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Increase in putrescine, amine oxidase, and acrolein in plasma of renal failure patients

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Abstract

Since polyamines have been suggested to be one of the uremic "toxins," the levels of each polyamine, its oxidized product, acrolein, and amine oxidase in plasma of patients with renal failure were investigated. The level of putrescine was increased, whereas the level of spermine was decreased in the plasma of patients with renal failure. The patients also had increased serum amine oxidase activity leading to increased degradation of spermine. Both levels of free and protein-conjugated acrolein were also increased in plasma of patients with renal failure. The accumulated acrolein found as protein conjugates was equivalent to 180 μM, which was 6-fold higher than in plasma of normal subjects. It was found that acrolein is mainly produced by polyamine oxidase in plasma. A cell lysate containing polyamine oxidase was cytotoxic in the presence of spermine. Our results indicate that the level of acrolein is well correlated with the degree of seriousness of chronic renal failure.

Keywords: Spermine; Putrescine; Amine oxidase; Acrolein; Renal failure

It is well established that polyamines (putrescine, spermidine, and spermine) are necessary for cell growth [1,2]. However, the addition of spermidine or spermine to culture medium containing ruminant serum inhibits cellular proliferation [3,4]. This effect is caused by the products of oxidation of polyamines that are generated by serum amine oxidase [5]. Ruminant serum amine oxidase catalyzes the oxidative deamination of spermidine and spermine to produce, respectively, an aminoaldehyde [*N*′-(4-aminobutyl)-aminopropionaldehyde] or an aminodialdehyde [*N*,*N*′-bis(3-propionaldehyde)-1,4-butanediamine], with H_2O_2 and ammonia [6]. Acrolein (CH_2=CHCHO) is then spontaneously formed from these two aminoaldehydes [7]. Thus, we recently compared the toxicity of acrolein, spermidine, spermine, 3-aminopropanal, formaldehyde, and H_2O_2, and found that acrolein is a major toxic compound produced from spermidine and spermine by amine oxidase, and spontaneously from 3-aminopropanal [8]. It has also been reported that 3-aminopropanal is produced from spermidine and spermine by polyamine oxidase [9].

Polyamines have been suggested to be one of the uremic "toxins," which accelerate the progression of uremia [10]. However, this idea has not been carefully explored. In a previous report dealing with uremia, the total polyamine levels in serum, estimated using an antibody against polyamines [10], were higher in patients with advanced adult uremia than in ambulatory uremic children. In this study, we determined the levels of each polyamine, i.e., putrescine, spermidine, and spermine, together with amine oxidase activity and the level of acrolein in plasma of patients with renal failure. We found that acrolein produced from spermidine and spermine by amine oxidase is accumulated in the plasma of these patients, and that the main amine oxidase that produces acrolein from spermidine and spermine is polyamine oxidase. The level of acrolein produced from

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spermine was well correlated with the degree of seriousness of chronic renal failure. Our results suggest that acrolein may function as the uremic "toxin."

Materials and methods

Plasma samples. With written consent, blood was obtained from normal subjects (14 male and 6 female; average age 30) and from patients with chronic renal failure who have never had hemodialysis (12 male and 8 female; average age 65). Plasma was carefully prepared to avoid the contamination of erythrocytes. Blood containing 1 mg/ml disodium ethylenediaminetetraacetic acid (EDTA) was centrifuged at 1500*g* for 30 min at 4 °C. The serum levels of creatinine and blood urea (BUN) were determined by standard method for blood chemistry, the CRTNase-POD (creatinase–peroxidase) for creatinine and urease-GLDH (glutamate dehydrogenase) for BUN. Hematocrit (Hct) was measured using automated hematology analyzer (Beckman Coulter).

Measurement of polyamines in plasma. Amino acids were removed by cellulose phosphate column chromatography before polyamine analysis because polyamine levels in plasma were very low. To 1.8 ml plasma, 0.2 ml of 50% trichloroacetic acid (TCA) was added and centrifuged for 10 min at 12,000*g*. The supernatant thus obtained was neutralized with 6 N KOH and applied to a cellulose phosphate column (1 ml) previously equilibrated with a buffer containing 0.1 M boric acid–Na_2CO_3 and 0.025 M NaCl (pH 8.0). Amino acids were eluted with 10 ml of the same buffer and polyamines were then eluted with 3 ml of a buffer containing 0.2 M boric acid–Na_2CO_3 and 0.8 M NaCl (pH 8.0). Polyamine contents were measured by HPLC as described previously [11].

