increase in $[Ca^{2+}]_i$ in Krebs solution in control CPAEs (Fig. 3A). Ach (1 μM) showed $[Ca^{2+}]_i$ increase also in MCTP-treated cells (Fig. 3B), but the net increment of $[Ca^{2+}]_i$ (Fig. 3Ca) and time integral of $[Ca^{2+}]_i$ elevation (Fig. 3Cb) were significantly smaller in MCTP-treated cells than in control cells. Therefore this might be one of the causes for the impaired NO production in MCTP-treated CPAEs.

Expression of eNOS protein in MCTP-treated CPAEs

We then examined the expression of eNOS protein in MCTP-treated cells with Western blotting. As shown in Fig. 4, expression of eNOS protein was observed both in control and MCTP-treated CPAEs. However, densitometric analysis of the band revealed that the expression level of eNOS protein was significantly lower in MCTP-treated CPAEs than in control CPAEs. Thus, it seems that the reduced eNOS expression may be another reason for the decreased NO production in MCTP-treated CPAEs.

Cellular uptake of L-[^{3}H]arginine in MCTP-treated and control CPAEs

We then examined the cellular uptake of L-arginine in control and MCTP-treated CPAEs. As shown in Fig. 5A, cellular uptake of L-arginine was significantly lower in MCTP-treated CPAEs than in control cells in the presence of 1 μCi/ml L-[^{3}H]arginine. However, cellular L-arginine uptake was linearly increased according to its extracellular concentration in MCTP-treated CPAEs, and the uptake of control level of L-[^{3}H]arginine was obtained with three times higher concentration of extracellular L-[^{3}H]arginine (3 μCi/ml) in MCTP-treated CPAEs.

Effects of excess L-arginine on endothelium-dependent responses in model vessel

Above results suggest that impaired Ca^{2+} mobilization, reduced eNOS protein expression and lower cellular uptake of L-arginine are responsible for the impairment of NO production in MCTP-treated CPAEs. However, the cellular L-arginine uptake was linearly increased according to its extracellular concentration (Fig. 5A). Therefore, assuming that the expressed eNOS protein was intact, it would be rational to speculate that the sufficient supply of L-arginine may restore NO production even in MCTP-treated CPAEs.

We then performed gel contraction assay in the presence of excess concentration of extracellular L-arginine. As expected, in the presence of 10 mM L-arginine, Ach induced relaxation of the gels that were overlaid with MCTP-treated

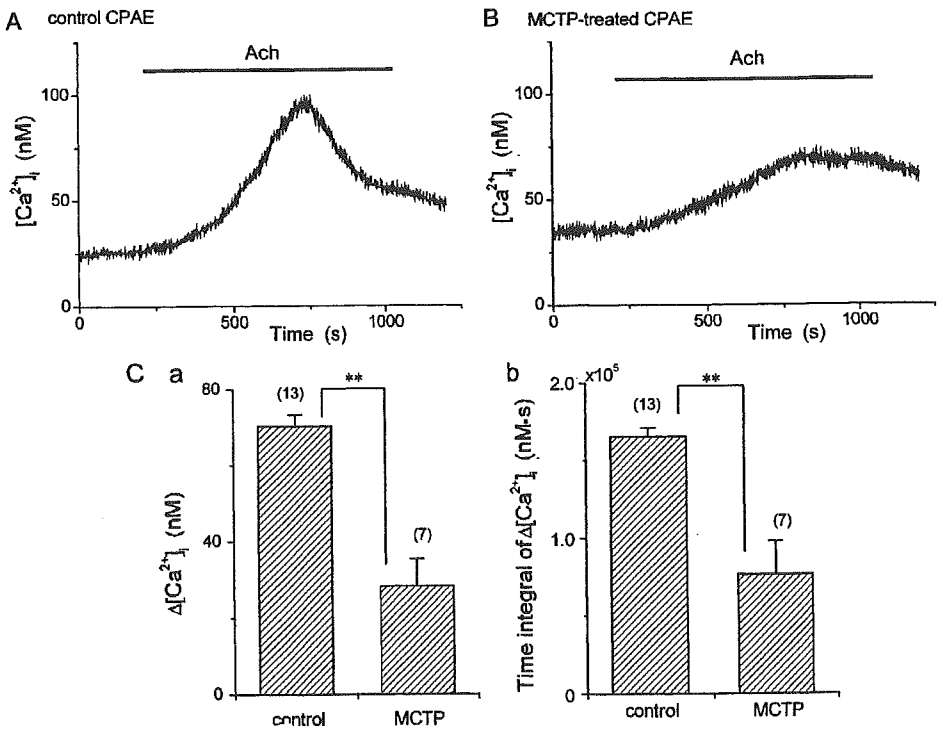

Fig. 3. Ach-induced Ca^{2+} mobilization in control and MCTP-treated CPAEs. (A) Control CPAEs showed gradual increase in $[Ca^{2+}]_i$ in response to 1 μM Ach. Representative $[Ca^{2+}]_i$ trace is shown. (B) Ach also showed an increase in $[Ca^{2+}]_i$ in MCTP-treated CPAEs, but the degree of elevation was smaller than in control cells. Representative $[Ca^{2+}]_i$ trace is shown. (C) Statistical analysis of net peak increment of Ach-induced elevation of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$), (a) and time integral of $\Delta[Ca^{2+}]_i$ for 15 min (b). MCTP-treated CPAEs showed significantly lower increase in $[Ca^{2+}]_i$ than control cells. $^{**}P < 0.01$.

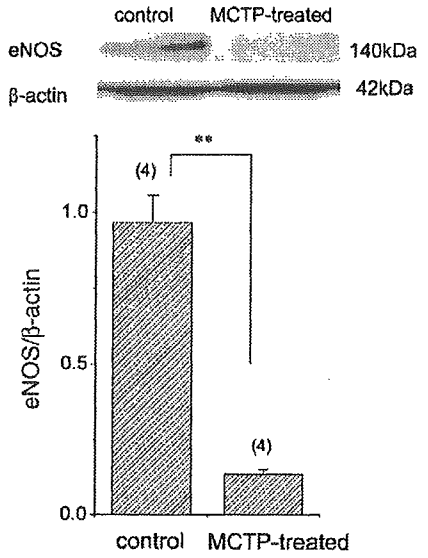

Fig. 4. Expression of endothelial NO synthase (eNOS) protein in control and MCTP-treated CPAEs. Note that the expression of 140 kDa protein is markedly reduced in MCTP-treated cells. Expression of β-action is shown as an internal control. Measurement was repeated for four times, and the representative band images are shown. Mean ± SEM values of the band analysis are shown. $^{**}P < 0.01$.

CPAEs, and the degree of relaxation was comparable to those observed with control CPAEs (Fig. 5B).

Discussion

Various methods have been introduced so far to detect the NO production in vitro, including porphyrinic-based microsensor (Malinski and Taha, 1992), Griess method (Wang et al., 1996), and DAF-2 fluorescence (Kimura et al., 2000). Each method has advantages and disadvantages in its convenience, sensitivity and selectivity. We have recently shown that endothelium-overlaid, smooth muscle-embedded model vessels can be used for detecting NO production in cultured endothelial cells (Kimura et al., 2004). The major advantage of this in vitro model vessel is that it can directly examine the effects of endothelium-derived substances on smooth muscle tonus, and therefore, it enables the detection of not only NO but also other endothelium-derived vasoactive substances. We observed in the present study that control CPAEs-overlaid gels showed relaxation in response to Ach, and L-NAME suppressed it almost completely (Fig. 2A). Therefore, it can be supposed that NO is the main vasorelaxant that was secreted by Ach in CPAEs. We have shown with this model vessel study that MCTP-treated CPAEs did not induce the relaxation of pre-contracted gels (Fig. 2B), thereby indicating that MCTP impairs the NO production in CPAEs. There have been only a few methods for studying PH, and most studies have used disease model animals including MCTP- and hypoxia-treated rats. In contrast, this novel in vitro model vessel is convenient and applicable for examining the effects of various drugs on impaired endothelium.

We have confirmed that MCTP induces the enlargement of pulmonary endothelial cells and the reduction in cell number (Fig. 1). It was reported in CPAEs that these phenomena are due to the inactivation of cdc2 kinase and consequent G2/M phase arrest (Thomas et al., 1998) or to the blockade of Golgi trafficking and subsequent impairment of cav-1α chaperone function (Mathew et al., 2004; Shah et al., in press), and megalocytic change of pulmonary endothelium would induce the prolonged vascular remodeling and causes PH in model animals. The impairment of NO production in MCTP-treated CPAEs, therefore, may be in part due to cell cycle arrest and loss of cav-1α function, which plays an important role in NO production (Minshall et al., 2003). We have further revealed in this study that the impairment of NO production is due to various other cellular mechanisms, including the impaired Ca^{2+} mobiliz-

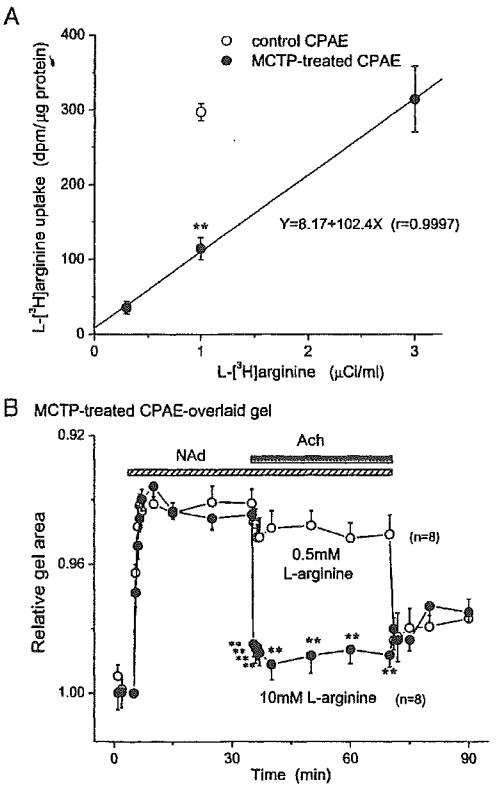

Fig. 5. Effects of high concentration of extracellular L-arginine on its cellular uptake and NO production in MCTP-treated CPAEs. (A) Cellular uptake of L-[^{3}H]arginine was linearly increased according to its extracellular concentration in MCTP-treated CPAEs (closed circles). Note that MCTP-treated CPAEs showed markedly lower uptake of L-[^{3}H]arginine than control CPAEs (open circles). Each symbol represents mean ± SEM value from 6 measurements. $^{**}P < 0.01$ vs. control. (B) High concentration of L-arginine (10 mM) reversed the Ach-induced relaxation of the pre-contracted gels (closed circles). The relaxation was significantly larger than that in the presence of 0.5 mM L-arginine (open circles). $^{**}P < 0.01$ vs. 0.5 mM L-arginine.

ing properties (Fig. 3), lower expression level of eNOS protein (Fig. 4) and reduced cellular uptake of L-arginine (Fig. 5A). Previous study has shown that the expression of eNOS mRNA was rather augmented, but its protein level was decreased in MCTP-treated rat lungs (Tyler et al., 1999). We have also observed that the expression of eNOS mRNA, assessed with RT-PCR, was not different between control and MCTP-treated CPAEs (data not shown). This suggests that the reduced expression of eNOS protein may be due to the impairment of translation but not transcription of its mRNA. Therefore, though it was reported that MCTP binds to DNA and inhibits mRNA transcription (Thomas et al., 1996), we suppose that this is not the only mechanism of action of MCTP on endothelial damage. Actually, recent reports have proposed another possible mechanism for the MCTP-induced impairment of cellular functions, that is, blockade of Golgi transport (Mathew et al., 2004; Shah et al., in press), that would affect cellular integrity including eNOS functions. Therefore, we suppose that the MCTP-induced impairment of cellular functions is due to various cellular and molecular mechanisms.

