tients who received 3.75-15 mg/day of TLV on a de novo basis for more than 1 week were retrospectively analyzed. The initial dose of TLV was determined by the attending physician taking into consideration the hemodynamics and degree of congestion of the patient, and was maintained during the study period.

Eligible patients had either lower limb edema, pulmonary congestion, or jugular venous distension due to fluid retention despite receiving tolerable amounts of conventional diuretics that included loop diuretics and/or thiazides, in addition to appropriate restriction of sodium and water intake. All patients had one or more previous hospitalizations due to decompensated HF during the past 12 months. All patients were assigned to New York Heart Association (NYHA) class III or IV.

Patients were excluded and did not receive TLV if they had hypovolemia, severe stenotic valvular disease, severe systemic infection or inflammation, end-stage renal failure on hemodialysis, acute coronary syndrome within 1 month, or hypernatremia with S-Na > 145 mEq/L. Patients who were dependent on any mechanical supports such as ventricular assist devices, intra-aortic balloon pumping, extracorporeal membranous oxygenation, mechanical ventilation, or any combination of these devices, were also excluded.

During the study period, restrictions on water intake were loosened according to the weight loss of the patients, but salt restriction was continued at 6 g/day (equivalent to 2.4 g/day of sodium). Patients with impaired consciousness who lacked a sense of thirst were also excluded from this study. Concomitant use of intravenous agents including human atrial natriuretic peptide, phosphodiesterase III inhibitors, dobutamine, or dopamine as well as i.v. furosemide was continued if present, and the doses were not changed during the study period.

The present study complied with the Declaration of Helsinki and the institutional review board of University of Tokyo approved the research protocol [the application number, 779(1)]. Informed consent was obtained from all patients before enrollment.

Measures: The following variables were collected (1) < 24 hours before the introduction of TLV and (2) at 1 week after the administration of TLV: demographic characteristics; blood laboratory parameters; and symptom parameters due to HF. Echocardiographic parameters were obtained < 24 hours before the administration of TLV by using a standard, comprehensive M-mode and 2D echocardiogram by expert echo-cardiologists. Left ventricular ejection fraction was calculated using the biplane Simpson method from apical 4- and 2-chamber views. No patient underwent urinary catheter placement, but daily UV was measured during the study period. Urine samples were obtained (1) in the early morning just before the administration of any medication including TLV and other diuretics on day 1, (2) at 4 hours after the administration of TLV on day 1, and (3) in the early morning just before the administration of any medication including TLV on day 7. Estimated amount of urinary sodium excretion during 24 hours ($U\text{-}NaEx_{24}$) was calculated by the following formula, ie [$U\text{-}NaEx_{24}$ (mg/day)] = 2.3 × [urinary sodium concentration (U-Na) (mg/dL)] × [predicted amount of urinary creatinine excretion during 24 hours (mg/day)] / [urinary creatinine concentration (mg/dL)]. Predicted amounts of urinary creatinine excretion during 24 hours were calculated in males and females separately by the following formula, ie (1) for males, 15.12 × [body weight (kg)] + 7.39 × [body height (cm)] - 12.63 × [age (years)] -79.9, and (2) for females, 8.58 × [body weight (kg)] + 5.09 × [body height (cm)] -4.72 × [age (years)] -74.9.[13]

Patients in whom UV on day 1 accomplished an increase compared with that of day 0 were defined as "responders" to TLV and the reverse as "non-responders", as we previously demonstrated its validity and reliability in association with various clinical parameters including amelioration of congestion.[14,15] Hyponatremia was defined as S-Na < 132 mEq/L considering the results of the receiver operating characteristics (ROC) analysis discussed below, and hypernatremia was defined as S-Na > 145 mEq/L. The HF symptom score was calculated as the summation of scores that were assigned to each symptom due to HF, such as (1) pitting edema in the lower extremities [1 point], (2) pulmonary congestion [1 point], (3) jugular venous distension [1 point], (4) dyspnea [1 point], and (5) degree of NYHA (assigned 1-4 points to each class, ex. assigned 4 points to NYHA class IV). The above score was constructed on the basis of the Minnesota Living with Heart Failure Questionnaire, which is one of the most widely used questionnaires to evaluate HF specific quality of life.[16,17] The internal consistency among each item of the score was sufficient (Cronbach's alpha, 0.741). The HF symptom score was calculated just before the administration of TLV and at 1 week after TLV treatment in all patients.

Statistical analysis: Categorical parameters are presented as frequencies and percentages, and continuous variables as the mean ± standard deviation. The patient characteristics were compared using the unpaired *t*-test or Mann-Whitney test for continuous variables, and the chi-square test or Fisher's exact test for categorical variables as appropriate. Pearson's product-moment correlation coefficient was also calculated to assess the relations between baseline parameters and changes in $U\text{-}NaEx_{24}$. The cut-off value of the baseline sodium concentration for any increases in urine sodium excretion during the one week of TLV treatment was analyzed by ROC analyses. S-Na and $U\text{-}NaEx_{24}$ measured on day 7 were compared with those of baseline by the Wilcoxon signed-rank test.

All statistical tests were 2-tailed, with $P < 0.05$ regarded as being statistically significant. All statistical analyses were performed using PASW Statistics 18 (SPSS Inc, Chicago, IL, USA).

Results

Baseline characteristics of responders to TLV (Table I): Among 97 patients with decompensated HF who were enrolled in the present study, 68 (70.1%) were responders with increased UV at day 1 compared with day 0. Among all 68 responders, TLV was initiated at 13 days after patient admission on average. Nine patients (13.2%) had ischemic etiology, and 27 (39.7%) had been diagnosed with dilated cardiomyopathy. All patients, unless contraindicated, had received standard medical therapy for HF that included β-blockers (91.2%), and angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers (82.4%) at the maximum tolerable doses. All patients had been dependent on a type of diuretic, including furosemide (mean, 61.9 ± 37.3 mg/day; range, 20-240 mg/day), spironolactone (32.9 ± 22.3 mg/day; 0-100 mg/day), and trichlormethiazide (0.4 ± 0.8 mg/day; 0-4 mg/day). Thirty-one patients (45.6%) were dependent on continuous infusion of

Table I. Demographic, Laboratory, and Echocardiographic Parameters Before the Administration of TLV in Responders With and Without Hyponatremia