Determination of amine oxidase activity in plasma. The reaction mixture (0.075 ml) containing 10 mM Tris–HCl (pH 7.5), 0.2 mM spermine, and 0.065 ml plasma was incubated at 37 °C for 48 h. To 0.02 ml of the reaction mixture, 0.55 ml of 5% TCA was added and centrifuged for 10 min at 12,000*g*. A 10 μl aliquot of the supernatant was used for the polyamine measurement by HPLC. The activity of amine oxidase was expressed as nmol spermine degraded/ml plasma. Where indicated, 1 mM pargyline, a specific inhibitor of monoamine oxidase, 0.1 mM semicarbazide, an inhibitor of monoamine and diamine oxidases, or 0.25 mM MDL72527 [N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine], a specific inhibitor of mammalian polyamine oxidase (a generous gift from Aventis Pharma) was added [12,13].

Measurement of free acrolein in plasma. Acrolein was measured according to the method of Alarcon [14]. The reaction mixture (0.2 ml) containing 0.1 ml plasma, 23 mM *m*-aminophenol, 43 mM hydroxylamine hydrochloride, and 1.5 N HCl was boiled for 10 min. The acrolein was determined by HPLC according to the method of Bohnenstengel et al. [15] using 0.08 ml supernatant after centrifugation. Fluorescence of 7-hydroxyquinoline (acrolein derivative) was measured at an excitation wavelength of 358 nm and an emission wavelength of 510 nm.

Measurement of protein-conjugated acrolein in plasma. Protein-conjugated acrolein [FDP-Lys; N^{ε}-(3-formyl-3,4-dehydropiperidino)-lysine] was determined by the method of Uchida et al. [16] using ACR-LYSINE ADDUCT ELISA SYSTEM (NOF Corporation, Tokyo) and 0.05 ml plasma. After the reaction was terminated, absorbance at 450 nm was measured by a microplate reader Bio-Rad Model 550.

Transfection of a cDNA for polyamine oxidase or amine oxidase into NIH3T3 cells. NIH3T3 cells were cultured in DMEM (Nissui Pharmaceutical, Tokyo) supplemented with 2 mM glutamine, 50 U/ml streptomycin, 100 U/ml penicillin G, and 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% CO_2. Plasmid pcDNA3.1(-)-hPAO1, encoding human polyamine oxidase [12], a kind gift of Dr. R.A. Casero, pcDNA3-hVAP-1, encoding human amine oxidase [17], a kind gift of Dr. M. Salmi, or pcDNA3, an empty vector (8 μg each) was transfected into 2×10^7 NIH3T3 cells (150 mm diameter dish) by the Lipofectamine plus reagent (Invitrogen) according to the accompanying manual.

Measurement of amine oxidase and polyamine oxidase in NIH3T3 cell lysate and acrolein produced by the lysate. At 24 h after transfection of the plasmid as described above, cells were suspended in 10 volumes of ice-cold lysis buffer containing 10 mM Tris–HCl (pH 7.5), 50 μM FUT-175 [6-amino-2-naphthyl-4-guanidinobenzoate, an inhibitor of protease [18], a generous gift from Torii Pharmaceutical], and 1 mM dithiothreitol, and kept on ice for 30 min. The lysate was centrifuged for 10 min at 12,000*g* and the supernatant was used for enzyme assays. Amine oxidase activity was measured using benzylamine as substrate by the method of Holt et al. [19]. The reaction mixture (0.1 ml) for polyamine oxidase activity containing 10 mM Tris–HCl (pH 7.5), 0.25 mM spermine, and 10 μl cell lysate (50 μg protein) was incubated at 37 °C for 10 min. The level of spermine was measured by HPLC as described above. Protein was determined by the method of Lowry et al. [20], and polyamine oxidase activity was expressed as nmol spermine degraded/min/mg protein. The reaction mixture (0.1 ml) for measurement of free acrolein produced from spermine containing 10 mM Tris–HCl (pH 7.5), 0.25 mM spermine, 5 mM *m*-aminophenol, and 10 μl cell lysate (50 μg protein) was incubated at 37 °C for 12 h. Free acrolein content was determined as described above.

Cell culture in the presence of spermine and/or polyamine oxidase. Mock transfected NIH3T3 cells or NIH3T3-PAO cells transfected with the cDNA for polyamine oxidase (1×10^4 cells/ml) were cultured as described above except that 10% horse serum, which is deficient in serum amine oxidase, was used instead of 10% FBS. Where indicated, cell lysate from NIH3T3-PAO cells, spermine, MDL72527, and semicarbazide were added to the medium at the final concentrations of 25 μg/ml, 50 μM, 0.1 mM, and 0.1 mM, respectively. The viable cell number was counted in the presence of 0.05% trypan blue. Mouse mammary carcinoma FM3A cells (5×10^4 cells/ml) were cultured as described previously [8].