In control gels, it seems that L-arginine was supplied from DMEM, which contains 0.5 mM L-arginine and was used for constructing the model vessel. We supposed that this L-arginine concentration in the gels might not be sufficient for NO production in MCTP-treated CPAEs. This was indeed the case, that is, endothelium-dependent relaxation was observed in MCTP-treated CPAEs in the presence of excess concentration of L-arginine (10 mM, Fig. 5B). It is well known as "arginine paradox" that excess concentration of L-arginine induces NO production in a concentration-dependent manner at far above the *K*d values of NOS, though the precise mechanisms are still not clarified (Kurz and Harrison, 1997; McDonald et al., 1997; Tsikas et al., 2000). Therefore, we suppose that the restoration of NO production in MCTP-treated CPAEs by excess L-arginine was due to both the arginine paradox phenomenon and the increased cellular uptake of L-arginine.

Dysfunction of pulmonary vascular endothelial cells is thought to play a central role in the pathogenesis of PH not only in experimental animals but also in human (Higenbottam and Laude, 1998; Michelakis, 2003). It is also supposed that the reduction of NO production is responsible in PH caused by MCTP (Mathew et al., 1995) or hypoxia (Millatt et al., 2003) and congenital PH model rat (Le Cras et al., 2000). Therefore, the treatment of PH with drugs modifying NO production has been recently highlighted (Michelakis, 2003). Oral intake and venous infusion of L-arginine have been reported to induce the reduction of pulmonary artery pressure in animal PH models (Mitani et al., 1997) and in PH patients (Mehta et al., 1995), but its detailed mechanism of action has not been clarified and its general use for the treatment of PH is still limited. The present study further revealed that the excess L-arginine would restore NO productivity in damaged endothelial cells with very low level of expression of eNOS protein and impaired Ca^{2+} mobilization. Therefore, other than NO gas inhalation (Gianetti et al., 2002) or oral intake of sildenafil (Wilkens et al., 2001), we suppose that excess supply of cheaper L-arginine might be another considerable maneuver for treating PH.

In conclusion, we have introduced a novel in vitro vessel model with MCTP-treated CPAEs in the present study and revealed that NO production can be restored by excess L-arginine even in damaged endothelial cells.

References

Campbell, A.I., Kuliszewski, M.A., Stewart, D.J., Frasch, H.F., Marshall, C., Marshall, B.E., Tyler, R.C., Muramatsu, M., Abman, S.H., Stelzner, T.J., Rodman, D.M., Bloch, K.D., McMurtry, I.F., 1999. Cell-based gene transfer to the pulmonary vasculature: endothelial nitric oxide synthase overexpression inhibits monocrotaline-induced pulmonary hypertension. Am. J. Respir. Cell Mol. Biol. 21, 567–575.

Chamley, J.H., Campbell, G.R., McConnell, J.D., Groschel Stewart, U., 1977. Comparison of vascular smooth muscle cells from adult human, monkey and rabbit in primary culture and in subculture. Cell Tissue Res. 177, 503–522.

Champion, H.C., Bivalacqua, T.J., Greenberg, S.S., Giles, T.D., Hyman, A.L., Kadowitz, P.J., 2002. Adenoviral gene transfer of endothelial nitric-oxide synthase (eNOS) partially restores normal pulmonary arterial pressure in eNOS-deficient mice. Proc. Natl. Acad. Sci. U.S.A. 99, 13248–13253.

Gianetti, J., Bevilacqua, S., De Caterina, R., 2002. Inhaled nitric oxide: more than a selective pulmonary vasodilator. Eur. J. Clin. Invest. 32, 628–635.

Higenbottam, T.W., Laude, E.A., 1998. Endothelial dysfunction providing the basis for the treatment of pulmonary hypertension: Giles F. Filley lecture. Chest 114, 72S–79S.

Ito, K.M., Sato, M., Ushijima, K., Nakai, M., Ito, K., 2000. Alterations of endothelium and smooth muscle function in monocrotaline-induced pulmonary hypertensive arteries. Am. J. Physiol. 279, H1786–H1795.

Kimura, C., Koyama, T., Oike, M., Ito, Y., 2000. Hypotonic stress-induced NO production in endothelium depends on endogenous ATP. Biochem. Biophys. Res. Commun. 274, 736–740.

Kimura, C., Cheng, W., Hisadome, K., Wang, Y.P., Koyama, T., Karashima, Y., Oike, M., Ito, Y., 2002. Superoxide anion impairs contractility in cultured aortic smooth muscle cells. Am. J. Physiol. 283, H382–H390.

Kimura, C., Oike, M., Ohnaka, K., Nose, Y., Ito, Y., 2004. Constitutive nitric oxide production in bovine aortic and brain microvascular endothelial cells: a comparative study. J. Physiol. (London) 554, 721–730.

Kurz, S., Harrison, D.G., 1997. Insulin and the arginine paradox. J. Clin. Invest. 99, 369–370.

Le Cras, T.D., Kim, D.H., Markham, N.E., Abman, A.S., 2000. Early abnormalities of pulmonary vascular development in the Fawn-Hooded rat raised at Denver's altitude. Am. J. Physiol. 279, L283–L291.

Malinski, T., Taha, Z., 1992. Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. Nature 358, 676–678.

Mathew, R., Zeballos, G.A., Tun, H., Gewitz, M.H., 1995. Role of nitric oxide and endothelin-1 in monocrotaline-induced pulmonary hypertension in rats. Cardiovasc. Res. 30, 739–746.

Mathew, R., Yuan, N., Rosenfeld, L., Gewitz, M.H., Kumar, A., 2002. Effects of monocrotaline on endothelial nitric oxide synthase expression and sulfhydryl levels in rat lungs. Heart Dis. 4, 152–158.

Mathew, R., Huang, J., Shah, M., Patel, K., Gewitz, M., Sehgal, P.B., 2004. Disruption of endothelial-cell caveolin-1α/raft scaffolding

during development of monocrotaline-induced pulmonary hypertension. Circulation 110, 1499–1506.

Mattocks, A.R., Jukes, R., Brown, J., 1989. Simple procedures for preparing putative toxic metabolites of pyrrolizidine alkaloids. Toxicon 27, 561–567.

McDonald, K.K., Zharikov, S., Block, E.R., Kilberg, M.S., 1997. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox". J. Biol. Chem. 272, 31213–31216.

Mehta, S., Stewart, D.J., Langleben, D., Levy, R.D., 1995. Short-term pulmonary vasodilation with L-arginine in pulmonary hypertension. Circulation 92, 1539–1545.

Michelakis, E.D., 2003. The role of the NO axis and its therapeutic implications in pulmonary arterial hypertension. Heart Failure Rev. 8, 5–21.

Millatt, L.J., Whitley, G.S., Li, D., Leiper, J.M., Siragy, H.M., Carey, R.M., Johns, R.A., 2003. Evidence for dysregulation of dimethylarginine dimethylaminohydrolase I in chronic hypoxia-induced pulmonary hypertension. Circulation 108, 1493–1498.

Minshall, R.D., Sessa, W.C., Stan, R.V., Anderson, R.G., Malik, A.B., 2003. Caveolin regulation of endothelial function. Am. J. Physiol.: Lung Cell. Mol. Physiol. 285, L1179–L1183.

Mitani, Y., Maruyama, K., Sakurai, M., 1997. Prolonged administration of L-arginine ameliorates chronic pulmonary hypertension and pulmonary vascular remodeling in rats. Circulation 96, 689–697.

Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109–142.

Reindel, J.F., Roth, R.A., 1991. The effects of monocrotaline pyrrole on cultured bovine pulmonary artery endothelial and smooth muscle cells. Am. J. Pathol. 138, 707–719.

Rubin, L.J., 1997. Primary pulmonary hypertension. N. Engl. J. Med. 336, 111–117.

Shah, M., Patel, K., Sehgal, P.B., in press. Monocrotaline pyrrole-induced endothelial cell megalocytosis involves a Golgi-blockade mechanism. Am. J. Physiol. Cell Physiol.

Thomas, H.C., Lame, M.W., Wilson, D.W., Segall, H.J., 1996. Cell cycle alterations associated with covalent binding of monocrotaline pyrrole to pulmonary artery endothelial cell DNA. Toxicol. Appl. Pharmacol. 141, 319–329.

Thomas, H.C., Lame, M.W., Morin, D., Wilson, D.W., Segall, H.J., 1998. Prolonged cell-cycle arrest associated with altered cdc2 kinase in monocrotaline pyrrole-treated pulmonary artery endothelial cells. Am. J. Respir. Cell Mol. Biol. 19, 129–142.

Tsikas, D., Boger, R.H., Sandmann, J., Bode-Boger, S.M., Frolich, J.C., 2000. Endogenous nitric oxide synthase inhibitors are responsible for the L-arginine paradox. FEBS Lett. 478, 1–3.

Tyler, R.C., Muramatsu, M., Abman, S.H., Stelzner, T.J., Rodman, D.M., Bloch, K.D., McMurtry, I.F., 1999. Variable expression of endothelial NO synthase in three forms of rat pulmonary hypertension. Am. J. Physiol. 276, L297–L303.

Wang, Y., Shin, W.S., Kawaguchi, H., Inukai, M., Kato, M., Sakamoto, A., Uehara, Y., Miyamoto, M., Shimamoto, N., Korenaga, R., Ando, J., Toyo oka, T., 1996. Contribution of sustained Ca^{2+} elevation for nitric oxide production in endothelial cells and subsequent modulation of Ca^{2+} transient in vascular smooth muscle cells in coculture. J. Biol. Chem. 271, 5647–5655.

Wilkens, H., Guth, A., Konig, J., Forestier, N., Cremers, B., Hennen, B., Bohm, M., Sybrecht, G.W., 2001. Effect of inhaled iloprost plus oral sildenafil in patients with primary pulmonary hypertension. Circulation 104, 1218–1222.

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Liver, Pancreas and Biliary Tract

Urine diacetylspermine as a novel tumour maker for pancreatobiliary carcinomas

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Abstract

Background. Serum carcinoembryonic antigen (highly specific) and carbohydrate antigen 19-9 (highly sensitive) have been used as tumour markers for pancreatobiliary caners. A novel urine tumour marker, diacetylspermine, was compared with the two conventional serum tumour markers in 125 patients with pancreatobiliary diseases.

Results. When the diagnosis of benign or malignant condition was examined, the sensitivity of urine diacetylspermine (75%) was higher than that of serum carcinoembryonic antigen (44%; $P = 0.048$) and the same as that of serum carbohydrate antigen 19-9 (75%). The specificity of urine diacetylspermine (81%) was lower than that of serum CEA (92%) and as high as that of serum carbohydrate antigen 19-9 (80%). The efficiency of urine diacetylspermine (79%) was higher than that of serum carcinoembryonic antigen (74%) and the same as that of serum carbohydrate antigen 19-9 (79%).

Conclusion. These results suggest that urine diacetylspermine is a marker for pancreatobiliary carcinoma, which is as highly sensitive and specific as serum carbohydrate antigen 19-9.

Keywords: Carbohydrate antigen 19-9; Carcinoembryonic antigen; Diacetylspermine

1. Introduction

Polyamines are ubiquitous organic polycations that are synthesised in substantial amounts by rapidly growing and dividing cells, including cancer cells [1]. Polyamines excreted in the urine are mainly in the monoacetylated form. Among them, acetylputrescine is most abundant followed by acetylcadaverine, N^1-acetylspemidine and N^8-acetylspermine. Diacetylated forms are minor components and the average amounts of diacetylspermidine and diacetylspermine are only 1.4 and 0.46%, respectively [2]. These diacetylated polyamine species have not been identified because they are of limited quantity in the urine and are undetectable by conventional methods due to a lack of the primary amino groups.

Russell [3] first reported increased excretion of polyamines in the urine in cancer patients, which evoked a surge of studies on polyamine analysis in the urine in 1970s [1]. However, the following results were disappointing because there were too many false negative and false positive results as far as total polyamine levels and its monoacetyl compounds were examined [4], although their average urinary levels were certainly higher in patients with cancer than in healthy persons [4,5]. Therefore, diagnostic utility of urine polyamines has been abandoned for a long time.