	Total ($n = 68$)	Serum sodium concentration < 132 mEq/L ($n = 25$)	Serum sodium concentration ≥ 132 mEq/L ($n = 43$)	*P*
Demographic parameters				
Dose of TLV, mg daily	6.3 ± 3.8	6.0 ± 3.2	6.5 ± 4.2	0.580
Timing of TLV administration, day	13 (4 - 414)	12 (4 - 414)	15 (4 - 140)	0.264
Age, years	49.9 ± 18.9	44.3 ± 19.9	53.1 ± 17.6	0.061
Male, *n* (%)	46 (67.6)	20 (80.0)	26 (60.5)	0.097
Body mass index	22.0 ± 4.0	22.0 ± 3.6	22.0 ± 4.3	0.984
Body weight, kg	58.6 ± 13.8	59.2 ± 11.3	58.3 ± 15.1	0.805
Etiology of ischemia, *n* (%)	9 (13.2)	5 (20.0)	4 (9.3)	0.209
Systolic blood pressure, mmHg	94.2 ± 9.5	92.1 ± 10.4	98.4 ± 9.8	0.185
Heart rate, bpm	83.4 ± 9.4	85.4 ± 9.4	81.4 ± 10.4	0.243
Concomitant medication				
Furosemide, mg daily	61.9 ± 37.3	70.4 ± 40.9	57.0 ± 34.5	0.154
Spironolactone, mg daily	32.9 ± 22.3	39.0 ± 22.9	29.4 ± 21.4	0.088
Trichlormethiazide, mg daily	0.4 ± 0.8	0.6 ± 1.2	0.3 ± 0.5	0.136
Number of prescribed diuretics	2.1 ± 0.6	2.2 ± 0.6	2.0 ± 0.6	0.154
β-Blocker, *n* (%)	62 (91.2)	23 (92.0)	39 (90.7)	0.614
ACEI/ARB, *n* (%)	56 (82.4)	20 (80.0)	36 (83.7)	0.698
Catecholamine infusion, *n* (%)	31 (45.6)	19 (76.0)	12 (27.9)	< 0.001†
Laboratory parameters				
Serum sodium, mEq/L	132.6 ± 6.5	125.8 ± 5.0	136.6 ± 3.1	< 0.001*
Serum potassium, mEq/L	4.1 ± 0.4	4.2 ± 0.4	4.1 ± 0.4	0.340
Serum BUN, mg/dL	32.8 ± 15.2	33.2 ± 19.6	32.5 ± 12.3	0.874
Serum creatinine, mg/dL	1.3 ± 0.5	1.2 ± 0.5	1.3 ± 0.5	0.506
Serum albumin, g/dL	3.5 ± 0.4	3.6 ± 0.4	3.4 ± 0.5	0.532
Serum total bilirubin, mg/dL	1.5 ± 1.1	1.8 ± 0.9	1.3 ± 1.1	0.045*
Serum osmolality, mOsm/L	276.3 ± 13.3	263.8 ± 12.9	283.2 ± 7.3	< 0.001*
Plasma arginine vasopressin, pg/mL	5.2 ± 3.1	8.1 ± 4.0	4.7 ± 2.3	0.038*
Plasma BNP, $\log_{10}$ pg/mL	2.8 ± 0.4	2.9 ± 0.3	2.7 ± 0.5	0.013*
Urine parameters				
Urine volume of day 0, mL/day	1337 ± 357	1390 ± 332	1305 ± 372	0.350
U-OSM, mOsm/L	490.6 ± 151.5	466 ± 141	505 ± 157	0.310
U-sodium, mEq/L	54.6 ± 29.5	48.0 ± 27.0	58.4 ± 30.6	0.160
U-potassium, mEq/L	32.0 ± 15.3	32.0 ± 16.7	32.0 ± 14.6	0.992
U-urea nitrogen, mg/dL	756.2 ± 302.1	761.0 ± 312.9	753.7 ± 300.8	0.933
U-creatinine, mg/dL	95.9 ± 59.9	93.5 ± 64.4	97.1 ± 58.1	0.819
Estimated U-sodium excretion, mg/24 hours	2602 ± 2245	2767 ± 2703	2200 ± 1643	0.375
FE_{Na}, %	0.77 ± 0.77	0.91 ± 1.09	0.70 ± 0.54	0.410
Echocardiographic parameters				
LV diastolic diameter, mm	61.4 ± 11.7	62.4 ± 7.1	60.8 ± 13.5	0.581
Ejection fraction, %	30.0 ± 18.9	22.3 ± 9.2	33.9 ± 21.2	0.008*
Ejection fraction ≥ 50% *n* (%)	13 (19.1)	0 (0)	13 (30.2)	0.002†
Symptom				
Heart failure symptom score	6.4 ± 1.4	6.9 ± 1.3	6.1 ± 1.4	0.013*
NYHA class IV, *n* (%)	31 (45.6)	18 (72.0)	13 (30.2)	0.001†

TLV indicates tolvaptan; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; BUN, blood urea nitrogen; BNP, B-type natriuretic peptide; U-OSM, urine osmolality; U, urinary; FENa, fractional excretion of sodium; LV, left ventricle; and NYHA, New York Heart Association. *$P < 0.05$ by unpaired *t*-test or Mann-Whitney test as appropriate. †$P < 0.05$ by chi-square test or Fisher's exact test as appropriate.

catecholamine. Many of the enrolled responders had mild end-organ dysfunction; the mean serum creatinine concentration was 1.3 ± 0.5 mg/dL and the mean serum total bilirubin concentration was 1.5 ± 1.1 mg/dL. S-Na averaged 132.6 ± 6.5 mEq/L and the range was 112-144 mEq/L. No patient had hypernatremia with S-Na > 145 mEq/L before the administration of TLV as per the exclusion criteria. Plasma arginine vasopressin was detectable in all patients (mean, 5.2 ± 3.1 pg/mL; range, 1.5-13.9 pg/mL) despite their relatively lower levels of serum osmolality (276.3 ± 13.3 mOsm/L). Thirteen patients (19.1%) had preserved left ventricular systolic function with an ejection fraction ≥ 50%. Baseline U-$NaEx_{24}$ was approximately zero-balanced with sodium intake (~2.5 g/day).

Changes in S-Na and urinary sodium excretion in responders after one week of TLV treatment: The correlations between changes in U-$NaEx_{24}$ during one week of TLV treatment and baseline parameters are shown in Table II. The changes in U-$NaEx_{24}$ had no significant correlation with baseline parameters except S-Na ($P = 0.011$, $r = 0.325$) (Figure 1). The cut-off value of S-Na for any increase in U-$NaEx_{24}$ was 132 mEq/L (area under curve, 0.711; sensitivity, 0.769; specificity, 0.572). We defined hyponatremia as S-Na < 132 mEq/L based on this result.

As for the comparison between the hyponatremic and normonatremic groups (Table I), hyponatremic responders had a slight but significantly greater decompensated state, higher serum level of total bilirubin, higher plasma levels of arginine vasopressin and B-type natriuretic peptide, a lower left ven-

Table II. Correlation Between Changes in U-NaEx$_{24}$ During 1 Week of TLV Treatment and Baseline Parameters in Responders, Versus Changes in U-NaEx$_{24}$ During 1 Week of TLV Treatment

	P	*r*
Demographic parameters		
Dose of TLV, mg daily	0.274	0.145
Age, years	0.463	0.097
Body mass index	0.331	0.129
Body weight, kg	0.098	0.218
Concomitant medication		
Furosemide, mg daily	0.710	-0.049
Spironolactone, mg daily	0.846	0.026
Trichlormethiazide, mg daily	0.428	0.105
Laboratory parameters		
Serum sodium, mEq/L	0.011*	0.325
Serum potassium, mEq/L	0.546	-0.080
Serum BUN, mg/dL	0.190	-0.173
Serum creatinine, mg/dL	0.927	-0.012
Serum albumin, g/dL	0.522	0.085
Serum total bilirubin, mg/dL	0.143	-0.193
Serum osmolality, mOsm/L	0.198	0.170
Plasma arginine vasopressin, pg/mL	0.634	-0.113
Plasma BNP, $\log_{10}$ pg/mL	0.869	-0.022
Echocardiographic parameters		
LV diastolic diameter, mm	0.921	0.015
Ejection fraction, %	0.533	-0.094

U-NaEx$_{24}$ indicates urinary sodium excretion during 24 hours; TLV, tolvaptan; BUN, blood urea nitrogen; BNP, B-type natriuretic peptide; and LV, left ventricle. *$P < 0.05$ by Pearson's product-moment correlation coefficient.

