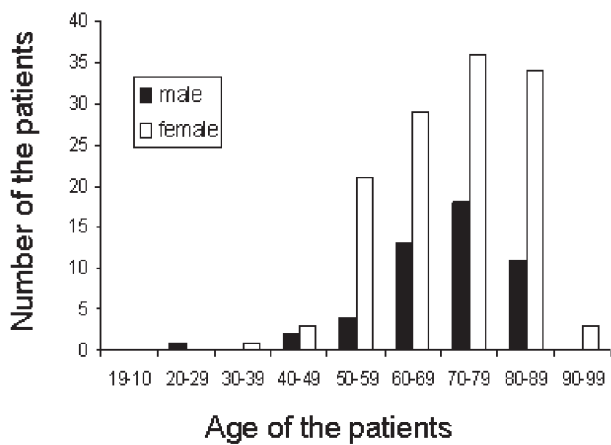


Fig. 4. Gender and age of the patients with gallstone ileus in Japan.

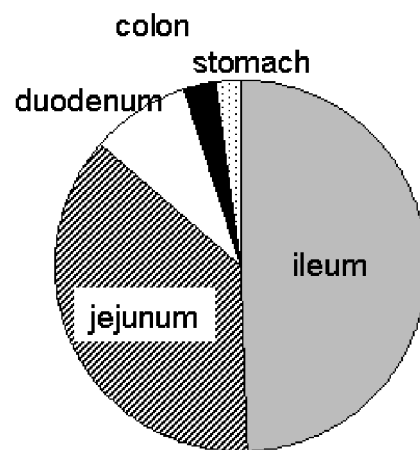


Fig. 5. The site of the impacted stones of 176 Japanese patients between 1985-2005.

(4.8%) [3]. In elderly patients, there may be concomitant disease such as colonic cancer, resulting in a greater frequency of episodes of disease onset.

Although clinical symptoms are not specific, most of the Japanese patients with gallstone ileus presented with abdominal pain (91.5%), accompanied by vomiting in 59.7% of all patients (Table 1). Jaundice (serum total bilirubin >2.0 mg/dl) was seen in 7.3% of Japanese patients. The use of diagnostic radiological tools has greatly facilitated the clinical diagnosis. The typical radiological images may include pneumobilia, intestinal obstruction, and ectopic gallstone. Pneumobilia may be secondary to bilioenteric fistula, endoscopic sphincterotomy, or an incompetent sphincter of Oddi, therefore it is not a definitive indication for gallstone ileus. Plain abdominal X-ray is a conventional, but useful strategy for detection of air-fluid levels and pneumobilia. Plain abdominal X-ray could visualize air-fluid level in 77.8%, bowel loop dilatation in 88.9%, pneumobilia in 37%, air in gallbladder in 3.7% and ectopic stone in 33% of patients according to the clinical study conducted in Italy [11]. Helical CT may improve the diagnosis of gallstone ileus providing important information [12,13]. Abdominal ultrasound may also prove to be a useful tool for the diagnosis [14,15]. Radiological upper gastrointestinal

tract imaging with barium may show an intraluminal stone or a fistula in the case of a choledocho-duodenal or choledocho-colonic fistula [16]. Our review of the Japanese literature disclosed that air-fluid levels and bowel loop dilatation were most frequently observed in our series [11]. Pneumobilia was observed in 50% of Japanese patients.

The therapeutic management of gallstone ileus remains controversial [17]. However, considering that a spontaneous passage of the impacted stone through the intestine is rare, a surgical approach is likely to be an appropriate therapeutic strategy if the condition of the patients permits such intervention [18]. Enterolithotomy alone was most frequently chosen as the routine surgical treatment for gallstone ileus in Japan. The mortality was higher in the patients with one-stage surgery including enterolithotomy, cholecystectomy and fistula closure, regardless of the patients' age [17]. Therefore simple enterolithotomy is considered to be appropriate in most patients. However, additional performance of a one-stage cholecystectomy and repair of fistula are desirable if the condition of the patient allows it, since there may be a concern that an enterolithotomy alone may cause the complications related to the persistence of the biliary-enteric fistula, which includes the possibility of recurrent of gallstone ileus and recurrent cholangitis [18]. Conservative therapy may also be considered for the treatment of gallstone ileus when the obstructing gallstone is smaller than 2 cm based on radiological measurement. Among 176 Japanese patients, 14.2% (25/176) of patients were conservatively treated. Endoscopic lithotomy was available only when the stone was impacted in the stomach, duodenum or colon. In the present case, we elected to perform enterolithotomy considering the age of the patient and the impacted site of the stone.

The occurrence of a high mortality rate may be due to the frequency of relatively elderly patients with unstable clinical conditions, concomitant disease such as cardiorespiratory disease and/or diabetes mellitus, delayed initiation of the treatment because of uncommon symptoms, and associated with all of these, frequent surgery associated complications such as pneumonia or cardiac failure. Overall mortality of patients was as high as 60% before 1925, 40% in 1960 and 15% after 1970 [3]. Our Japanese literature review indicated that the mortality of this disease has been decreased even further to 8%.