Recently, Hiramatsu et al. [2,6,7] reported that diacetylspermine, one of spermine derivatives, is more frequently expressed in the urine of patients with malignant conditions compared to benign diseases using high-performance liquid

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chromatography (HPLC) procedure. However, HPLC is difficult, not so popular and cannot be used as a clinical laboratory test. Thereafter, Hiramatsu et al. [8] developed a sensitive and accurate enzyme-linked immunosorbent assay (ELISA) system with the use of an affinity-purified polyclonal antibody against diacetylspermine. ELISA is easy to use, and therefore, appropriate to be used in the clinical laboratory.

Serum carcinoembryonic antigen (CEA) [9,10] and carbohydrate antigen (CA) 19-9 [11] have been used as tumour markers for pancreatobiliary caners. Serum CEA is highly specific for pancreatobiliary cancers and serum CA19-9 is highly sensitive. Their positivity increases with the progression of the diseases. They are not useful in detecting early cancers but are used as a marker of the recurrence of the tumour after surgical resection.

There are few reports of urine diacetylspermine levels in patients with pancreatobiliary diseases. In this series, clinical implications of this novel urine tumour marker, diacetylspermine, were examined by comparing with two conventional tumour markers, serum CEA and CA19-9, in 125 patients with pancreatobiliary diseases.

2. Materials and methods

This series consisted of 125 patients with pancreatobiliary diseases who were treated in the Department of Surgery I, Kyushu University Hospital, Fukuoka, Japan from November 2002 to January 2003. The 125 patients were 70 men and 55 women and their age ranged from 28 to 86 years with a mean of 63.5 ± 12.2 years. The 125 patients included 52 patients in the preoperative or postoperative state of benign diseases (control group), 22 patients in the preoperative state of malignant diseases, and the other 51 patients in the postoperative state (more than 3 months) of malignant conditions. Of the 51 postoperative patients, 10 had unequivocal signs of recurrence as judged by the clinical findings including imaging and the others had no definite evidence of recurrence as proven by subsequent clinical follow-up as well. Therefore, a total of 32 patients were considered to have malignant pancreatobiliary tumours. The 52 patients in the control group consisted of 28 with benign inflammatory diseases [15 in the preoperative state (9 chronic pancreatitis, 3 hepatolithiasis, 1 cholecystolithiasis, 2 adenomyomatosis of the gallbladder) and 13 in the postoperative state (10 chronic pancreatitis, 1 cholecystolithiasis, 1 choledocholithiasis and 1 choledochal cyst)] and 24 with adenoma (8 in the preoperative state of intraductal papillary-mucinous adenoma of the pancreas, 15 in the postoperative state of the same disease, 1 in the postoperative state of adenoma of the papilla of Vater). Urine diacetylspermine is known to be elevated in acute inflammatory conditions including acute pancreatitis, acute cholecystitis and the early postoperative state due to the rapid turnover of cells (personal communication with Dr. M. Kawakita). All patients who suffered from acute inflammatory diseases and were within 3 months after the operation were excluded from the present series. The patients with pancreatic endocrine tumours and hepatocellular carcinomas were also excluded because we intended to compare urine diacetylspermine and serum CEA and CA19-9. Urine diacetylspermine and serum CEA and CA19-9 were examined in all these 125 patients.

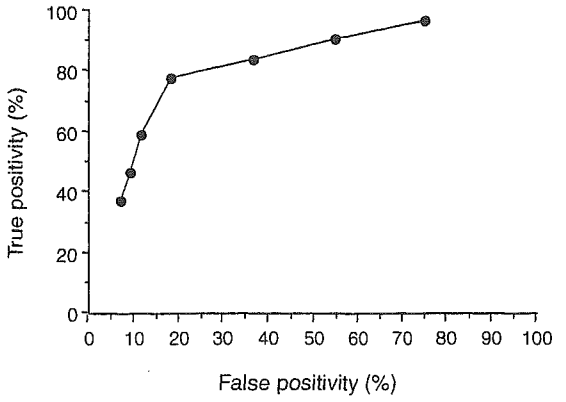

Fig. 1. Receiver operating characteristic curve indicating test performance for pancreatobiliary carcinoma for the following threshold values: 175, 225, 275, 325, 375, 425 and 475 nmol/g Creatinine. (●–●) Performance in distinguishing malignant from benign pancreatobiliary diseases.

Urine and peripheral blood were obtained in the morning. Diacetylspermine was measured by using ELISA system, which was created by Transgenic Co. Ltd, Kumamoto, Japan according to the Hiramatsu's method [8] in CRC Co. Ltd., Fukuoka, Japan. The cut-off level was set at 325 nmol/g Creatinine by constructing a receiver operating characteristics curve (Fig. 1). The serum levels of CEA and CA19-9 were measured in the Clinical Laboratory of Kyushu University Hospital. Their cut-off levels were 2.5 ng/ml and 37.4 IU/ml, respectively.

The clinical stage of malignant pancreatobiliary diseases was determined according to TNM Classification of Malignant Tumours issued by the UICC [12]. The 32 patients with primary or metastatic pancreatobiliary adenocarcinoma consisted of six in Stage IIb, two in Stage III and 24 in Stage IV.

Informed consent was obtained from each patient. The protocol was submitted to and preapproved by the Senior Staff Committee of the Department.

Values were expressed as mean ± standard deviation. Mean values were compared by Student's t test and the distribution of patients was measured by the chi-square test. The sensitivity, specificity, positive predictive value, negative predictive value and efficiency were measured. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Measurements of urine diacetylspermine

Urine diacetylspermine levels of the 52 patients in the control group and 32 with pancreatobiliary adenocarcinoma were 267.2 ± 143.0 and 621.5 ± 584.0 nmol/g Creatinine,

Table 1
Malignant and benign diagnosis by urinary diacetylspermine, serum CEA and CA19-9

	Diacetylspermine		CEA		CA19-9	
	Positive	Negative	Positive	Negative	Positive	Negative
Malignant	22	10	14	18	21	11
Benign	8	44	4	48	7	45
Sensitivity (%)	75[a]		44[a]		75	
Specificity (%)	81		92		80	
Positive predictive value (%)	71		78		86	
Negative predictive value (%)	84		73		87	
Efficiency (%)	79		74		79	

[a] $P = 0.044$.

respectively ($P < 0.001$). The serum CEA and CA19-9 values of the two groups were 1.2 ± 0.8 and 39.2 ± 158.5 ng/ml in the controls and 16.8 ± 21.3 and $7{,}825.2 \pm 23{,}996.8$ U/ml in the malignant group ($P = 0.0209$), respectively. The values of eight patients with intraductal papillary-mucinous adenoma were 243.2 ± 96.1 nmol/g Creatinine, 1.4 ± 1.2 ng/ml and 9.0 ± 5.0 U/ml, which were similar to those of the control group and were lower than those in patients with adenocarcinoma, but the differences were not statistically significant.

3.2. Diagnosis of benign and malignant conditions

Data concerning benign and malignant conditions are shown in Table 1. The sensitivity of urine diacetylspermine for malignant conditions was 75%, which was higher than 44% ($P = 0.044$) of serum CEA and as high as 75% of serum CA19-9. The specificity of urine diacetylspermine was 81%, which was lower than 92% of serum CEA and as high as 82% of serum CA19-9. Efficiency of urine diacetylspermine was 79%, which was similar to 74% of serum CEA and 79% of serum CA19-9.

3.3. Detection of recurrence of pancreatobiliary carcinoma

Data are present in Table 2. The sensitivity of urine diacetylspermine for the presence of recurrence of malignant pancreatobiliary diseases was 80%, which was higher than 70% of serum CEA and 70% of serum CA19-9. The specificity of urine diacetylspermine was 66%, which was lower than 78% of CEA and 83% of CA19-9.

Table 3
Tumour stage and urine diacetylspermine, serum CEA and CA19-9

Stage	Diacetylspermine		CEA		CA19-9	
	Positive	Negative	Positive	Negative	Positive	Negative
IIb	3	3	0	6	4	2
III	2	0	1	1	1	1
IV	19	5	11	13	16	8

3.4. Tumour stage and three markers

The mean values of urine diacetylspermine of patients with Stages IIb, III and IV carcinoma were 347.8 ± 139.7, 605.1 ± 375.9, 691.3 ± 651.7 nmol/g Creatinine, serum CEA 0.9 ± 0.1, 15.6 ± 20.9 and 50.8 ± 182.4 ng/ml and serum CA19-9 134.2 ± 154.6, 1726.1 ± 2438.2 and $10{,}256.3 \pm 27{,}405.1$ U/ml, respectively. The positivity of the three markers increased with the progression of tumours (Table 3). Three of the six patients with Stage IIb carcinoma showed a positive urine diacetylspermine result. None of them showed elevation of serum CEA, but four of them did show elevation of serum CA19-9.

3.5. Predictivity for unresectability of carcinoma by three markers

The sensitivity for prediction of unresectability of urine diacetylspermine (81%) was higher than that of serum CEA

Table 2
Recurrence and urinary diacetylspermine, serum CEA and CA19-9

	Diacetylspermine		CEA		CA19-9	
	Positive	Negative	Positive	Negative	Positive	Negative
Recurrence						
Yes	8	2	7	3	7	3
No	14	27	9	32	7	34
Sensitivity (%)	80		70		70	
Specificity (%)	66		78		83	
Positive predictive value (%)	36		44		50	
Negative predictive value (%)	93		91		92	
Efficiency (%)	69		76		80	

Table 4
Unresectability and three markers

	Diacetylspermine		CEA		CA19-9	
	Positive	Negative	Positive	Negative	Positive	Negative
Unresectability						
Yes	21	5	14	12	17	9
No	3	3	0	6	4	2
Sensitivity (%)	81[a]		54[a]		65	
Specificity (%)	50		100		33	
Positive predictive value (%)	88		100		81	
Negative predictive value (%)	38		33		18	
Efficiency (%)	75		63		59	

[a] $P = 0.039$.

(54%, $P = 0.039$) and serum CA19-9 (65%) (Table 4). The specificity of serum CEA was highest of the three markers. The efficiency of urine diacetylspermine (75%) was higher than that of serum CEA (63%) and serum CA19-9 (59%), but the differences were not significant.

4. Discussion

Serum levels of CA19-9 (highly sensitive) and CEA (highly specific) have been used as tumour markers for pancreatobiliary diseases. Usefulness of urine diacetylspermine as a tumour marker was studied in 125 patients with pancreatobiliary diseases. The sensitivity of urine diacetylspermine was higher than that of serum CEA and as high as that of CA19-9 and the specificity of urine diacetylspermine was also as high as those of serum CEA and CA19-9. A half of the patients with Stage IIb pancreatobiliary carcinoma gave a positive result for urine diacetylspermine. Urine diacetylspermine is a novel tumour marker for pancreatobiliary carcinoma, being as highly sensitive and specific as serum CA19-9.

It is true that ideal tumour markers should have high specificity and sensitivity for target malignant conditions. This urine diacetylspermine showed sensitivity and specificity similar to serum CA19-9 and CEA, respectively. Because the urine sample is easy to obtain and the measurement could be done by the ELISA kit, urine diacetylspermine is a novel convenient tumour marker for pancreatobiliary carcinomas.

The clinical outcome of patients with pancreatobiliary carcinoma remains dismal despite the recent progress of diagnostic and therapeutic modalities. Therefore, early diagnosis of pancreatobiliary carcinoma is mandatory. Conventional tumour markers such as serum CA19-9 and CEA have been reported to be not useful in detecting early pancreatic carcinoma. Urine diacetylspermine was positive in a half of patients with Stage IIb pancreatobiliary carcinoma, although the total number of patients examined was small. Therefore, this marker may be of value to detect early pancreatobiliary cancer.

Clinical differentiation between benign inflammatory diseases and malignant diseases is important as well as that of benign and malignant neoplasms. The urine diacetylspermine level is increased in the conditions where cell turnover is accelerated. Thus, patients in the phase of acute inflammation and in the early postoperative state were excluded from the present series. Urine diacetylspermine in patients with benign pancreatobiliary neoplasms were as low as that in those with chronic inflammatory pancreatobiliary diseases, and both the levels were lower than that of pancreatobiliary carcinoma. Therefore, urine diacetylspermine seems to be useful to distinguish malignant pancreatobiliary diseases from benign ones.