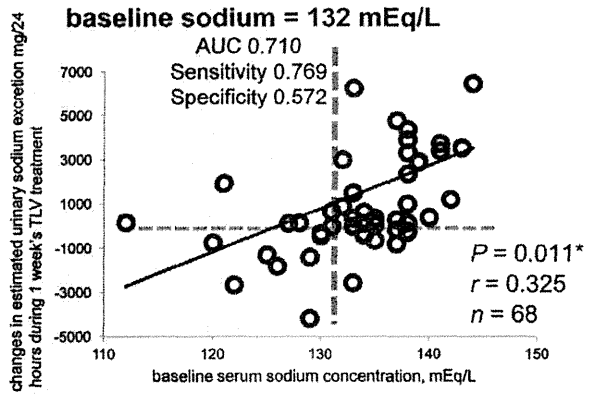

Figure 1. Relationship between baseline S-Na and changes in U-NaEx$_{24}$ during one week TLV treatment in responders. *$P < 0.05$ by Pearson's product-moment correlation coefficient.

tricular ejection fraction, and more frequent requirement for inotrope infusion. Baseline U-Na, U-NaEx$_{24}$, and FE$_{Na}$ were not different between the two groups.

TLV treatment increased S-Na significantly in hyponatremic responders (n = 25), whereas S-Na remained unchanged in normonatremic responders (n = 43) (Figure 2). U-NaEx$_{24}$ increased significantly in normonatremic responders, but remained unchanged in hyponatremic responders after one week of TLV treatment (Figure 3).

Clinical course in hyponatremic and normonatremic responders: Clinical parameters obtained at 4 hours after the administration of TLV on day 1 and day 7 were compared in hyponatremic and normonatremic groups. U-Na concentration

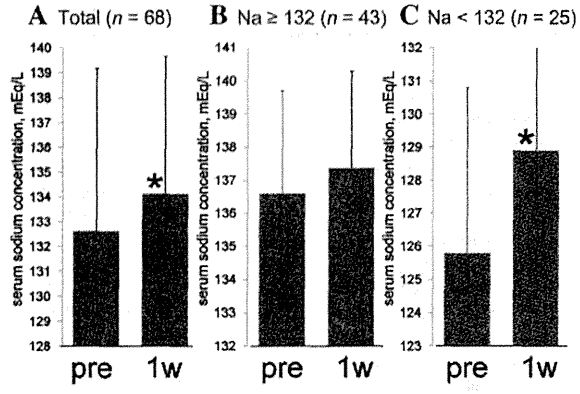

Figure 2. Changes in S-Na after administration of TLV in responders. pre, baseline; 1w, at 1 week after the administration of TLV. *$P < 0.05$ by Wilcoxon signed-rank test compared with baseline.

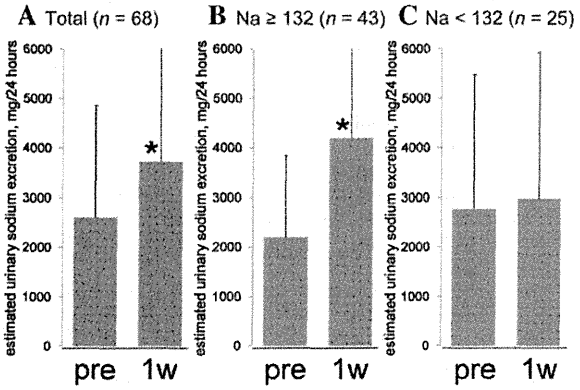

Figure 3. Changes in U-NaEx$_{24}$ after administration of TLV in responders. pre, baseline; 1w, at 1 week after the administration of TLV. *$P < 0.05$ by Wilcoxon signed-rank test compared with baseline.

obtained at 4 hours after the administration of TLV was significantly higher in normonatremic responders than that in hyponatremic responders (Table III). Urine osmolality was equally decreased compared with baseline in both the normonatremic and hyponatremic groups at 4 hours after TLV treatment.

On day 7, U-NaEx$_{24}$ was significantly higher in the normonatremic responders than in the hyponatremic responders (Table IV). U-NaEx$_{24}$ was increased by approximately 2-fold in the normonatremic responders, which means they had a minus balance since their food contained ~2.5 g/day of sodium.

As for the changes in HF parameters on day 7, serum total bilirubin, plasma B-type natriuretic peptide, and body weight exhibited better improvement in normonatremic than in hyponatremic responders, although the differences were statistically insignificant (Table IV). As a result, hyponatremic responders still had higher serum concentrations of total bilirubin and higher concentrations of plasma B-type natriuretic peptide (Table IV). The serum potassium level was slightly increased on day 7 in the normonatremic responders compared with the hyponatremia responders (Table IV).

Effects of TLV in nonresponders: In the nonresponders (n = 29), S-Na remained unchanged during the study period regard-

Table III. Urine Parameters at 4 Hours After TV Initiation in Responders With and Without Hyponatremia

	Total ($n = 68$)	Serum sodium concentration < 132 mEq/L ($n = 25$)	Serum sodium concentration ≥ 132 mEq/L ($n = 43$)	P
Urine parameters				
U-OSM, mOsm/L	276.8 ± 97.0	255 ± 111	289 ± 87	0.157
U-sodium after 4 hour, mEq/L	52.7 ± 30.3	48.0 ± 27.0	58.4 ± 30.6	0.001*
U-potassium after 4 hour, mEq/L	25.9 ± 15.2	26.8 ± 14.9	25.3 ± 15.5	0.703
U-urea nitrogen after 4 hour, mg/dL	330.9 ± 189.6	334.0 ± 234.4	329.4 ± 165.7	0.932
U-creatinine after 4 hour, mg/dL	39.8 ± 30.1	40.1 ± 32.8	39.6 ± 28.9	0.948

TLV indicates tolvaptan; U-OSM, urine osmolality; and U, urinary. *$P < 0.05$ by unpaired t-test or Mann-Whitney test as appropriate.

Table IV. Characteristics at 1 Week After TLV Treatment in Responders With and Without Hyponatremia.

	Total ($n = 68$)	Serum sodium concentration < 132 mEq/L ($n = 25$)	Serum sodium concentration ≥ 132 mEq/L ($n = 43$)	P
Clinical parameters				
Demographic parameters				
Body weight, kg	57.7 ± 13.4	57.9 ± 11.7	57.5 ± 14.5	0.903
Laboratory parameters				
Serum sodium, mEq/L	134.1 ± 5.5	128.6 ± 4.3	137.4 ± 2.9	< 0.001*
Serum potassium, mEq/L	4.2 ± 0.4	4.2 ± 0.5	4.3 ± 0.5	0.834
Serum BUN, mg/dL	28.7 ± 14.3	26.3 ± 14.0	30.0 ± 14.4	0.310
Serum creatinine, mg/dL	1.3 ± 0.6	1.1 ± 0.4	1.4 ± 0.7	0.074
Serum total bilirubin, mg/dL	1.6 ± 1.1	2.1 ± 1.0	1.2 ± 1.1	0.002*
Plasma BNP, $\log_{10}$ pg/mL	2.7 ± 0.4	2.8 ± 0.3	2.6 ± 0.5	0.001*
Urine parameters				
U-OSM, mOsm/L	367.5 ± 137.1	337.6 ± 142.5	383.4 ± 133.5	0.268
U-sodium, mEq/L	57.3 ± 31.2	53.2 ± 36.3	59.6 ± 28.2	0.491
U-potassium, mEq/L	25.9 ± 13.5	24.9 ± 11.9	26.5 ± 15.0	0.696
U-urea nitrogen, mg/dL	512.8 ± 320.3	450.6 ± 353.8	552.4 ± 298.7	0.360
U-creatinine, mg/dL	78.0 ± 54.8	75.5 ± 52.5	79.3 ± 56.6	0.826
Estimated U-sodium excretion, mg/24 hours	3730 ± 3608	2972 ± 2950	4198 ± 3550	< 0.001*
FE_{Na}, %	1.13 ± 1.13	1.16 ± 1.22	1.11 ± 1.10	0.894
Symptom				
Heart failure symptom score	4.5 ± 1.8	5.4 ± 1.7	4.0 ± 1.6	0.001*
Changes of clinical parameters				
Body weight, kg	-1.24 ± 1.66	-0.87 ± 1.05	-1.45 ± 1.90	0.165
Serum total bilirubin, mg/dL	-0.08 ± 0.54	0.23 ± 0.94	-0.13 ± 0.65	0.248
Serum potassium, mEq/L	0.06 ± 0.49	0.05 ± 0.41	0.16 ± 0.46	0.265
Plasma BNP, pg/mL	-154.1 ± 315.7	-167.2 ± 283.3	-144.1 ± 547.6	0.451