Statistics. Values are expressed as means ± SE. Groups were compared using a two-tailed unpaired Student's *t* test or simple linear regression analysis.

Results

Decrease in spermine and increase in putrescine, amine oxidase, and acrolein in plasma of patients with chronic renal failure

The polyamine content and amine oxidase activity in plasma of patients with renal failure, who have never had hemodialysis, were measured. As shown in Fig. 1, the level of putrescine in plasma of patients with renal failure was higher than that in normal subjects, whereas the level of spermine was lower than normal. The level of spermidine was slightly lower than normal, but the difference was not significant.

We determined the activity of amine oxidase in plasma as the ability of the enzyme to degrade spermine. As shown in Fig. 2A, amine oxidase activity in plasma of patients with renal failure was increased compared to normal subjects. The levels of free acrolein and protein-conjugated acrolein produced from free acrolein in plasma were determined, respectively, by HPLC and ELISA. As shown in Figs. 2B and C, both types of acrolein were increased in plasma of patients. The concentration of acrolein that causes 50% inhibition of cell

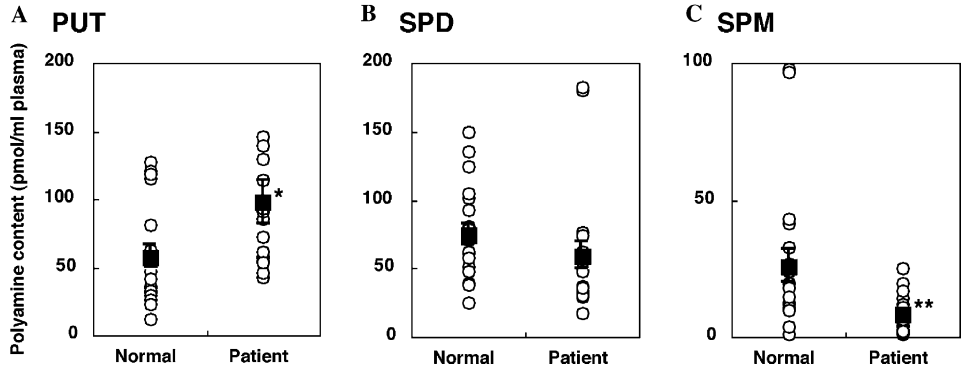

Fig. 1. Comparison of putrescine (A), spermidine (B), and spermine (C) contents in plasma of normal subjects and patients with chronic renal failure. Polyamines were measured after removal of amino acids as described in Materials and methods. Normal ($n = 20$); patients ($n = 20$). *, $P < 0.05$; **, $P < 0.01$.

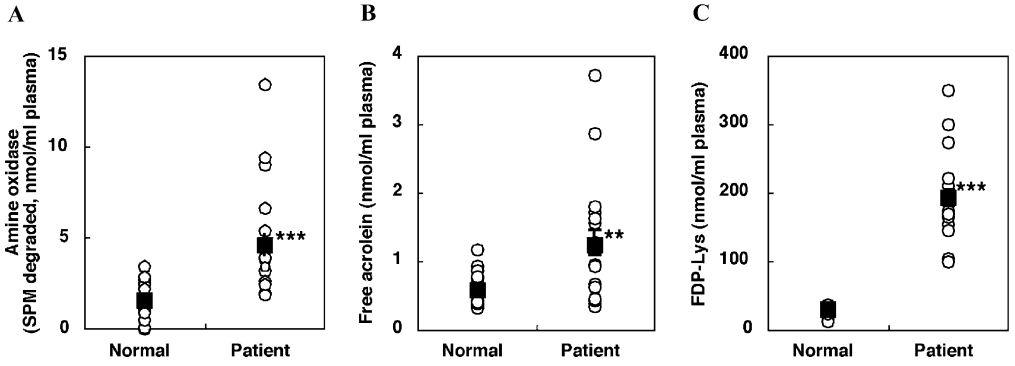

Fig. 2. Comparison of amine oxidase (A), free (B), and protein-conjugated (C) acrolein in plasma of control subjects and patients with chronic renal failure. Amine oxidase and free and protein-conjugated acrolein were measured as described in Materials and methods. Normal ($n = 20$); patients ($n = 20$). **, $P < 0.01$, ***, $P < 0.001$.

growth (IC_{50}) is 5–10 μM in a cell-culture system [8]. The mean value for free acrolein in the plasma of uremic patients was 1.3 μM, whereas that in normal subjects was 0.5 μM. Acrolein found as protein conjugates in the plasma of patients was equivalent to 180 μM, which is 6-fold higher than that in plasma of normal subjects. It is thought that the function of some proteins in plasma would be lost through the covalent linkage with acrolein.