It is true that the surgical decision is made by imaging and/or macroscopic findings, but the prediction of resectability by serum tumour markers, if possible, is of great value for surgeons. The positivity and values of urine diacetylspermine increased with the progression of the malignant diseases. Therefore, the urine diacetylspermine level may be of some value to predict the choice of the treatment.

One of the other purposes of tumour marker determination is to predict the clinical outcome of patients with pancreatobiliary carcinoma. This series is a preliminary report on urine diacetylspermine in patients with pancreatobiliary diseases and there is no long-term information. Therefore, further examination is mandatory to identify the usefulness of this novel maker as a prognostic indicator.

Theoretically, urine diacetylspermine is considered to be not specific for a special type of malignancy but for all malignant conditions of any organs. Sugimoto et al. [6] reported that urine diacetylpolyamine was markedly increased in patients with urogenital malignancies and van den Berg et al. [13] reported a high concentration of diacetylspermine in the urine of patients with non-Hodgkin's lymphoma by capillary gas chromatography. In this series, a high concentration of urine diacetylspermine was proved in patients with pancreatobiliary carcinoma. Concerning pancreatic cancer, we previously reported a high incidence of pancreatic cancer in selected patients with diabetes mellitus [14] and intraductal papillary-mucinous neoplasm of the pancreas [15]. This novel marker may be of great value in mass screening and follow-up of patients at high risks of the development of malignancy such as familial adenomatosis coli, ulcerative coli-

tis, pancreatobiliary malunion, congenital choledochal cyst, diabetes mellitus, intraductal papillary-mucinous tumour of the pancreas [16], Barret's oesophagus and so on.

Although this is a preliminary report on urine diacetylspermine in patients with pancreatobiliary diseases, this novel urinary marker is as sensitive and specific for pancreatobiliary carcinomas as serum CA19-9. Urine diacetylspermine may be a universal tumour marker for malignant conditions and useful in mass screening and follow-up of high-risk groups.

Conflict of interest statement

None declared.

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References

[1] Tabor C, Tabor H. 1-4-Diaminobutane (putrescine), spermidine and spermine. Annu Rev Biochem 1976;45:285–306.
[2] Hiramatsu K, Sugimoto M, Kamei S, Hoshino M, Kinoshita K, Iwasaki K, et al. Determination of amounts of polyamines excreted in urine: demonstration of *N*1,*N*8-diacetylspermidine and *N*1,*N*12-diacetylspermine as components commonly occurring in normal human urine. J Biochem 1995;117:107–12.
[3] Russell D. Increased polyamine concentrations in the urine of human cancer patients. Nat New Biol 1971;233:144–5.
[4] Scalabrino G, Ferioli E. Polyamines in mammalian tumors. Part II. Adv Cancer Res 1982;36:1–102.
[5] Bachrach U. Polyamines as markers of malignancy. Prog Drug Res 1992;39:9–33.
[6] Sugimoto M, Hiramatsu K, Kamei S, Kinoshita K, Hoshino M, Iwasaki K, et al. Significance of urinary *N*1,*N*8-diacetylspermidine and *N*1,*N*12-diacetylspermine as indicators of neoplastic diseases. J Cancer Res Clin Oncol 1995;121:317–9.
[7] Hiramatsu K, Sugimoto M, Kamei S, Hoshino M, Kinoshita K, Iwasaki K, et al. Diagnostic and prognostic usefulness of *N*1,*N*8-diacetylspermidine and *N*1,*N*12-diacetylspermine in urine as novel markers of malignancy. J Cancer Res Clin Oncol 1997;123:539–45.
[8] Hiramatsu K, Miura H, Kamei S, Iwasaki K, Kawakita M. Development of a sensitive and accurate enzyme-linked immunosorbent assay (ELISA) system that can replace HPLC analysis for the determination of *N*1,*N*12-diacetylspermine in human urine. J Biochem 1998;124:231–6.
[9] Gold P, Freedman S. Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. J Exp Med 1965;121:439–62.
[10] Gold P, Freedman S. Specific carcinoembryonic antigens of the human digestive system. J Exp Med 1965;121:467–81.
[11] Koprwoski H, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, Fuhrer P. Colorectal carcinoma antigens detected by hybridoma antibodies. Somatic Cell Genet 1979;5:957–72.
[12] International Union Against Cancer: TNM classification of malignant tumours. 6th ed. International Union Against Cancer. New York: John Willey & Sons, Inc., Publication; 2002.
[13] van den Berg GA, Muskiet FA, Kingma AW, van der Slik W, Halie MR. Simultaneous gas-chromatographic determination of free and acetyl-conjugated polyamines in urine. Clin Chem 1986;32:1930–7.
[14] Ogawa Y, Tanaka M, Inoue K, Yamaguchi K, Chijiiwa K, Mizumoto K, et al. A prospective pancreatographic study on prevalence of pancreatic cancer in diabetic patients. Cancer 2002;94:2344–9.
[15] Yamaguchi K, Ohuchida J, Ohtsuka T, Nakano K, Tanaka M. Intraductal papillary-mucinous tumor of the pancreas concomitant with ductal carcinoma of the pancreas. Pancreatology 2002;2:484–90.
[16] Yamaguchi K, Yokohata K, Noshiro H, Chijiiwa K, Tanaka M. Mucinous cystic neoplasm of the pancreas or intraductal papillary-mucinous tumor of the pancreas. Eur J Surg 2000;166:141–8.

Late Restenosis of the Balloon-Dilated Site
—— Serial Angiographic Observations Beyond 7 Years ——

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Background The present retrospective study was performed to assess the long-term (>7 years) fate of stabilized balloon-dilated sites.

Methods and Results Between February and April 1986, 171 patients underwent successful percutaneous balloon angioplasty. Early restenosis (<1 year) occurred in 53%, but repeat balloon angioplasty stabilized the balloon-dilated site. The early period was defined as 6 months, late years as 3–5 years and long-term years as 7–12 years. Angiographic evaluation at both early year or late year periods (mean=4.7 years) and long-term (mean=10.4 years) periods following stabilization was available in 71 patients (94 lesions) with mean age of 61.7±8.5 years. Of the 71 patients 69.6% were male. Restenosis occurring after 1 year was defined as late restenosis. The mean diameter stenosis changed from 6 months (50.3±12.4%) to late-period (44.2±13.2%; $p<0.05$) and long-term period (50.3±16.1%; $p<0.001$); but the reference vessel diameter did not change significantly. Late restenosis occurred in 28% (3–5 years) and 33% (7–12 years) of 94 lesions, and 13.8% of lesion required repeat target lesion revascularization. During this period, 5.3% of patients (5 lesions) underwent revascularization for new proximal or distal lesions.

Conclusions Decrease of luminal diameter during the early 6 months, was followed by regression after stabilization of the balloon-dilated site up to 5 years, but luminal re-narrowing occurs again over 7 years after balloon angioplasty. (*Circ J* 2005; **69:** 380–385)

Key Words: Angioplasty; Coronary disease; Follow-up studies; Restenosis

Percutaneous coronary balloon angioplasty (PTBA) has become a popular alternative to bypass surgery as a less invasive revascularization therapy for chronic coronary artery disease (CAD) since the 1980s. However, published documents have been reported where a high percentage of patients with occuring restenosis within 6 months of angioplasty and this has limited its use![1-10] Early clinical investigations reported that restenosis rarely occurs beyond 6 to 12 months after balloon angioplasty. Experimental studies in a number of species have shown that the initial myointimal thickening response composed of proliferation of smooth muscle cells and extracellular matrix to injury reaches a maximum within a few months; in the absence of further injury, luminal enlargement ensues reported as 'late regression'![1] Further angiographic investigations have reported preservation of balloon-dilated site for a decade if the patient is stable after 6 months after balloon angioplasty![12] In contrast, our pathological findings suggest that atherosclerosis progresses again after 7 years from balloon angioplasty.

In the present study, we report re-progression after late regression occurring more than 7 years after balloon angioplasty.

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Methods

Patient Population

Between February and April 1986, 203 consecutive patients underwent clinically uneventful coronary balloon angioplasty for de novo native coronary artery lesions at Kokura Memorial Hospital, Kitakyushu, Japan. According to angiographic criteria (>20% increase of minimal luminal diameter by visual estimation) and clinical criteria (improvement of symptom status without major complications), the procedure was considered successful in 171 patients (84.2%). Angiographic follow-up studies at 6 months (0.6 years=early), 3–5 years (4.7 years=late) and beyond 7 years (7–12 years; 10.4 years=long-term) in 71 (41.5%) patients with 94 lesions (group A), 23 of whom were followed up to 15 years, were available for retrospective analysis. Lesions with restenosis that were amenable to conservative treatment or restenosis treated by repeat angioplasty within 1 year after balloon angioplasty were eligible for enrollment in the study. Lesions with no need for bypass surgery, repeat-balloon angioplasty or death for 1 year were defined as 'stabilized'.

Follow-up Angiography

Coronary angiography at the early, late and long-term periods after PTBA was performed regardless of symptoms in these 71 patients. Quantitative angiographic analysis was performed with the Cardiovascular Angiography Analysis System II. Minimal lesion lumen diameter and lesion length was measured; percentage diameter stenosis as well as the interpolated reference diameter was calculated. Care

Table 1 Derivation of the Study Patients (Serial Late Angiographic Follow-up)

First time balloon angioplasty in 1986 (n=203)	
Unsuccessful	*19*
CABG	*7*
Death	*6*
Successful (n=171)	
Angiographic follow-up beyond 7 years (study group) (n=71)	
Angiographic follow up <7 years	*74*
CABG <7 years	*12*
Death <7 years	*13*
TLR after stabilization <7 years	*1*
Total (n=71)	*100*

CABG, coronary artery bypass graft; TLR, target lesion revascularization.

Table 3 Symptomatic Status at Late and Long-Term Angiographic Follow-up Period

	Late (n=71)	*%*	*Long-term (n=71)*	*%*
Asymptomatic	*43*	*60.6*	*29*	*41.4*
Symptomatic	*28*	*39.4*	*41*	*58.6*
CCS 3/4	*12*	*16.9*	*19*	*27.1*
Acute MI	*1*	*1.4*	*3*	*4.3*
Vasospasm	*2*	*2.8*	*0*	*0.0*
New leison related	*25*	*35.2*	*24*	*34.3*
Balloon-dilated lesion related	*1*	*1.4*	*9*	*12.9*
Unclear	*0*	*0.0*	*8*	*11.4*

CCS, angina classification of Canadian Cardiovascular Society; MI, myocardial infarction.

was taken by the operator to select the same reference points for the post-intervention and follow-up studies. The minimal lumen diameter was determined by computer at the same ballooned site taking the most severe narrowing, even if it was far from the initial narrowest point providing it was within the balloon-dilated site. The measurements of 2 angiographic views were averaged. Isosorbide dinitrate (5 mg) was injected intracoronally before each angiography. The diameter of 5F to 8F catheter tips free of contrast medium filmed at the center of the image were caliper measured and used for calibration in each study. Early restenosis was defined as more than 50% diameter stenosis observed at the 6-month angiography; late restenosis (LR) was defined as restenosis occurring beyond 1 year after angioplasty.

Statistical Analysis

Continuous variables are expressed as mean ± 1SD and compared with paired t-test for matched observations, or unpaired t-test if non-matched. Categorical variables were compared by the chi-square or Fisher's exact test. All tests of significance were two-tailed, and p-values of less than 0.05 were considered to be statistical significant. A change of stenosis diameter by >0.5 mm was defined as significant between late and long-term periods.

Results

Patient Characteristics

Seventy-one of 171 patients (group A) had follow-up angiography beyond 7 years, 23 of whom were followed beyond 12 years (Table 1), but in 74 patients (group B) angiography beyond 7 years was not available. Table 2 describes and compares the baseline characteristics of both groups.