BUN indicates blood urea nitrogen; BNP, B-type natriuretic peptide; U-OSM, urine osmolality; U, urinary; and FE_{Na}, fractional excretion of sodium. *$P <$ 0.05 by unpaired t-test or Mann-Whitney test as appropriate.

less of the baseline serum sodium level (normonatremic group, 136.5 ± 3.8 versus 135.4 ± 4.6 mEq/L; hyponatremic group, 125.6 ± 4.0 versus 126.4 ± 3.1 mEq/L, $P = 0.428$ and $P = 0.645$, respectively, by paired t-test). In the same manner, there were no statistically significant changes in $U\text{-}NaEx_{24}$ during the study period in nonresponders (normonatremic; 3120 ± 2478 versus 2410 ± 1788 mg/day, hyponatremic; 3084 ± 2806 versus 2500 ± 1610 mg/day, $P = 0.265$ and $P = 0.385$, respectively, by paired t-test).

Discussion

In the present study, we demonstrated that hyponatremia improved at 1 week after the administration of TLV in responders, whereas such an improvement was not observed at all in nonresponders. Hypernatremia was not observed in any participants. $U\text{-}NaEx_{24}$ increased significantly on day 7 only in normonatremic responders.

Patients with advanced HF are often complicated with severe congestion refractory to considerable amounts of diuretics, which favors the development of hyponatremia by enhanced sodium excretion and increases in a variety of neurohormonal secretions.[18] In Europe and the United States, many authors have previously reported the advantage of TLV in amelioration of such hyponatremia, which results in not only disturbance of consciousness but also poor prognosis.[7,19,20] As we reported,[14,15,21] the efficacy of TLV is limited to responders. Nonresponders are associated with reduced concentrating and diluting ability of urine probably due to dysfunction of the collecting ducts and/or loss of the medullar osmotic gradient.[14,21] Consistently, nonresponders had a larger amount of estimated sodium excretion in urine together with higher FE_{Na} at baseline, which indicated nephrogenic renal dysfunction.

Thus far, there have been no reports discussing the administration of TLV in HF patients with normonatremia, probably due to the fact that TLV has not been approved for HF patients with normonatremia in Europe and the United States. In

contrast, the Japanese government has approved TLV for use in normonatremic patients. Based on our experience, TLV treatment is highly effective for ameliorating congestion in normonatremic patients in clinical practice, although hypernatremia must be avoided. As shown in Figure 2, S-Na increased only in hyponatremic responders, whereas it remained unchanged in normonatremic responders. As a result, there were no patients who developed hypernatremia after the administration of TLV in our practice, including this study population.

Why did TLV treatment not increase S-Na in normonatremic responders? Congestion was effectively treated also in normonatremic responders as well as hyponatremic responders. We observed significant decreases in urine osmolality at 4 hours after the administration of TLV in both groups, which indicated sufficient aquaresis. Recently, it has been demonstrated that TLV enhances excretion of sodium in urine in addition to aquaresis as we previously discussed.[14,21] Plasma arginine vasopressin stimulates reabsorption of sodium through activation of epithelial Na-channels (ENaC) in the distal nephrons.[22] Inhibition of V2 receptors by TLV represses ENaC activity and may possibly increase excretion of sodium in urine. As shown in Figure 3, U-$NaEx_{24}$ increased significantly after TLV treatment in normonatremic responders. The mechanism can be a safety net against hypernatremia after TLV treatment in normonatremic responders. The slight increase in serum potassium concentration in normonatremic responders can also be explained by ENaC inhibition by TLV. Although sodium intake is an important factor that determines sodium excretion, salt restriction was continued at 6 g/day during the study period in all in-hospital patients, and there were no differences in the amount of daily salt intake between the hyponatremic and normonartemic groups.

ENaC is an active transporter and may require a substantial energy supply. ENac is expressed in cortical collecting ducts, where ATP is normally abundant. In sharp contrast to normonatremic responders, we observed that U-$NaEx_{24}$ remained unchanged in hyponatremic responders. Hemodynamic recovery was insufficient even after one week of TLV treatment in patients with hyponatremia (Table IV). ENaC activity may be attenuated by reduced ATP-supply due to such impaired circulation, and attenuated ENaC activity leads to a lesser response to TLV in terms of increases in sodium excretion. In contrast, ENaC activity may be more preserved due to the relatively higher supply of ATP under less impaired hemodynamics in the normonatremic group, and a higher amount of sodium is in turn excreted in urine after TLV administration.

Another reason why hypernatremia did not emerge during TLV treatment may be the enhanced natriuretic effect of concomitant diuretics due to amelioration of renal congestion by TLV. Patients with advanced HF are often refractory to conventional natriuretic agents owing to renal congestion, which recovers more easily in normonatremic patients because of their relatively stable hemodynamics. In contrast, volume overload may continue in patients with hyponatremia even after one week of TLV treatment considering insufficient recovery of body weight and serum total bilirubin concentration. The natriuretic effect of concomitant diuretics may still be repressed under persistent renal congestion in patients with hyponatremia.

We acknowledge that our study has several limitations.

1. It was conducted retrospectively in a single center, and consequently included a limited number of patients and might have patient selection bias. Doses of TLV were determined by attending physicians after determining the hemodynamic stability and degree of congestion of the patient.
2. We observed the clinical courses for 1 week after the administration of TLV under fixed doses of other medication including diuretics, and the improvement in hyponatremia was not sufficient. Longer administration of TLV along with reduction of concomitant natriuretic diuretics may better improve the hyponatremia. Resolution of hyponatremia has been reported to be associated with improved in-hospital and 1-year mortality.[23] Longer prognosis under improved hyponatremia by TLV treatment would be a future concern.
3. In this study, TLV was initiated at almost 2 weeks after admission because we tried our best to optimize conventional treatment before the administration of TLV. Therefore, the results may not be adapted to patients in an acutely decompensated phase.
4. The formula to estimate U$NaEx_{24}$ used in this study was originally developed from urine samples in a healthy population. Although we would like to validate the formula among patients with cardiorenal failure or those who received diuretics, 24-hour urine collection is prohibited at many institutes, including our hospital, to avoid in-hospital infection. Our calculation, therefore, might not be accurate in terms of absolute values, although the trend of sodium excretion should still be valid.
5. We did not demonstrate direct involvement of ENaC or data on aldosterone concentration in the present study, although several previous reports demonstrated a significant relationship between TLV and ENaC in sodium excretion in urine. Further investigation of TLV and ENaC would be necessary.

In conclusion, we have demonstrated that TLV can improve hyponatremia only in responders, and that hypernatremia rarely emerges in patients with normonatremia by TLV treatment, which is explained by enhanced excretion of not only free water but also sodium, especially in patients with normonatremia. We believe that the above mechanism contributes to the safety of TLV when applied to normonatremic patients.

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Reactive Oxygen Species and Scleroderma

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Contents

Abstract

Scleroderma is a fibrotic condition characterized by immunological abnormalities, vascular injury, and increased accumulation of extracellular matrix proteins in the skin. Although the etiology of scleroderma has not yet been fully elucidated, a growing body of evidence suggests that extracellular matrix overproduction by activated fibroblasts results from complex interactions between endothelial cells, lymphocytes, macrophages, and fibroblasts via a number of mediators, such as cytokines, chemokines, and growth factors.