The severity of renal failure is usually judged by the serum level of creatinine and BUN, and also the hematocrit value. The levels of creatinine and BUN were well correlated with the reduction in spermine in plasma (Figs. 3A and B). The decrease in spermine was also parallel with the decrease in hematocrit value (Fig. 3C). Furthermore, the serum level of creatinine was well correlated with the increase in amine oxidase, free-

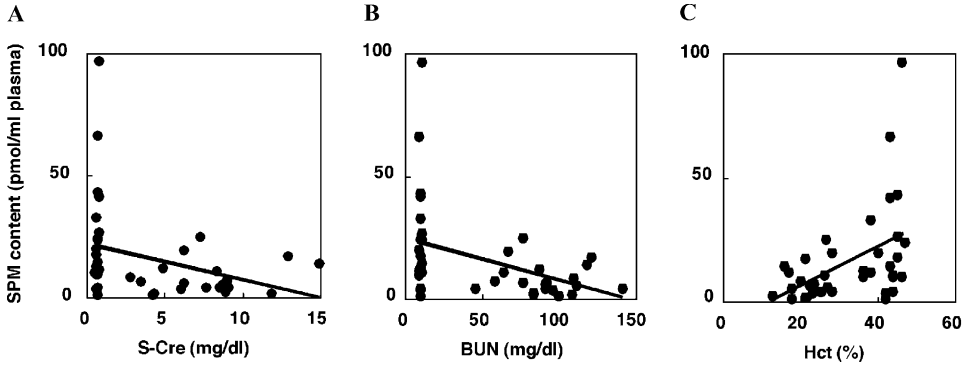

Fig. 3. Correlation between spermine content in plasma and serum creatinine (A), blood urea (B), and hematocrit (C). Each point was taken from the data of normal subjects and patients with renal failure. (A) $r = 0.340$, $P < 0.05$; (B) $r = 0.408$, $P < 0.01$; (C) $r = 0.471$, $P < 0.01$.

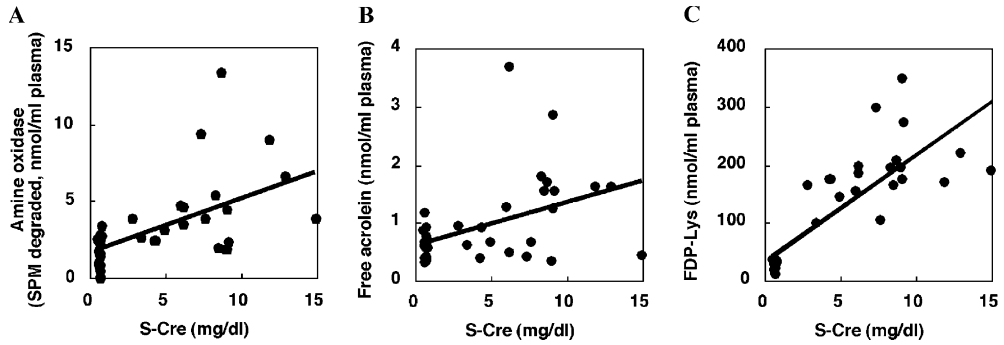

Fig. 4. Correlation between creatinine in serum and amine oxidase (A), free acrolein (B), and protein-conjugated acrolein (C). Each point was taken from the data of normal subjects and patients with renal failure. (A) $r = 0.352$, $P < 0.001$; (B) $r = 0.469$, $P < 0.01$; (C) $r = 0.834$, $P < 0.001$.

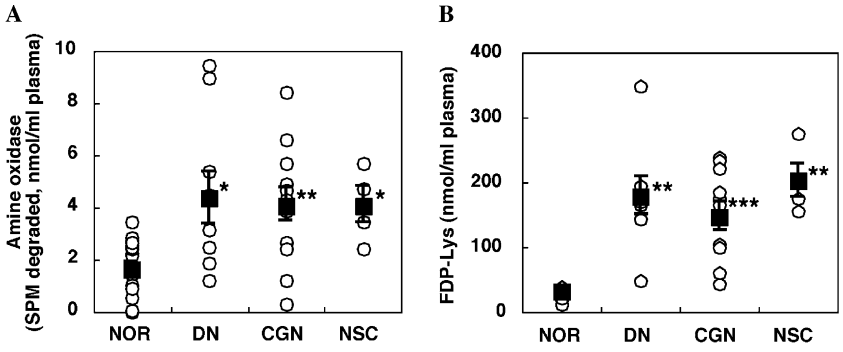

Fig. 5. Comparison of amine oxidase (A) and protein-conjugated acrolein (B) in plasma of normal subjects and patients with different kinds of chronic renal failure. NOR, normal subjects ($n = 20$); DN, diabetic nephropathy ($n = 7$); CGN, chronic glomerulonephritis ($n = 9$); NSC, nephrosclerosis ($n = 4$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