There were no significant differences between the 2 groups. Follow-up angiography of group A patients showed: (i) early period at a mean of 7.2 months (range: 1.5 to 11.6 months); (ii) late or 3–5 years at 4.7 years (range: 2.9 to 6.3 years) after stabilization; and (iii) long-

Table 2 Baseline Characteristics of Group A Study Group Patients and Group B Patients Without Late Angiographic Follow-up

	Group A	*%*	*Group B*	*%*	*p value*
Patients	*71*		*74*		
Lesions	*94*		*105*		
Old MI	*40*	*56.3*	*33*	*44.6*	*0.14*
CCS 3/4	*27*	*38.0*	*33*	*44.6*	*0.25*
Multivessel disease	*32*	*45.1*	*38*	*51.4*	*0.39*
Acute MI	*12*	*16.9*	*8*	*10.8*	*0.47*
Target vessel					
LAD	*37*	*39.4*	*45*	*42.9*	
LCX	*32*	*34.0*	*30*	*28.6*	
RCA	*25*	*26.6*	*29*	*27.6*	
LMT	*0*	*0.0*	*1*	*1.0*	
Poor LVEF	*2*	*2.8*	*2*	*2.7*	*0.99*
Age	*61.7±8.5*		*62.9±8.9*		*0.36*
Male	*49*	*69.0*	*51*	*68.9*	*0.99*
Hypertension	*30*	*42.3*	*25*	*33.8*	*0.30*
Hyperlipidemia	*17*	*23.9*	*21*	*28.4*	*0.58*
Diabetes mellitus	*15*	*21.1*	*15*	*20.3*	*0.86*
Smoking	*28*	*39.4*	*30*	*40.5*	*0.89*
Total cholesterol	*189±59*		*192.1±42*		*0.73*
Triglycerides	*143.2±83*		*137.1±60*		*0.61*
High-density cholesterol	*41.1±18*		*42.1±16*		*0.35*
HbA1c	*5.96±1.8*		*5.97±1.6*		*0.98*
Creatinine	*1.38±1.6*		*1.20±0.2*		*0.56*
TLR <1 year	*18*	*25.4*	*16*	*21.6*	*0.49*

MI, myocardial infarction; CCS, angina classification of Canadian cardiovascular society; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; RCA, right coronary artery; LMT, left main trunk; HbA1c, Hemoglobin A1c.

Table 4 Quantitative Coronary Angiographic Analysis at Various Follow-up Periods

	Pre (n=94)	Immediate (n=94)	Early 6 months (n=94)	Late 3–5 years (n=94)	Long-term 7–12 years (n=94)	Long-term 12–15 years (n=23)
Minimal lumen diameter, mm	0.75±0.32	1.49±0.39	1.38±0.43	1.54±0.45	1.41±0.54	1.24±0.54
p-value	<0.0001	0.0671	<0.0001	0.0007	0.0862	
Reference diameter, mm	2.74±0.56	2.72±0.57	2.77±0.59	2.76±0.51	2.79±0.58	2.48±0.51
p-value	0.1254	0.9734	0.9815	0.4192	0.2345	
Diameter stenosis, %	70.9±11.4	48.9±11.3	50.7±12.1	44.2±13.2	51.6±16.9	48.7±18.9
p-value	<0.0001	0.3839	<0.0001	0.0005	0.573	
Lesion length, mm	13.1±8.3	10.7±4.9	12.1±6.4	10.9±4.6	11.4±6.3	11.7±7.2
p-value	0.0016	0.1453	0.0295	0.526	0.2553	
%Diameter stenosis >50	100%	33%	52%	28%	33%	48%

Statistical analysism paired t-test used.

Table 5 Need of Repeat Percutaneous Coronary Intervention (PCI) Beyond 7 Years Post-Stabilization

	n=94	%
LR at long-term	31	33.0
LR (%DS >70)	11	11.7
Total occlusion at long-term	7	7.4
AMI	3	3.2
Silent occlusion	2	2.1
Proximal total occlusion	2	2.1
PCI of new lesion in non-dilated vessel	25	26.6
PCI of same vessel	18	19.1
PCI of LR	13	13.8
PCI of new proximal or distal lesion	5	5.3
Proximal	3	3.2
Distal	2	2.1

LR, late restenosis; %DS, percentage diameter stenosis.

term or beyond 7 years between 6.9 to 11.8 years after stabilization, or 12 to 14.6 years for the subgroup of 23 patients.

There were fewer symptomatic patients at the late follow-up period than at the long-term follow-up. The incidence of unstable angina and acute myocardial infarction was slightly more frequent at the long-term follow-up period. While the incidence of new lesion-related ischemia was the same for both late and long-term follow-up periods, balloon-dilated site-related ischemia was greater at the long-term period (Table 3). One patient required repeat angioplasty for LR by the late-follow-up period, and was therefore excluded from the long-term follow-up. Repeat percutaneous coronary interventions (PCI) were required for disease progression in 22 (31%) patients with 25 (27%) new lesions, and 13 (18%) patients with 13 (14%) late restenotic lesions.

Angiographic Results

The mean minimal lumen diameter attained at the initial balloon angioplasty decreased, although not significantly during the first 6 months, and subsequently increased up to the late follow-up period, but then decreased significantly to the long-term follow-up period. The derived parameters changed accordingly. In contrast the reference diameter remained practically unchanged (Table 4).

While the incidence of lesions with percentage diameter stenosis >50 decreased from the early to the late follow-up period, it increased significantly to the long-term period. This resulted in an LR rate of 26 lesions (28%) for the late period, and 31 lesions (33%) for the long-term follow-up, the stenosis being severe (percentage diameter stenosis >70) in 11 lesions (12%). Seven lesions resulted in total occlusion (Table 5).

Regression and Progression After Stabilization

There was significant lesion regression in 23 of 94 lesions (24.5%), while in 6 (6.4%) there was significant progression occurred up to the late follow-up period; thus regression being dominant during the first 5 years (Fig 1).

In contrast lesion-progression dominated at the long-term follow-up period and was observed in 22 patients (23.4%; Fig 2).

Discussion

Many clinical investigations have reported that restenosis in balloon-dilated sites mainly occurred during the first 6 months after successful angioplasty, and rarely beyond that time, for the phenomenon of regression occurrs thereafter up to 5 years![11,13–16] In a previous clinical study the stenotic diameter did not change significantly between 6 months and 10 years after angioplasty![12] The results from the present study confirmed the phenomenon of late regression up to 5 years after balloon angioplasty, but also late progression at 10 years. Interestingly lesions with more than 40% of diameter stenosis at the late follow-up time did not progress by the long-term period, but those with less than 40% progressed significantly with no further enlargement of vessel diameter, in contrast to the reported regression after 5 years of balloon angioplasty![11]

Our early restenosis rate of 52%, somewhat higher than published data![17–20] may be due to the relatively small mean luminal diameter of the dilated reference arteries (2.74± 0.56, n=94). However, there was no significant differences about patient characteristics between with and without late angiographic follow-up. Disease progression in CAD is an insidious phenomenon, but often responsible for the late recurrence of cardiac symptoms and events. Angioplasty per-se may trigger the appearance of a new lesion(s) proximal to the balloon-dilated site![21–23] Five (5.3%) of our study lesions needed repeat PCI. Three (60%) lesions were proximal, but 2 (40%) occurred in the same vessel, distal and unrelated to the dilated lesion several years after balloon angioplasty. Therefore, this indicates that this phenomenon may or may not be secondary to the endothelial injury by the guidewire or balloon catheter, but rather suggests disease progression.

A rigorous and quantitatively evaluated serial angiographic study at 1, 3, 6 and 12 months after angioplasty showed that most of the reduction in mean minimum

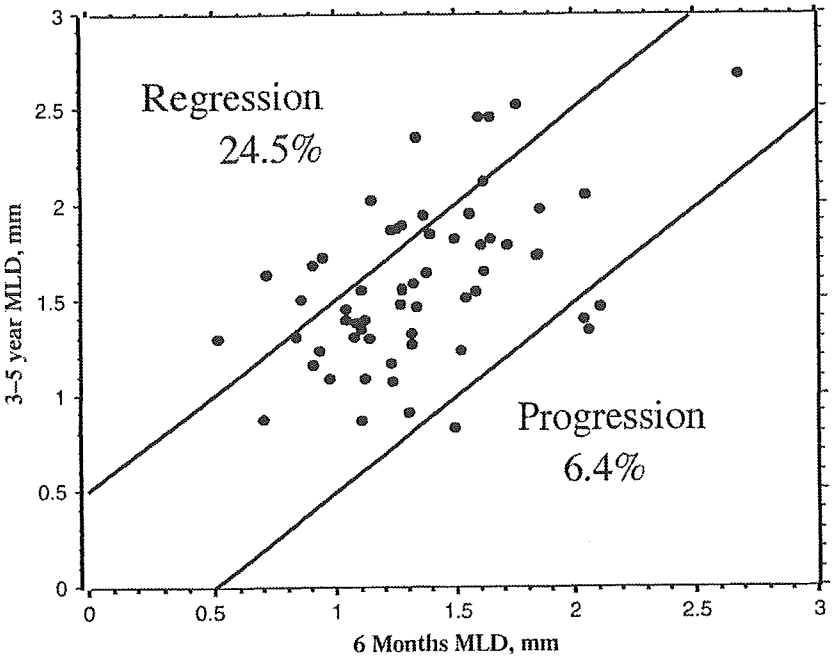

Fig 1. Lesion progression during the early (6 months) period post-balloon angioplasty, is substituted by regression up to the late (3–5 years) period.

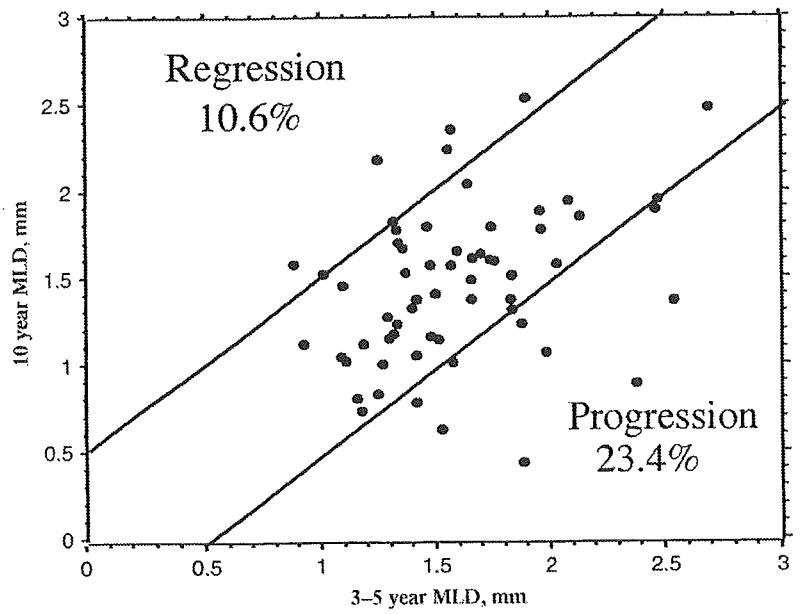

Fig 2. Progression of coronary stenosis is the prevailing feature by the long-term follow-up period (7–12 years) over the regression dominant late period (3–5 years).

luminal diameter occurred between 1 and 3 months after angioplasty, and that there was no change from 6 to 12 months! Lesion regression between 6 months and 3 years[3] or between 1 and 2 years[24] have been reported, but the actual consensus is that late lesion regression occurs between 7 months and 4.5 years after angioplasty![11]

Early restenosis is caused by the combination of neointimal thickening, consisting of smooth muscle cells and extracellular matrix, and arterial remodeling leading to vessel shrinkage. Experimental, pathological and intracoronary ultrasound studies suggest that the role of this constriction or shrinkage of the arterial in restenosis is greater than had previously been appreciated![25–30] In animals, the myointimal thickening in response to balloon injury reaches a maximum before 6 months and then regresses![13–16] This regression has been confirmed in pathological studies in humans dying at various time intervals after intracoronary metallic stent implantation; evidence of neointimal proliferation decreases with time and was difficult to find in patients who had died more than 2 years after angioplasty?[31] Although the role of remodeling is not known, regression of the intimal hyperplasia after PTBA might be also the major contributor to late regression.