Recent investigations have further suggest that reactive oxygen species (ROS) are play a role in autoimmunology, vasculogenesis, and fibrogenesis in scleroderma. In addition, ROS exert effects on cellular signaling pathways in the induction of scleroderma. In this chapter, current findings on the role of ROS in the pathophysiology of human scleroderma, as well as animal models of scleroderma, are described. These may strengthen our understanding of the pathogenesis of, and assist in exploring new treatments targeting ROS for, scleroderma.

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Introduction

Scleroderma is a connective tissue disease involving fibrosis of the skin, which is characterized by the excessive accumulation of extracellular matrix (ECM) proteins, vascular injury, and immunological abnormalities (Yamamoto 2009). During the early stages of scleroderma, activated fibroblasts in the affected areas produce high amounts of collagen. Histological analysis of the initial stages of scleroderma reveals perivascular infiltrates of mononuclear cells in the dermis, which is associated with increased collagen synthesis in surrounding fibroblasts. Although the pathogenesis of systemic sclerosis (SSc) has not been fully elucidated, a number of studies demonstrate a crucial role of several fibrogenic cytokines released from immunocytes in initiating the sequence of events leading to fibrosis. Oxidative stress may be involved in the pathogenesis of SSc, exerting various effects on vasculogenesis and fibrogenesis. In this chapter, current findings on the autoimmune mechanisms and roles of reactive oxygen species (ROS) in SSc are discussed.

Autoimmune Mechanisms in Human SSc

T cells, macrophages, and mast cells are present in increased numbers or in an activated state in the lesional scleroderma skin and are thought to play an active role in the pathogenesis of SSc. In particular, type 2 helper T cells and/or M2-type macrophages play an important role. Additionally, activated peripheral B cells are found in abnormally large numbers in patients with SSc (Sato et al. 2001). B cells contribute not only to antibody production but also to T cell activation and differentiation and the production of various cytokines. Circulating antibodies to platelet-derived growth factor (PDGF) receptors, which stimulate ROS and collagen (Baroni et al. 2006), have been identified in patients with SSc. The ROS-Ras-ERK1/2 cascade results in fibroblast activation and the formation of a myofibroblastic phenotype. By contrast, a recent study failed to detect stimulatory anti-PDGF receptor autoantibodies (Classen et al. 2009).

Transforming growth factor-β (TGF-β) increases the synthesis of ECM by fibroblasts, modulates cell-matrix adhesion protein receptors, and regulates the production of proteins such as plasminogen activator, an inhibitor of plasminogen, or procollagenase, which can modify the ECM by proteolytic action. In addition, TGF-β is capable of stimulating its own synthesis by fibroblasts through autoinduction. TGF-β increases TGF-β receptor (TGF-βR) levels in fibroblasts (Kawakami et al. 1998), and thus the maintenance of increased TGF-β production may lead to the progressive deposition of ECM, resulting in fibrosis. Indeed,

TGF-β mRNA levels are elevated in the lesional skin of SSc and shown to co-localize with type I collagen. Overexpression of TGF-βR, which is regulated at the transcriptional level (Yamane et al. 2002), is recognized in fibroblasts in the skin of scleroderma patients (Kubo et al. 2001). Signaling by TGF-β elicits potent profibrotic responses in fibroblasts, and thus, blocking endogenous TGF-β signaling eradicates the scleroderma phenotype (Ihn et al. 2001). Signaling occurs predominantly by phosphorylation of cytoplasmic mediators belonging to the Smad family. In scleroderma fibroblasts, phosphorylation and nuclear translocation of Smad2/3 are increased, suggesting activation of the Smad pathway (Mori et al. 2003). Smad7 acts as an intracellular antagonist of TGF-β signaling and an inhibitor of TGF-β-induced transcriptional responses. In scleroderma skin and cultured scleroderma fibroblasts, the basal level and the TGF-β-inducible expression of Smad7 are selectively decreased, whereas Smad3 expression is increased (Dong et al. 2002). On the other hand, Smad7 expression levels in scleroderma fibroblasts are uncertain. Smad7-Smurf-mediated negative regulation of TGF-β signaling is impaired in scleroderma fibroblasts (Asano et al. 2004). Other signaling pathways besides the Smad proteins, such as the p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), c-Myb, Ets, and Egr pathways, have also been shown to mediate TGF-β signaling in scleroderma fibroblasts. Recent studies have suggested that upregulation of ECM production by TGF-β stimulation in fibroblasts and subsequent activation of signaling pathway is mediated by ROS. On the other hand, ROS can cause activation of TGF-β either directly or indirectly via proteases.

Connective tissue growth factor (CTGF) is selectively induced in fibroblasts after activation by the active form of TGF-β. Recombinant CTGF protein stimulates DNA synthesis and upregulates collagen, fibronectin, and integrin expression in fibroblasts (Frazier et al. 1996). CTGF functions as a downstream mediator of TGF-β and may coordinate the action of TGF-β, such as fibroblast proliferation, adhesion, and ECM production (Igarashi et al. 1995). Overexpression of CTGF is known to occur in cultured scleroderma fibroblasts (Shi-wen et al. 2000; Sonnylal et al. 2010). The constitutive overexpression of CTGF in scleroderma fibroblasts is independent of TGF-β signaling but dependent on Sp1 (Holmes et al. 2003). Moreover, serum levels of CTGF are elevated in patients with SSc (Sato et al. 2000). Dermal fibroblasts exposed to hypoxia (1 % O_2) or $CoCl_2$ (1–100 μM) enhance expression of CTGF mRNA (Ishibuchi et al. 2010). Skin fibroblasts transfected with hypoxia-inducible factor (HIF)-1α show increased levels of CTGF protein and mRNA, as well as nuclear staining of HIF-1α, which was enhanced further by treatment with $CoCl_2$. These data may suggest that hypoxia, caused possibly by microvascular alterations, upregulates CTGF expression through the activation of HIF-1α in dermal fibroblasts of SSc patients and thereby contributes to the progression of skin fibrosis. HIF induces the release of VEGF, which drives angiogenesis by activating endothelial cells. A recent study has demonstrated that variations in the promoter region of the CTGF gene (G-945C polymorphism) are linked to susceptibility to SSc (Fonseca et al. 2007).

An imbalance exists between the type 1 and type 2 cytokine response in the pathogenesis of scleroderma. IL-13 is a pleiotropic cytokine, elaborated in significant quantities by appropriately stimulated type 2 cells. IL-13 has the ability to suppress proinflammatory cytokine production in monocytes/macrophages and is known to enhance the growth and differentiation of B cells and to promote immunoglobulin synthesis. In addition, in vitro studies demonstrate that IL-13 is a potent stimulator of fibroblast proliferation and collagen production. The profibrotic effect of IL-13 is thought to involve irreversible fibroblast activation, triggered either directly or indirectly through TGF-β (Jinnin et al. 2004; Lee et al. 2001). Serum levels of IL-13 are elevated in patients with SSc, correlated with the number of plaque lesions (Hasegawa et al. 1997) or nailfold capillaroscopic features (Riccieri et al. 2003).