The present case was the oldest patient which has been reported in Japanese scientific literature. Considering the poor conditions due to bowel obstructions and the potential complications related to surgery and

TABLE 1.  
*Clinical symptoms*

Abdominal pain	91.5% (161/176)
Vomiting	59.7% (105/176)
Fever (>38°C)	40.9% (72/176)
Jaundice	7.3% (13/176)
Abdominal distension	84.7% (149/176)

TABLE 2.  
*Radiological findings*

Pneumobilia	50% (88/176)
Air-fluid level	88.1% (155/176)
Detection of stone	31.3% (55/176)

TABLE 3.  
*Treatments for gallstone ileus*

Surgical treatment	
Enterolithotomy	74
Enterolithotomy & fistula closure (one-stage)	44
Enterolithotomy & fistula closure (two-stage)	21
Non-surgical treatment	
Conservative	25
Endoscopic lithotomy	11
Extracorporeal Shock Wave Lithotripsy (ESWL)	1



anesthesia, we performed enterotomy and surgical lithotripsy under spinal anesthesia, which is likely to be most appropriate and minimally invasive for our patient. During approximate 1-year follow up period, the patient has been well without related to the persistence of the biliary-enteric fistula such as cholangitis.

In summary, the successful treatment of the oldest case of gallstone ileus in Japan was reported. Although the clinical symptoms are not specific, it is not difficult to make an accurate diagnosis of gallstone ileus using radiological imaging. The literature review of Japanese patients between 1985 and 2005 has definitively revealed the improved outcome of the disease in the recent years.

#### REFERENCES

1. Kirchmayr W, Muhlmann G, Zitt M, Bodner J, Weiss H et al. Gallstone ileus: rare and still controversial. *ANZ J Surg* 2005; 75:234-238.
2. Kasahara Y, Umemura H, Shiraha S, Kuyama T, Sakata K et al. Gallstone ileus. Review of 112 patients in the Japanese literature. *Am J Surg* 1980; 140:437-440.
3. Reisner RM, and Cohen JR. Gallstone ileus: a review of 1001 reported cases. *Am Surg* 1994; 60:441-446.
4. Atli AO, Coskun T, Ozenc A, and Hersek E. Biliary enteric fistulas. *Int Surg* 1997; 82:280-283.
5. Stagnitti F, Mongardini M, Schillaci F, Dall'Olio D, De Pascalis M et al. Spontaneous biliodigestive fistulae. The clinical considerations, surgical treatment and complications. *G Chir* 2000; 21:110-117.
6. Dittrich K, and Weiss H. Ileus of the small intestine caused by a lost gallstone! A late complication of laparoscopic cholecystectomy. *Chirurg* 1995; 66:443-445.
7. Yoshida H, Tajiri T, Mamada Y, Taniai N, Hirakata A et al. Diagnosis of gallstone ileus by serial computed tomography. *Hepatogastroenterology* 2004; 51:33-35.
8. Chipman JK. Bile as a source of potential reactive metabolites. *Toxicology* 1982; 25:99-111.
9. Syme RG. Management of gallstone ileus. *Can J Surg* 1989; 32:61-64.
10. Ihara E, Ochiai T, Yamamoto K, Kabemura T, and Harada N. A case of gallstone ileus with a spontaneous evacuation. *Am J Gastroenterol* 2002; 97:1259-1260.
11. Lassandro F, Gagliardi N, Scuderi M, Pinto A, Gatta G et al. Gallstone ileus analysis of radiological findings in 27 patients. *Eur J Radiol* 2004; 50:23-29.
12. Pavlidis TE, Atmatzidis KS, Papaziogas BT, and Papaziogas TB. Management of gallstone ileus. *J Hepatobiliary Pancreat Surg* 2003; 10:299-302.
13. Doko M, Zovak M, Kopljar M, Glavan E, Ljubicic N et al. Comparison of surgical treatments of gallstone ileus: preliminary report. *World J Surg* 2003; 27:400-404.
14. Ripolles T, Miguel-Dasit A, Errando J, Morote V, Gomez-Abril SA et al. Gallstone ileus: increased diagnostic sensitivity by combining plain film and ultrasound. *Abdom Imaging* 2001; 26:401-405.
15. Lasson A, Loren I, Nilsson A, Nirhov N, and Nilsson P. Ultrasonography in gallstone ileus: a diagnostic challenge. *Eur J Surg* 1995; 161:259-263.
16. Pangan JC, Estrada R, and Rosales R. Cholecystoduodenocolic fistula with recurrent gallstone ileus. *Arch Surg* 1984; 119:1201-1203.
17. Rodriguez-Sanjuan JC, Casado F, Fernandez MJ, Morales DJ, and Naranjo A. Cholecystectomy and fistula closure versus enterolithotomy alone in gallstone ileus. *Br J Surg* 1997; 84:634-637.
18. Clavien PA, Richon J, Burgan S, and Rohner A. Gallstone ileus. *Br J Surg* 1990; 77:737-742.