Other pathological studies reported that endothelial cells regenerate as the result of the healing process maturation following balloon angioplasty, during which the initially proliferated and migrated smooth muscle cells are replaced by collagen fibers or extracellular matrix?[31] This process leads to luminal enlargement and result in late lesion regression, which might explain the low incidence of symptoms ascribable to the balloon-dilated site. In fact repeat angioplasty to the balloon-dilated site was required in only

1 patient up to the late (3–5 years follow-up) period in the current study.

However, while CAD progression-related ischemia did not increase from late to long-term follow-up (35.2% vs 34.3%), balloon-dilated site-related ischemia increased from 1.4% to 12.9%.

In an another pathological study of 7 years or more after balloon angioplasty demonstrated infiltration of lipid-laden macrophages mainly in the shoulder regions of the subendothelial space. After more than 10 years, thin fibrous caps heavily infiltrated by foam-cells were observed around the circumference of the lumen at balloon-dilated sites. These changes are suggestive of re-atherosclerotic process, which might be the pathogenetic mechanism of late progression or restenosis that may result in plaque vulnerability and even cause acute coronary syndromes.[32] In a few lesions (n=23) analyzed at 13.3 years (range: 11.7–14.6 years) after balloon angioplasty this tendency of late progression continued further.

Inward remodeling in addition to atherosclerotic changes might have contributed to late progression in 23.4% of lesions. In a small number of lesions (10.6%), outward remodeling might have accounted for the luminal enlargement.[33–36] Long-term studies using intravascular ultrasound[37,38] are needed to further elucidate the mechanism of arterial remodeling.

Study Limitations

The major limitation is the retrospective nature of the study and the potential bias by enrolling only 71 of 171 patients with successful angioplasty and serial angiographic investigation. However, there were no significant background differences between patients followed with or without angiography. Because of the retrospective nature there was a wide dispersion in the follow-up interval between late and long-term angiography.

Conclusions

The current study confirmed the lesion regression occurring up to 5 years after balloon angioplasty, but lesion progression occurred over 7 years after successful balloon angioplasty. Lesion progression is more likely to occur and be of greater magnitude in lesions with relatively little severity at 5 years. Pathologically late progression represents atheroscrelotic changes and might become symptomatic beyond 7 years after the late regression phase of balloon angioplasty, but clinical events are more often caused by new non-dilated lesions at 10 or more years of observation.

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References

1. Nobuyoshi M, Kimura T, Nosaka H, Mioka S, Ueno K, Yokoi H, et al. Restenosis after successful percutaneous transluminal coronary angioplasty: Serial angiographic follow-up of 229 patients. *J Am Coll Cardiol* 1988; **12:** 616–662.
2. Mabin TA, Holmes DR, Smith HC, Vlietstra RE, Reeder GS, Bresnahan JF, et al. Follow-up clinical results in patients undergoing percutaneous transluminal coronary angioplasty. *Circulation* 1985; **71:** 754–760.
3. Rosing DR, Cannon RO, Watson RM, Bonow RO, Mincemoyer R, Ewels C, et al. Three year anatomic, functional and clinical follow-up after successful percutaneous transluminal coronary angioplasty. *J Am Coll Cardiol* 1987; **9:** 1–7.
4. Talley JD, Hurst JW, King SB III, Douglas JS, Roubin GS, Gruentzig AR, et al. Clinical outcome 5 years after attempted percutaneous coronary angioplasty in 427 patients. *Circulation* 1988; **77:** 820–829.
5. Cequier A, Bonan R, Crepeau J, Cote G, Se Guise P, Joly P, et al. Restenosis and progression of coronary atherosclerosis after coronary angioplasty. *J Am Coll Cardiol* 1988; **12:** 49–55.
6. Grigg LE, Valentine PA, Manolas EG, Flower DJ, Hunt D. Long-term clinical and angiographic results following percutaneous transluminal coronary angioplasty. *Aust NZ J Med* 1988; **18:** 689–692.
7. Kamp O, Beatt KJ, De Feyter PJ, van den Brand M, Suryapranata H, Luijten HE, et al. Short-, medium-, and long-term follow-up after percutaneous transluminal coronary angioplasty for stable and unstable angina pectoris. *Am Heart J* 1989; **117:** 991–996.
8. Sharaf B, Riley RS, Drew TM, Williams DO. Late (five to eight years) clinical and angiographic assessment of patients undergoing successful percutaneous transluminal coronary angioplasty. *Am J Cardiol* 1992; **69:** 965–967.
9. Weintraub WS, Ghazzal ZMB, Douglas JS, Liberman HA, Morris DC, Coohen CL, et al. Long-term clinical follow-up in patients with angiographic restudy after successful angioplasty. *Circulation* 1993; **87:** 831–840.
10. King SB 3rd, Schlumpf M. Ten-year completed follow-up of percutaneous transluminal coronary angioplasty: The early Zurich experience. *J Am Coll Cardiol* 1993; **22:** 353–360.
11. Ormiston JA, Stewart FM, Roche AHG, Webber BJ, Whitlock RML, Webster MWI. Late regression of the dilated site after coronary angioplasty: A 5-year quantitative angiographic study. *Circulation* 1997; **96:** 468–474.
12. Guiteras-Val P, Varas-Lorenzo C, Garcia-Picart J, Marti-Claramunt V, Auge-Sanpera JM. Clinical and sequential angiographic follow-up six months and 10 years after successful transluminal coronary angioplasty. *Am J Cardiol* 1999; **83:** 868–874.
13. Inoue K, Nakamura N, Shiode N, Suyama H. Pathologic studies of late vascular responses to successful balloon angioplasty. *Circulation* 1999; **100:** I-145 (abstract).
14. Ross R, Glomset JA. The pathogenesis of atherosclerosis (first of two parts). *N Engl J Med* 1976; **295:** 369–377.
15. Stemerman MB, Ross R. Experimental atherosclerosis. I: Fibrous plaque formation in primates: An electron microscope study. *J Exp Med* 1972; **136:** 769–789.
16. Stemerman MB, Spaet TH, Pitlick F, Cintron J, Lejnieks I, Tiell ML. Intimal healing: The pattern of reendothelialization and intimal thickening. *Am J Pathol* 1977; **87:** 125–143 (abstract).
17. Fishman JA, Ryan GB, Karnovsky MJ. Endothelial regeneration in the rat carotid artery and the significance of endothelial denudation in the pathogenesis of myointimal thickening. *Lab Invest* 1975; **32:** 339–351.
18. Gruentzig AR, King SB III, Schlumpf M, Siegenthaler W. Long term follow-up after percutaneous transluminal coronary angioplasty. *N Engl J Med* 1987; **316:** 1127–1132.
19. Kaltembach M, Kober G, Scherer D, Vallbracht C. Recurrence rate after successful coronary angioplasty. *Eur Heart J* 1985; **6:** 276–281.
20. Leimgruber PP, Roubin GS, Hollman J, Cotsonis GA, Meier B, Douglas JS, et al. Restenosis after successful coronary angioplasty in patients with single-vessel disease. *Circulation* 1986; **73:** 710–717.
21. Serruys PW, Luijten HE, Beatt KJ, Geuskins R, de Feyter PJ, van den Brand M, et al. Incidence of restenosis after successful coronary angioplasty: A time-related phenomenon. *Circulation* 1988; **77:** 361–371.
22. Hwang MH, Sihdu P, Pacold I, Johnson S, Scanlon PJ, Loeb HS. Progression of coronary artery disease after percutaneous transluminal coronary angioplasty. *Am Heart J* 1988; **115:** 297–301.
23. Hamby RI, Amin HS, Gulotta SJ, Ricciardi R. Coronary artery stenosis complicating coronary angioplasty: Report of six cases. *Am Heart J* 1987; **114:** 639–643.
24. Bruce F, Waller BF, Pinkerton CA, Foster LN. Morphorogic evidence of accerated left main coronary artery stenosis: A late complication of percutaneous balloon angioplasty of the proximal left anterior descending coronary artery. *J Am Coll Cardiol* 1987; **9:** 1019–1023.
25. Mikuriya Y, Wakayama K, Kadota M, Matsumoto Y, Tamura A, Yano S, et al. Long-term angiographic follow-up results in patients undergoing percutaneous transluminal coronary angioplasty. *Jpn Circ J* 1989; **53:** 728–734.
26. Post MJ, Borst C, Kuntz R. The relative importance of arterial

remodeling compared with intimal hyperplasia in lumen renarrowing after balloon angioplasty: A study in the normal rabbit and the hypercholesterolemic Yucatan micropig. *Circulation* 1994; **89:** 2816–2821.
27. Glagov S, Weisenberg E, Zarins CK, Stanlunavicrus R, Kolettis G. Compensatory enlargement of human atherosclerotic coronary arteries. *N Engl J Med* 1987; **316:** 1371–1375.
28. Glagov S. Intimal hyperplasia, vascular modeling, and the restenosis problem. *Circulation* 1994; **89:** 2888–2891.
29. Isner JM. Vascular remodeling: Honey, I think I shrunk the artery. *Circulation* 1994; **89:** 2937–2941.
30. Mintz GS, Picard AD, Kent KM, Satler LF, Popma JJ, Leon MB. Intravascular ultrasound comparison of restenotic and de novo coronary artery narrowings. *Am J Cardiol* 1994; **74:** 1278–1280.
31. Mintz GS, Popma JJ, Pichard AD, Kent KM, Satler LF, Bucher TA, et al. Differing mechanisms of late arterial responses to transcatheter therapy: A serial quantitative angiographic and intravascular ultrasound study. *J Am Coll Cardiol* 1995; **25:** 155A (abstract).
32. Katsumi I, Nobuaki N, Nobuo S, Hiromi S. Pathologic studies of late vascular responses to successful balloon angioplasty. *Circulation* 1999; **100:** I-145 (abstract).
33. Mintz GS, Pichard AD, Kent KM, Satler LF, Popma JJ, Wong CS, et al. Endovascular stents reduce restenosis by eliminating geometric arterial remodeling: A serial intravascular ultrasound study. *J Am Coll Cardiol* 1995; **25:** 35A (abstract).
34. Nobuyoshi M, Kimura T, Ohishi H, Horiuchi H, Nosaka H, Hamasaki N, et al. Restenosis after percutaneous transluminal coronary angioplasty. *J Am Coll Cardiol* 1991; **17:** 433–439.
35. Ward MR, Pasterkamp G, Yeung AC. Cornelius borst arterial remodeling: Mechanisms and clinical implications. *Circulation* 2000; **102:** 1186–1191.
36. Schoenhagen P, Ziada KM, Vince DG, Nissen SE, Tuzcu EM. Arterial remodeling and coronary artery disease: The concept of "dilated" versus "obstructive" coronary atherosclerosis. *J Am Coll Cardiol* 2001; **38:** 297–306.
37. Tamada H, Nishikawa H, Mukai S, Setsuda M, Nakamura M, Suzuki H, et al. Impact of diabetes mellitus on angiographically silent coronary atherosclerosis. *Circ J* 2003; **67:** 423–426.
38. Yamagishi M, Hosokawa H, Saito S, Kanemitsu S, Chino M, Koyanagi S, et al. Coronary disease morphology and distribution determined by quantitative angiography and intravascular ultrasound. *Circ J* 2002; **66:** 735–740.

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Research Article

Evidence for a novel racemization process of an asparaginyl residue in mouse lysozyme under physiological conditions

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Abstract. We examined chemical reactions in mouse lysozyme after incubation under physiological conditions (pH 7 and 37 °C). After incubation for 8 weeks, racemization was observed specifically at Asn127 among the 19 Asp/Asn residues in mouse lysozyme. Furthermore, analysis of the primary structure showed that the racemized residue was not Asp, but Asn, which demonstrates that deamidation and isomerization did not occur. These results mean that this racemization occurs without forming a succinimide intermediate. This is the first example of D-asparaginyl formation in a protein occurring during the racemization process under physiological conditions.