CCL2/monocyte chemoattractant protein-1 (MCP-1) gene expression is upregulated in human fibrosis, as well as in animal models of fibrosis (Yamamoto 2006). In vitro studies show that CCL2 upregulates type I collagen mRNA expression in rat fibroblasts, which is indirectly mediated by endogenous upregulation of TGF-β gene expression (Gharaee-Kermani et al. 1996). CCL2 enhances expression of matrix metalloproteinase-1 (MMP-1), MMP-2, as well as tissue inhibitor of metalloproteinase-1 (TIMP-1) in cultured skin fibroblasts (Yamamoto et al. 2000). Previous studies demonstrated increased expression of CCL2 in patients with SSc. Serum levels and spontaneous production levels of CCL2 by peripheral blood mononuclear cells are elevated in patients with SSc, compared with normal controls, and are correlated with pulmonary fibrosis (Hasegawa et al. 1999). Increased expression of CCL2 is demonstrated in scleroderma skin (Hasegawa et al. 1999; Distler et al. 2001; Galindo et al. 2001a), and scleroderma fibroblasts express increased levels of CCL2 mRNA and protein (Distler et al. 2001; Galindo et al. 2001a). Stimulation with PDGF results in a significant increase in CCL2 mRNA and protein (Yamamoto et al. 2001a). Furthermore, the autoinduction of CCL2 is observed in scleroderma fibroblasts, but not in normal fibroblasts (Yamamoto et al. 2001b). CCL2 levels may also be increased by IL-13, a potent stimulator of CCL2 (Zhu et al. 2002). These in vivo and in vitro results suggest an important involvement of CCL2 in the pathogenesis of scleroderma. Increased numbers of mast cells are noted in scleroderma skin. CCL2 also recruits mast cells, in addition to monocytes. Human mast cells are shown to be a rich source of chemokines, including CCL2, CCL3/macrophage inflammatory protein-1α (MIP-1α), CCL4/MIP-1β, and CCL5/RANTES (Selvan et al. 1994), as well as a number of cytokines/growth factors and mediators capable of activating fibroblasts or endothelial cells. Expression of SCF is upregulated in scleroderma fibroblasts (Yamamoto et al. 1998a) and is thought to contribute to the increase of mast cells in scleroderma. SCF enhances CCL2 expression in human mast cells (Yamamoto et al. 2001c). Because CCL2 enhances type I collagen mRNA expression in skin fibroblasts, the interaction between mast cells and fibroblasts via SCF/CCL2 may play an important role in the development of fibrosis. CCR2 is a major CCL2 receptor. CCR2 upregulation in vascular structures, perivascular inflammatory

infiltrates, and fibroblasts has recently been demonstrated in SSc (Carulli et al. 2005). In particular, CCR2-positive fibroblasts in early-stage dSSc showed a profibrotic phenotype, with overexpression of α-smooth muscle actin (α-SMA), CTGF, and CCL2 (Carulli et al. 2005). Their results suggest potential autocrine regulation of key fibrotic properties via the CCL2/CCR2 loop in the early phases of scleroderma. A novel protein, MCPIP (MCP-induced protein), upregulates members of the apoptotic gene family involved in the induction of cell death (Zhou et al. 2006) and may provide a novel molecular pathway by which CCL2/CCR2 signal transduction is linked to transcriptional gene regulation leading to apoptosis. CCL2 promoter polymorphism is associated with SSc (Karrer et al. 2005). CCL2 may contribute to the induction of dermal sclerosis directly, via its upregulation of mRNA expression of ECM on fibroblasts, as well as indirectly through the mediation of a number of cytokines released from immunocytes recruited into the lesional skin.

PDGF has mitogenic activity for mesenchymal cells, regulates matrix metabolism, has chemotactic and vasoactive properties, and produces inflammatory cytokines (Yamakage et al. 1992). Overexpression of PDGF has been reported in a number of fibrotic diseases. Elevated levels of PDGF-A chain are demonstrated in scleroderma skin (Gay et al. 1989). In addition, TGF-β upregulates PDGF-α mRNA and protein levels in scleroderma fibroblasts, in comparison with the control (Gay et al. 1989). On the other hand, increased expression of the PDGF B-chain and β-receptor in scleroderma skin has also been reported (Klareskog et al. 1990; Xue-yi et al. 1998; Makhluf et al. 1996).

IL-4 is known to promote fibroblast proliferation, gene expression, and synthesis of ECM proteins such as collagen and tenascin. IL-4 has been shown to upregulate TIMP-2 in dermal fibroblasts via the MAPK pathway (Ihn et al. 2002) as well as to upregulate TGF-β production. Increased IL-4 production is detected in the sera or in activated peripheral blood mononuclear cells of patients with SSc (Needlemann et al. 1985). Scleroderma fibroblasts express more IL-4 receptor α and produce more collagen after IL-4 stimulation (Serpier et al. 1997).

TGF-β can contribute to the differentiation of both regulatory T cells (Tregs) and inflammatory Th17 cells. IL-17 is a T cell-derived cytokine and functions to secrete various cytokines and chemokines by different cell types. Elevated levels of IL-17 have been observed in patients with SSc, especially in the early stages (Kurasawa et al. 2000), and limited SSc (Murota et al. 2008). IL-17 has been reported to induce fibroblast proliferation, but not collagen production in SSc fibroblasts (Kurasawa et al. 2000). IL-17 induces ROS production (Pietrowski et al. 2011). Also, Th17 promotes inflammation in SSc. IL-23 is associated with the activation and proliferation of Th17 cells. Increased serum IL-23 levels are shown in patients with SSc, in association with the disease duration and prevalence of pulmonary fibrosis (Komura et al. 2008). IL-21/IL-21R signaling has recently been shown to promote fibrosis by facilitating the development of the CD4+ Th2 response (Pesce et al. 2006). IL-21 increases IL-4 and IL-13 receptor expression in macrophages (Pesce et al. 2006), thereby possibly enhancing fibrosis, and is abundantly expressed in the epidermis in SSc (Distler et al. 2005).

Oxidative Stress

Oxidative stress is an imbalance between oxidants (reactive oxygen and nitrogen species (ROS/RNS)) and antioxidants which affect lipids, DNA, carbohydrates, and proteins. ROS generated during various metabolic and biochemical reactions have multifarious effects that include oxidative damage to DNA. ROS can cause several abnormalities, such as endothelial cell damage or enhanced platelet activation, leading to upregulation of the expression of adhesion molecules or secretion of inflammatory or fibrogenic cytokines including PDGF and TGF-β. In addition, free radicals stimulate fibroblast proliferation with narrowing of the vessel walls and ischemia (Gabrielli et al. 2009). Thus, excessive oxidative stress has been implicated in the pathogenesis of scleroderma (Sambo et al. 1999). Reduced levels of micronutrient antioxidants and increased susceptibility of serum lipoproteins to oxidation have been reported in patients with SSc (Bruckdorfer et al. 1995). Free radicals are produced by several mechanisms such as hypoxanthine-xanthine oxidase system and activation of polymorphonuclear leukocytes. Several markers which reflect free radical formation, i.e., 8-isoprostane and N(epsilon)-(hexanoyl) lysine, are elevated in the serum of patients with SSc (Ogawa et al. 2006; Shimuzu et al. 2008). Marked oxidative stress is shown in urinary levels of patients with SSc (Avouac et al. 2010). Also, autoantibodies against antioxidant enzymes such as peroxiredoxin I and methionine sulfoxide reductase A (MSRA) are elevated in the serum of patients with SSc (Iwata et al. 2007; Ogawa et al. 2010).

Scleroderma fibroblasts produce ROS constitutively (Sambo et al. 2001). Other effects of oxygen radicals include the stimulation of skin fibroblast proliferation at low concentrations (Murrel et al. 1990) and the production of increased amounts of collagen (Falanga et al. 1993), suggesting that low oxygen tension may contribute to the increased fibrogenic properties of scleroderma fibroblasts. Furthermore, several of the autoantigens targeted by scleroderma autoantibodies fragment in the presence of ROS and specific metals such as iron or copper (Casciola-Rosen et al. 1997). The authors suggest that tissue injury by ischemia-reperfusion generates ROS, which in turn induces the fragmentation of specific autoantigens. On the other hand, oxidative stress transiently induces CCL2 mRNA and protein expression in cultured skin fibroblasts (Galindo et al. 2001b), suggesting that ROS may play a regulatory role in inflammation by modulating monocyte chemotactic activity. Sera of SSc patients stimulated ROS production in particular in cultured endothelial cells (Servettaz et al. 2007).