Key words. Deterioration; mouse lysozyme; protein aging; racemization.

Many protein and peptide pharmaceuticals have been developed and are utilized in medical practice, including erythropoietin, interferon and granulocyte colony-stimulating factor. Because deterioration during the preservation of protein and peptide pharmaceuticals can lead to autoimmune responses [1] as well as functional disorders, we must elucidate the mechanisms of protein deterioration and propose methods to prevent it [2–4]. Protein deterioration is reportedly caused by spontaneous reactions, enzymatic reactions or environmental stresses such as high temperature, salt and ultraviolet irradiation [5]. Notable examples of spontaneous modification are pyroglutamylation of Glu/Gln residues, deamidation of Asn/Gln residues, isomerization of Asp/Asn residues cleavage of the peptide bond in Asp/Asn-Pro sequences [6] and racemization of other amino acids.

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Proteins in living tissues are comprised exclusively of L-amino acids; however, Asp/Asn residues in peptides and proteins are prone to racemize to D-Asp residues through deamidation and isomerization [7–13]. There are numerous examples of proteins that exhibit racemization in aged human tissues: dentin in teeth, membrane proteins in erythrocytes, elastin in aortic tissue, myelin basic protein in the brain, beta amyloid peptides in Alzheimer's disease plaques and αA-crystallin in the eye lens [7–15]. Racemization of αA-crystallin in the eye lens has also been reported to be site specific [16]. Considering the relationship between racemization and protein structure, we decided to investigate site-specific racemization of Asp/Asn residues.

Mouse lysozyme (ML) is secreted by macrophages, monocytes and polymorphonuclear leukocytes, and is widely distributed in body fluids and tissues [17, 18]. ML, a globular protein, is composed of 130 amino acid residues and contains all the usual 20 amino acids. The

three-dimensional structure has already been analyzed using nuclear magnetic resonance (NMR) spectroscopy [19]. Therefore, it is a suitable model to study protein deterioration under physiological conditions. Moreover, ML is a self protein for mice, and the deteriorated ML is an ideal protein for immunological studies. Here, we examined the deterioration of ML and found that Asn127 in ML was preferentially racemized after 8 weeks of incubation under physiological conditions.

Materials and methods

Materials

Sephadex G-25 was obtained from Pharmacia (Uppsala, Sweden). CM-Toyopearl 650S was obtained from Tohsoh (Tokyo, Japan). All other reagents were of analytical grade for biochemical use. Amino acid analyses were performed on a Hitachi L-8500 amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis of protein peptide samples in 6 N HCl under a vacuum at 110 °C for 20 h. Mass analyses were performed using a matrix-assisted laser desorption-time-of-flight mass spectrometer (MALDI-TOF/MS) Voyager (Applied Biosystems, Framingham, Mass.).

Preparation of ML

ML was obtained using a *Pichia pastoris* transformant according to our previous method [20]. The protein was purified by cation exchange (CM-Toyopearl 650S) chromatography.

Preparation of peptides

ML was reduced with 2-mercaptoethanol (2-ME) and S-alkylated with 3-bromopropyl trimethylammonium bromide (TAP-Br) according to a previous method [21]. Reduced S-alkylated ML was dissolved in 5 ml 0.1 M phosphate buffer (pH 8). To this solution, N-tosyl-L-phenylalanine chloromethylketone (TPCK)-trypsin (1% lysozyme by weight) was added and the solution was incubated for 2 h at 40 °C. The digested sample was separated by RP-HPLC using a C18 column (Mightysil RP-18 GP, 250 × 4.6 mm; Kanto Chemical, Osaka, Japan) with a linear gradient of 0–50% acetonitrile containing 0.1% HCl, at a flow rate 0.4 ml/min. Peptides were detected based on absorbance at 210 nm.

Analysis of racemization

Analysis of racemization at Asp and Asn residues in incubated ML was carried out using the method of Aswad [22] with slight modification. In brief, 4 mg o-phthaldialdyde (OPA) was dissolved in 300 µl methanol. To this solution was then added 250 µl 0.4 M sodium borate (pH 9.4), 390 µl distilled water and 60 µl 1.0 M N-acetyl-L-cysteine (NAC) solution at pH 5.5 (OPA-NAC solution). The OPA-NAC solution was stored at 4 °C until use. ML or peptides were hydrolyzed in 6 N HCl under a vacuum at 110 °C for 4 h. The hydrolysate was freeze-dried, and distilled water was added to the residue (Asp solution). Twenty microliters of the Asp solution was mixed with 10 µl OPA-NAC solution and this was left to stand for 3 min before 470 µl 0.05 M sodium acetate (pH 5.2) was added to the solution. The sample solution was centrifuged at 15,000 g for 1 min, and 20 µl of supernatant was subjected to RP-HPLC on a Vydac Protein & Peptide C18 (250 × 4.6 mm; Vydac, Columbia, Md.). The column was isocratically eluted with 0.05 M sodium acetate (pH 5.8) containing 4% acetonitrile at a flow rate of 0.4 ml/min. Column effluents were monitored based on absorbance at 350 nm.

Identification of N-terminal residue in T18 tryptic peptide

After reduced S-alkylated ML had been digested by TPCK-trypsin, the digested sample was separated by RP-HPLC as described above. The obtained tryptic peptide T18 was subjected to gas-phase protein sequencer N-terminal sequence analyses performed using an ABI 473A peptide sequencer (Applied Biosystems, Foster City, Calif.).

Preparation and separation of epimers in T18 tryptic peptides

Three types of peptide, in which the Asn127 residues were L-Asn, D-Asn and L-Asp, were synthesized as 11-mer peptides (D-L-S-Q-Y-I-R-N-C-G-V; N: one of the studied residues) using a peptide synthesizer (Pioneer peptide synthesizer; Applied Biosystems) [23–25]. Reduced and S-carboxymethylated peptides were digested with TPCK-trypsin as describe above, resulting in authentic tetramer peptides (L-N-carboxymethyl C-G-V, D-N-carboxymethyl C-G-V and L-D-carboxymethyl C-G-V). Confirmation of the purities of authentic peptides was made by amino acid analysis. Separation of each of the T18 tryptic peptide epimers was performed by RP-HPLC using a C18 column (Shiseido Capcellpack C18, 250 × 4.6 mm; Shiseido, Tokyo, Japan). The column was isocratically eluted with 3% acetonitrile containing 0.1% HCl at a flow rate 0.5 ml/min.

Results

Analyses of racemization of Asp/Asn residues in ML

In this experiment, we used ML obtained from *P. pastoris* transformants as described previously by our group [20]. We investigated racemization of Asp/Asn residues in ML after incubating at pH 7 and 37 °C in a glass tube. After incubating for 5 or 8 weeks, ML was partially hydrolyzed for 4 h to produce free aspartic acid, and we analyzed the racemization of the resulting aspartic acid. The D-Asp ratio in ML increased slightly but significantly with incu-

Table 1. Formation of D-Asp/Asn in ML after incubation in 0.05 M PBS at pH 7 and 37 °C.

	Incubation time		
	0 h	5 weeks	8 weeks
D-Asp (%)	1.95	3.75	4.35
SD	± 0.07	± 0.35	± 0.07

Data include all 19 Asp/Asn residues. Values represent the average and SD (n = 2).

bation when compared with that in ML without incubation (table 1). The D-Asp ratio in ML after incubation for 8 weeks was 2.4% after a correction of 1.95% for the racemization occurring during partial hydrolysis.

Specific racemization occurred at Asn127 in ML

Figure 1 shows the primary structure of ML. ML has 11 Asn and 8 Asp residues. To analyze the racemized Asp/Asn residues after incubating for 8 weeks, we performed tryptic digestion of reduced S-alkylated ML. Individual tryptic peptides were identified based on amino acid composition and mass analysis. The RP-HPLC elution patterns of tryptic peptides from ML incubated for 8 weeks and from ML without incubation are shown in figure 2A and B, respectively. Similar patterns could be seen irrespective of incubation. We examined the racemization at Asp/Asn residues in individual tryptic peptides. As shown in the left column of table 2, in ML incubated for 8 weeks, the D-Asp ratio of tryptic peptide T18 containing an Asn127 residue was extremely high (39.6%), whereas those of other peptides were about 2%. In ML without incubation, the D-Asp ratio of tryptic peptide T18 was 1.85%. These results indicated that, among all the Asp/Asn residues of ML, Asn127 was the only residue that racemized after 8 weeks of incubation under physiological conditions.

Confirmation of racemization at Asn127 in ML

To confirm the racemization at Asn127, we established a separation system of tryptic peptide T18 depending on

T1+2 :K^{1}-V-Y-E-R^{5}
T3 :C^{6}-E-F-A-R^{10}
T4+5 :T^{11}-L-K-R^{14}
T6 :N^{15}-G-M-A-G-Y-Y-G-V-S-L-A-D^{27}-W-V-C-L-A-Q-H-E-S-N^{37}-Y-N^{39}-T-R^{41}
T7 :A^{42}-T-N^{44}-Y-N^{46}-R^{47}
T8 :G^{48}-D^{49}-Q-S-T-D^{53}-Y-G-I-F-Q-I-N^{60}-S-R^{62}
T9 :Y^{63}-W-C-N^{66}-D^{67}-G-K^{69}
T10 :T^{70}-P-R^{72}
T11+12 :A^{73}-V-N^{75}-A-G-G-I-N^{80}-C-S-A-L-L-Q-D^{87}-D^{88}-I-T-A-A-I-Q-C-A-K-R^{98}
T13 :V^{99}-V-R^{101}
T14 :D^{102}-P-Q-G-I-R^{107}
T15 :A^{108}-W-V-A-W-R^{113}
T16 :A^{114}-H-C-Q-N^{118}-R^{119}
T17 :D^{120}-L-S-Q-Y-I-R^{126}
T18 :N^{127}-C-G-V^{130}

Figure 1. Primary structure of ML. T represents tryptic peptides, and peptide numbering is from the N-terminal peptide. Asparagine and aspartic acid residues are shown in bold.

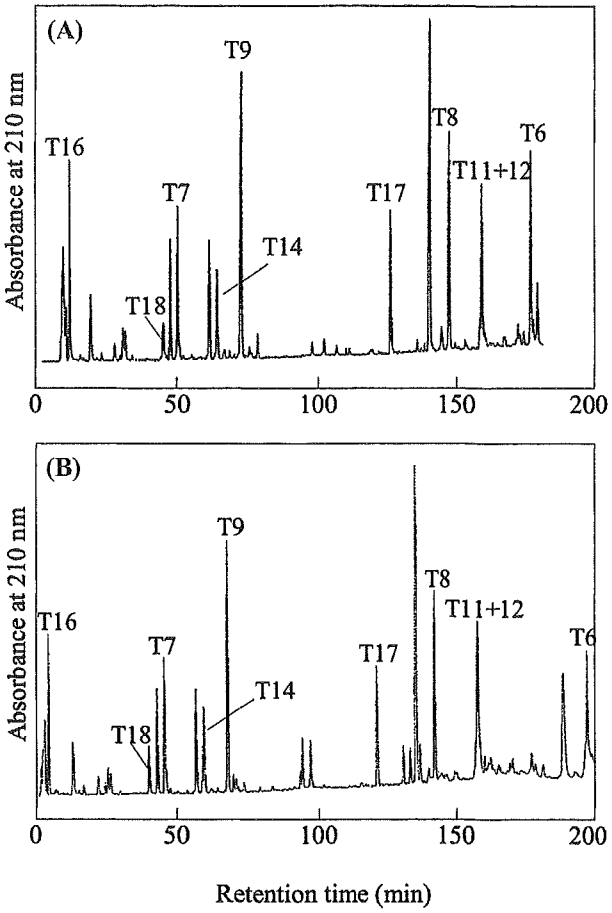

Figure 2. RP-HPLC patterns of tryptic peptides from reduced S-alkylated ML. ML was incubated for 8 weeks (*A*) or 0 h (*B*). Peptides were separated by RP-HPLC using a C18 column (RP-18 GP, 4.6 × 250 mm, Mightysil) with a linear gradient of 0–50% acetonitrile containing 0.1% HCl, at a flow rate of 0.4 ml/min. Peptides were detected by measuring their absorbance at 210 nm. Each peak was assigned based on amino acid composition and mass analysis. Tryptic peptides containing Asp/Asn residues are denoted.

the epimerism at Asn127. S-alkylated T18 peptides [Asn-carboxymethyl (CM)Cys-Gly-Val] were analyzed by RP-HPLC (see Materials and methods). Figure 3A shows that the epimers of the synthesized peptide could be clearly separated using RP-HPLC. When tryptic peptide T18 from ML after 8 weeks of incubation was analyzed using this system, we found two peptide peaks (fig. 3B). The results of co-chromatography of the tryptic peptide T18 and the synthesized peptides (fig. 3C–E) suggested that the tryptic peptide T18 from ML after 8 weeks of incubation contained D-Asn-(CM)Cys-Gly-Val. The former peak intensity in figure 3B indicated that 30–40% of the tryptic peptide was D-Asn-(CM)Cys-Gly-Val, which was consistent with the result in table 2.