Vascular Injury

Vascular injury causes endothelial cell activation, dysfunction, and altered capillary permeability as primary events. These are followed by increased expression of adhesion molecules leading to mononuclear cell infiltrates in the skin. Microvascular injury may be the result of direct or indirect injury by anti-endothelial cell antibodies (AECAs), which are frequently detected in sera of patients with SSc (Ihn et al. 2000).

AECAs can activate endothelial cells to express cell adhesion molecules which alter leukocyte attachment and can lead to endothelial cell damage and apoptosis. Kuwana et al. (2004), however, proposed that insufficient vascular repair machinery due to defective vasculogenesis contributes to the microvascular abnormality in SSc. Although circulating concentrations of angiogenic factors are high in SSc, the levels of bone marrow-derived circulating endothelial precursors (CEP) are low, suggesting a dysregulation of vasculogenesis in SSc. Also, ischemic condition induces hypoxia. ROS can be generated in the vessel walls. Gabrielli et al. (Gabrielli et al. 2009) proposed that uncontrolled production of ROS activates local mesenchymal cells to induce chemotaxis, proliferation, ECM production, and cytokine secretion. Further, excessive ROS production by an autocrine circuitry finally leads to the disease burn out.

Endothelin-1 (ET-1) is a prototypical endothelial cell-derived product. Since ET-1 is a vasoconstrictive agent, loss of normal vessel compliance and vasorelaxation may be induced by increased levels of ET-1. ET-1 promotes fibroblast synthesis of collagen (Horstmeyer et al. 2005; Shi-wen et al. 2007) and thus provides the link between vasculopathy and fibrosis. ET-1 can induce CTGF and may mediate the induction of collagen synthesis by activation of CTGF (Shephard et al. 2004). Further, ET-1 can also induce myofibroblast differentiation in fibroblasts (Shi-wen et al. 2001). Circulating ET-1 levels have been observed in patients with dSSc with widespread fibrosis and those with lSSc and hypertensive disease (Vancheeswaran et al. 1994), suggesting that soluble ET-1 levels may be a marker of fibrosis and vascular damage. These facts underscore the importance ET-1 in scleroderma.

Nitric oxide (NO) is a strong vasodilator substance produced by endothelium and inhibits the biochemical effect of ET-1. However, ET-1 induces inducible NO synthase (iNOS) expression in endothelial cells (Hirata et al. 1993), and iNOS expression is detected in the endothelial cells in the lesional skin of SSc (Yamamoto et al. 1998b). So far, several reports have shown impaired NO production in SSc (Yamamoto et al. 1998b; Takagi et al. 2003), which may contribute to the vascular pathogenesis of the arteriolar intimal proliferation in SSc. Thus, an imbalance between vasoconstriction and vasodilatation can lead to ischemia-reperfusion injury, endothelial damage, and subsequent increased collagen gene expression via hypoxia. Hypoxia induces ECM proteins in cultured fibroblasts, and vascular endothelial growth factor (VEGF) overexpression may be caused in response to chronic hypoxia condition (Beyer et al. 2009).

Scleroderma Fibroblasts

Fibroblasts are stimulated by inflammatory cells, such as activated T cells, monocytes/macrophages, mast cells, and eosinophils. Additionally, fibroblasts can be activated to perform new functions important for controlling ECM synthesis and for producing various cytokines, growth factors, chemokines, growth factor receptors, integrins, and oxidants. The phenotype and activation of fibroblasts is dependent on

both soluble factors and ECM-generated signals. Fibroblasts interact with the surrounding collagens via integrins. Aberrant signaling by ECM may disturb this interaction, thereby contributing to the persistent modulation of fibroblasts which results in fibrosis, as seen in the autocrine loops of cytokine production and excessive deposition of ECM proteins in the skin. Human skin fibroblasts are heterogeneous with regard to their synthesis of collagen, proliferative responses, and response to growth factors. Enhanced collagen synthesis is regulated at the transcriptional level. Some researchers think that scleroderma fibroblasts are the result of phenotypic changes in dermal fibroblasts caused by soluble factors; others contend that scleroderma fibroblasts are recruited from circulating or resting mesenchymal precursor cells as fibrocytes. Alternatively, they may be generated by clonal selection of high-collagen-producing fibroblasts.

Myofibroblasts represent activated and contractile phenotypes which exist in fibrotic lesions. Myofibroblasts express α-SMA and can produce various cytokines, growth factors, and chemokines. TGF-β1 is a central regulator of the phenotypic changes of fibroblasts into myofibroblasts; the modulators are mechanical tension and fibronectin involving the ED-A domain. The differentiation into myofibroblasts is also regulated by mast cell mediators, of which tryptase is one of the likely candidates (Gailit et al. 2001). Recent studies suggest that fibroblast-to-myofibroblast differentiation is mediated by ROS signaling, via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4).

Fibrocytes are derived from circulating monocytes (CD34+ bone marrow-derived progenitors) and enter into the tissues. Fibrocytes produce matrix proteins such as collagens I and III and participate in the remodeling process by secreting matrix metalloproteinases (Quan et al. 2004). Fibrocytes are also a source of inflammatory cytokines, growth factors, and chemokines. Although fibrocytes are involved in scleroderma, their role has yet to be fully elucidated.

Role of Apoptosis

Excessive ROS/RNS induce cell death. Autoreactive clones that survive the apoptotic process may lead to increased susceptibility to autoimmune disorders. Apoptosis causes typical cellular morphological changes including cell shrinkage, nuclear condensation, DNA fragmentation, and membrane alterations. This may in turn cause apoptotic cells to become a possible source of autoantigens (Mahoney and Rosen 2005). Scleroderma fibroblasts are thought to escape apoptosis because cultured scleroderma fibroblasts are resistant to Fas-induced apoptosis (Jelaska and Korn 2000; Santiago et al. 2001), and apoptosis of fibroblasts in SSc skin lesions has not been observed (Santiago et al. 2001). TGF-β protects myofibroblasts from undergoing apoptosis. Serum-starved rat lung fibroblasts treated with IL-1 result in apoptosis which can be reduced by concomitant treatment with TGF-β (Zhang and Phan 1999). Also, α-SMA-positive myofibroblasts increase in number following stimulation by TGF-β, which protects these myofibroblasts against apoptosis induction. Other studies have shown that pretreatment with TGF-β significantly

reduced apoptosis caused by serum starvation in myofibroblasts, whereas this was not the case with non-myofibroblasts (Santiago et al. 2001). Thus TGF-β1 may play a role in inducing apoptosis-resistant fibroblast populations in SSc. In scleroderma fibroblasts, the Bcl-2 level is significantly higher, whereas the Bax level significantly lower (Santiago et al. 2001).

On the other hand, endothelial cell apoptosis is thought to occur early in the pathogenesis of scleroderma. Endothelial cell apoptosis was first noted in the UCD-200/206 chickens, which develop hereditary systemic connective tissue disease resembling human SSc (Sgonc et al. 1996). This phenomenon occurs before perivascular mononuclear cell infiltration. Also, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) is shown to be positive on the endothelial cells in human scleroderma skin (Zhang and Phan 1999). A recent study showed that sera of patients with SSc induced apoptosis of endothelial progenitor cells, which is mediated by Akt-FOXO3a-Bim pathway (Zhu et al. 2008). On the other hand, apoptosis of endothelial cells induces resistance to apoptosis in fibroblasts largely through PI3K-dependent mechanisms (Laplante et al. 2005). Furthermore, fibroblasts exposed to a medium-conditioned by apoptotic endothelial cells present myofibroblast changes (Laplante et al. 2005).