Furthermore, we examined the N-terminal amino acids of the tryptic peptide T18 using an amino acid sequencer. As shown in figure 4, we detected mainly PTH-Asn. From the above results, we concluded that racemization oc-

Table 2. Formation of D-Asp/Asn in tryptic peptides containing Asp/Asn residues derived from ML after incubation for 8 weeks at pH 7 and 37 °C.

Peptide	D-Asp (%)	Number of Asp/Asn residues	Total amount of D-Asp [a]
T6	2.9	4	3.8
T7	2.2	2	0.5
T8	2.3	3	1.1
T9	2.4	2	1.0
T11 + 12	2.5	4	2.2
T14	1.9	1	−0.1
T16	2.4	1	0.5
T17	2.0	1	0.1
T18	39.6	1	37.7
0 h[b] (ML)	1.95	19	

[a] Total amount of D-Asp (%) = [(D-Asp) − 1.95] × corresponding number of Asp/Asn residues.
[b] See table 1.

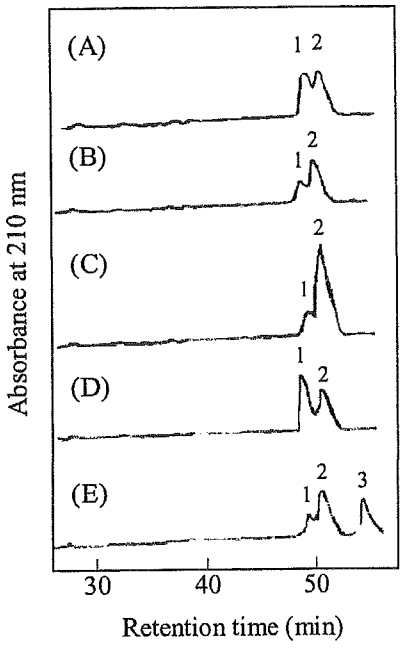

Figure 3. RP-HPLC patterns of tryptic peptides [Asn-(CM)Cys-Gly-Val]. Peptides were separated by RP-HPLC using a C18 column (Shiseido Capcellpack C18, 250 × 4.6 mm) with isocratic elution buffer (3% acetonitrile and 0.1% HCl) at a flow rate of 0.5 ml/min: mixture of the synthetic peptide [L-Asn-(CM)Cys-Gly-Val] and the synthetic peptide [D-Asn-(CM)Cys-Gly-Val] (*A*); tryptic peptide T18 derived from ML after incubation for 8 weeks (*B*); mixture of the tryptic peptide T18 derived from ML after incubation for 8 weeks and the synthetic peptide [L-Asn-(CM)Cys-Gly-Val] (*C*); mixture of the tryptic peptide T18 derived from ML after incubation for 8 weeks and the synthetic peptide [D-Asn-(CM)Cys-Gly-Val] (*D*); mixture of the tryptic peptide T18 derived from ML after incubation for 8 weeks and the synthetic peptide [L-Asp-(CM)Cys-Gly-Val] (*E*). Peaks 1, 2 and 3 correspond to the peptides D-Asn-(CM)Cys-Gly-Val, L-Asn-(CM)Cys-Gly-Val and L-Asp-(CM)Cys-Gly-Val, respectively.

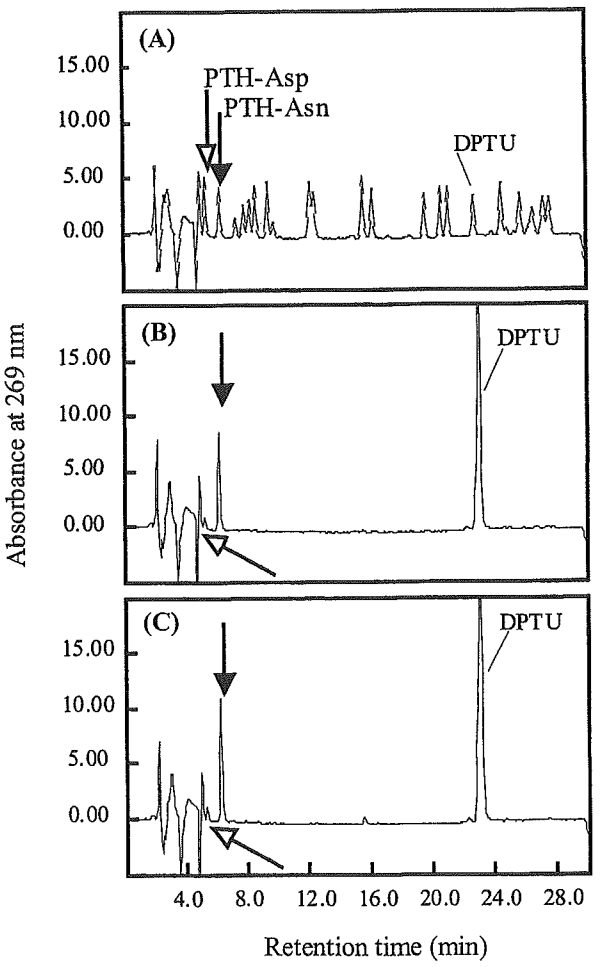

Figure 4. N-terminal analyses of the tryptic peptide T18 by a gas-phase protein sequencer. Closed arrows and open arrows indicate the retention time of phenyl thio hydantoin (PTH)-Asn and PTH-Asp, respectively; standard cycle (*A*); first cycle for mouse lysozyme incubated for 8 weeks (*B*) and 0 h (*C*).

curred at Asn127 in ML after 8 weeks of incubation under physiological conditions.

Discussion

As shown in table 1, 2.4 (= 4.35−1.95)% of Asp/Asn residues in ML were racemized after incubation for 8 weeks under physiological conditions, which means that 2.4% of D-Asp/Asn residues formed after 8 weeks of incubation. To determine the racemization site, we examined the formation of D-Asp/Asn in tryptic peptides derived from ML after 8 weeks of incubation. As shown in table 2, we found that D-Asn in the tryptic peptide T18 was preferentially racemized. Based on the total amount of D-Asp (table 2), we can calculate the average D-Asp ratio in ML by adding up the values the right column in table 2 and dividing by 19 (total number of Asp/Asn residues in ML) to give 2.45%. This value was similar to Asp ratio (2.4%) derived from ML after 8 weeks of incubation, suggesting that specific racemization occurred at Asn127 in ML after 8 weeks of incubation. Moreover, from a comparison of the retention time of the tryptic peptide T18 derived from ML after 8 weeks of incubation with those of the authentic peptides on RP-HPLC, and analysis of the N-terminal amino acids of the tryptic peptide T18 derived from ML after 8 weeks of incubation, we concluded that L-Asn at position 127 in ML was preferentially converted to D-Asn after 8 weeks of incubation under physiological conditions.

The mechanism of racemization at Asn residues is considered to involve the formation of an aminosuccinimidyl (Asu) peptide intermediate which is an obligatory first step in these reactions (see fig. 5) [26−29]. General base catalysis by nucleophilic attack, in which the lone pairs of electrons on the nitrogen of the carboxyl-side backbone attack the side chain carbonyl group, causes the formation of intramolecular succinimide, and the subsequent hydrolyzation of succinimide leads to D/L-aspartyl and D/L-isoaspartyl residues. The formation of succinimide thus plays an important role in the racemization of Asp/Asn residues [30]. However, as shown above, after 8 weeks of incubation of ML under physiological conditions, we found that the racemization occurred at Asn127 in ML, indicating that the racemization did not form by way of the succimide intermediate. This is the first example of racemization at an Asn residue in a protein occurring without formation of the succinimide intermediate.

In the mechanism with the succinimide intermediate, specific racemization in a protein depends on the primary structure, the secondary structure, the higher order structure [27, 28, 31−34] and the steric disorder of the neighboring C terminal residue [16]. Therefore, since the C terminus residue of Asn127 is Cys128 in ML, which forms a disulfide bond with Cys6, the succinimide formation mechanism cannot explain the specific racemization at Asn127 in ML during incubation of ML under physiological conditions. The reactive C-terminal (Val130) carboxylate in ML is one of the candidates for involvement in the racemization at Asn127. However, the solution structure of ML using NMR [19] and molecular dynamic simulation (data not shown), suggested that the C-terminal carboxylate is unlikely to access Asn127. Recently, Li et al. [35] examined the conversion from L-Asn to D-Asn using the peptide containing Asn, and found that this conversion in the peptide partially occurred without deamidation, indicating that succinimide formation at the Asn residue did not occur. Conversion from L-Asn to D-Asn in the peptide was considered to proceed by way of the tetrahedral intermediate (see fig. 6) [35]. Thus, in the present case, the racemization at Asn127 in ML during incubation under physiological conditions may have occurred by way of the tetrahedral intermediate.

For alternative mechanisms, we focus on the C-alpha radical at Asn127. The C-alpha radical at an amino acid residue in a peptide was reported to be stable in oxidative

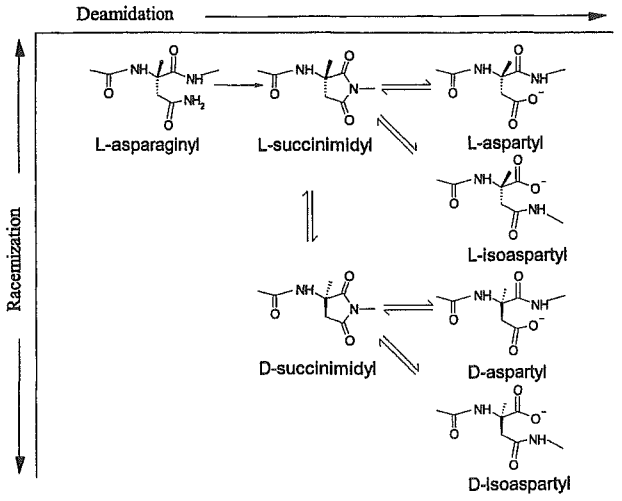

Figure 5. The conventional mechanism of the Asn racemization reaction at neutral or basic pH [16].

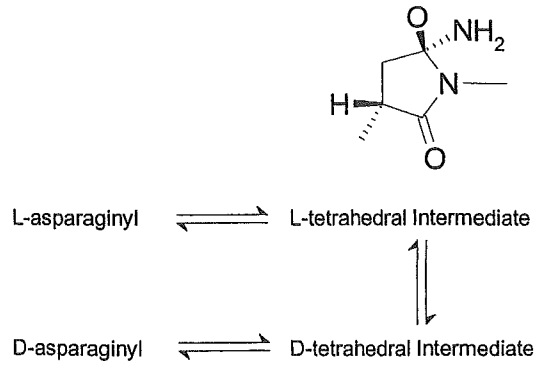

Figure 6. Possible conversion pathway from L-Asn to D-Asn. Cited from Li et al. [35].