The serum-soluble Fas (sFas) levels are higher in patients with SSc (Wetzig et al. 1998; Stummvoll et al. 2000; Bianchi et al. 2000). Untreated SSc patients have significantly higher serum sFas levels than the treated SSc patients and healthy controls (Ates et al. 2004). It has been suggested that increased sFas levels in the serum of SSc patients can protect autoreactive T cells from FasL-induced apoptosis (Kessel et al. 2004). Spontaneous apoptosis of CD8+ T cells in the peripheral blood is significantly higher in patients with SSc compared with normal controls, while spontaneous apoptosis in CD4+ T cells occur at similar rates in both SSc and controls (Kessel et al. 2004). Enhanced helper T cell function, resulting in the reduction CD8+ T cells, may lead to autoimmunity by modifying the immune balance.

Akt is one of the key enzymes inhibiting both spontaneous and stress-induced apoptosis. 3'-phosphorylated phosphoinositides bind to the pleckstrin domain of Akt. Akt activity may result in the inhibition of proapoptotic Bad, Bax, Bik, and caspase-9 by phosphorylation. It has recently been reported that Akt is active in scleroderma fibroblasts. Cultured scleroderma fibroblasts exhibited high levels of p-Akt, in comparison to control fibroblasts (Jun et al. 2005). TGF-β can activate Akt in fibroblasts and, by doing so, may also induce apoptosis resistance in scleroderma fibroblasts. These findings point to a potential role for Akt in the resistance of scleroderma fibroblasts to apoptosis.

ROS in Animal Models of SSc

Although animal models which reproduce all the aspects of SSc are not currently available, bleomycin-induced scleroderma mouse exhibits definite dermal sclerosis mimicking human scleroderma (Yamamoto 2010). In this model, features such as

definite dermal sclerosis with dermal thickening, pulmonary fibrosis, and the presence of autoantibody in the sera are induced; thus, this model is now widely accepted as a representative mouse model for scleroderma. Bleomycin is a frequently used antitumor antibiotic effective against various kinds of cancers. Bleomycin has a number of biochemical properties, such as blocking the cell cycle at G2, cleaving the single-strand and double-strand DNA, degrading cellular RNAs, production of free radicals, and induction of apoptosis. As mentioned above, ROS secreted by bleomycin is supposed to participate in the induction of dermal sclerosis in this model. Superoxide dismutase (SOD) is an enzyme which catalyzes the conversion of superoxide radicals to oxygen and hydrogen peroxide. Administration of SOD ameliorated the induction of dermal sclerosis (Yamamoto et al. 1999).

Vasculopathy in SSc involves several types of cells such as endothelial cells, vascular smooth muscle cells, and pericytes, depending on different phases. Progressive thickening of blood vessel walls with proliferation of vascular intima is the typical feature of SSc (Gabrielli et al. 2009). Proliferation of vascular smooth muscle cells and pericytes is suggested to lead to the vessel-wall thickening and occlusive changes by thickened intima. In the bleomycin model, α-smooth muscle actin (α-SMA)-positive myofibroblasts were observed in the dermis and gradually increased in tandem with the induction of dermal sclerosis. In addition, significant thickness of vascular wall was also observed in the deep dermis (Yamamoto and Katayama 2011). Elastica van Gieson stain revealed proliferation of vascular intima, and α-SMA stain suggests proliferation of vascular smooth muscle cells. Although the mechanism of intimal proliferation is uncertain, several factors such as chemical influence, virus, stress (e.g., oxidative or ischemia-reperfusion), immune-mediated cytotoxicity, apoptotic process, and anti-AECAs are suggested as possible initial triggers. An abnormal response of microvascular endothelial cells to bleomycin may result in vascular injury.

Bleomycin exerts various effects on skin-constituted cells such as fibroblasts, keratinocytes, and endothelial cells, as well as immunocytes. In vitro, bleomycin upregulates collagen and TGF-β1 mRNA expression in cultured rat lung and human skin fibroblasts. Also, bleomycin enhances gene expression of ECM proteins as well as fibrogenic cytokines, which may contribute to the induction of fibrosis. TGF-β is a mediator of the fibrotic effect of bleomycin at the transcriptional level and that the TGF-β response element is required for bleomycin stimulation of the proα1(I) collagen promoter (King et al. 1994). Endothelial cells have been reported to play an important role in the inflammatory as well as fibrotic process. In vitro studies showed a dose-dependent stimulation of endothelial cell secretion of collagen synthesis by bleomycin, which was inhibited by the anti-TGF-β antibody (Phan et al. 1991). The schematic proposal of the possible mechanisms of bleomycin-induced scleroderma is shown in Fig. 164.1.

On the other hand, recent studies have shown that pro-oxidative agents induced skin fibrosis (Servettaz et al. 2009). In particular, local injection of hypochlorite or hydroxyl radicals induced skin and lung fibrosis, as well as serum anti-DNA topoisomerase 1 autoantibodies.

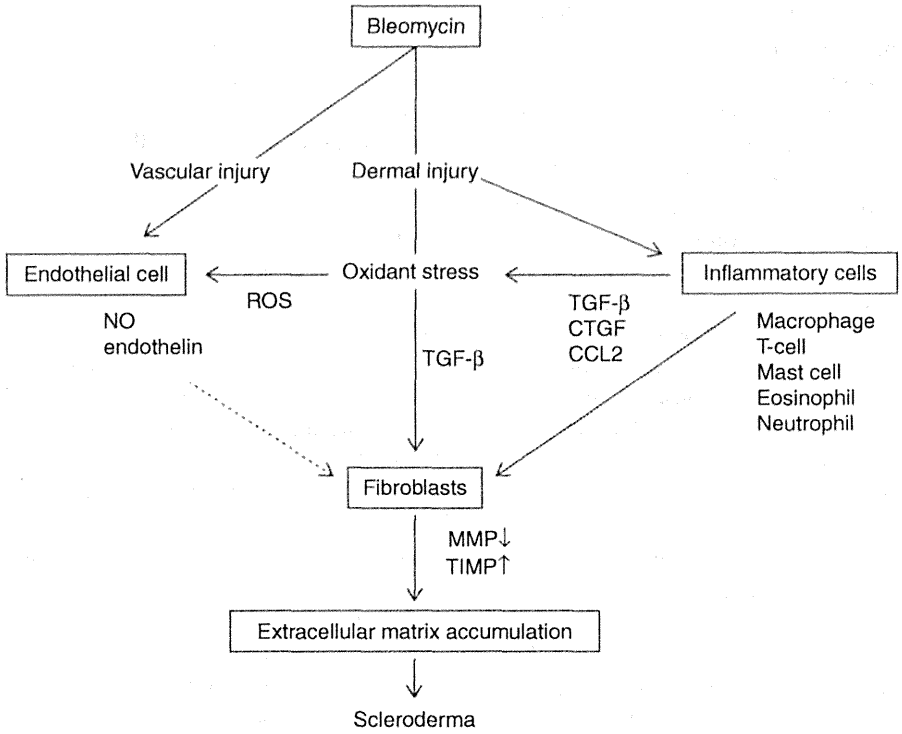

Fig. 164.1 Possible pathomechanism of bleomycin-induced scleroderma

Conclusion

Oxidative influenced pathways are implicated to play an important role in the induction of scleroderma, which drives inflammation, vascular damages, and fibrosis. Antioxidants aiming at targeting or controlling of oxygen radicals or ROS-induced cellular events may lead to the novel therapeutic trials for scleroderma.

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