acrolein, and protein-conjugated acrolein (Fig. 4). These results suggest that acrolein produced from spermine may function as the uremic "toxin," which accelerates the progression of uremia.

The uremic patients had diseases with different primary etiologies. Thus, we compared amine oxidase activity and acrolein levels between patients with different diseases (Fig. 5). The changes in amine oxidase activity and protein-conjugated acrolein were each very similar among the different diseases (diabetic nephropathy, chronic glomerulonephritis, and nephrosclerosis). The results suggest that production of acrolein is a common feature of patients with chronic renal failure, irrespective of the primary disease that leads to renal failure.

Properties of amine oxidase in plasma of patients

We examined the properties of amine oxidase in plasma of patients with renal failure. For this purpose, we studied the effects of an inhibitor of monoamine oxidase, pargyline, an inhibitor of monoamine and diamine oxidases, semicarbazide, and an inhibitor of polyamine oxidase, MDL72527 [12]. As shown in Fig. 6, amine oxidase activity in plasma of all eight patients examined was inhibited by MDL72527, and the activity in four patients was inhibited by semicarbazide. Pargyline only inhibited activity in plasma of one patient. The results indicate that acrolein is produced mainly by polyamine oxidase and partly by diamine oxidase in the plasma of these patients.

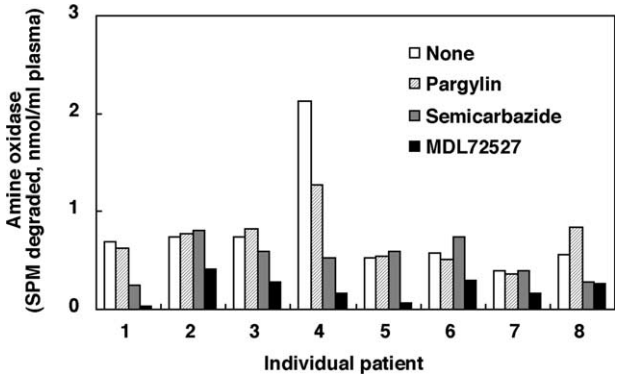

Fig. 6. Effects of inhibitors of amine oxidases on the activity in plasma of patients with chronic renal failure. Degradation of spermine by plasma was measured in the presence and absence of inhibitors of amine oxidases. The concentrations of pargyline, semicarbazide, and MDL72527 were 1, 0.1, and 0.25 mM, respectively. Each value is the average of duplicate determinations.

Cytotoxicity by spermine and polyamine oxidase

The relationship between induction of amine oxidase and production of acrolein was examined using NIH3T3-PAO cells transfected with a cDNA for human polyamine oxidase [12]. Spermine was rapidly degraded by the cell lysate from NIH3T3-PAO cells (Fig. 7A) and acrolein was produced (Fig. 7B). Control NIH3T3 cells, not transfected with a cDNA for polyamine oxidase, had a low level of amine oxidase activity and generated only small amounts of acrolein. Degradation of spermine and production of acrolein by the cell lysate from NIH3T3-PAO cells were inhibited by MDL72527, but not by semicarbazide. Production of acrolein from spermine by polyamine oxidase increased with incubation time up to 24 h (data not shown). Using NIH3T3 cells transfected with the cDNA for another amine oxidase, human amine oxidase VAP-1 [17], spermine was not degraded significantly by the cell lysate nor was acrolein produced, although benzylamine (a VAP-1 substrate) was degraded greatly (data not shown). These results confirmed that acrolein is produced by polyamine oxidase, but not by VAP-1.

We next determined whether polyamine oxidase, like bovine serum amine oxidase, causes cell toxicity in the presence of spermine. As shown in Fig. 7C, addition of a cell lysate prepared from NIH3T3 cells expressing high levels of polyamine oxidase (NIH3T3-PAO cells) caused cytotoxicity of NIH3T3 cells in the presence of 50 μM spermine. Addition of MDL72527, but not semicar-

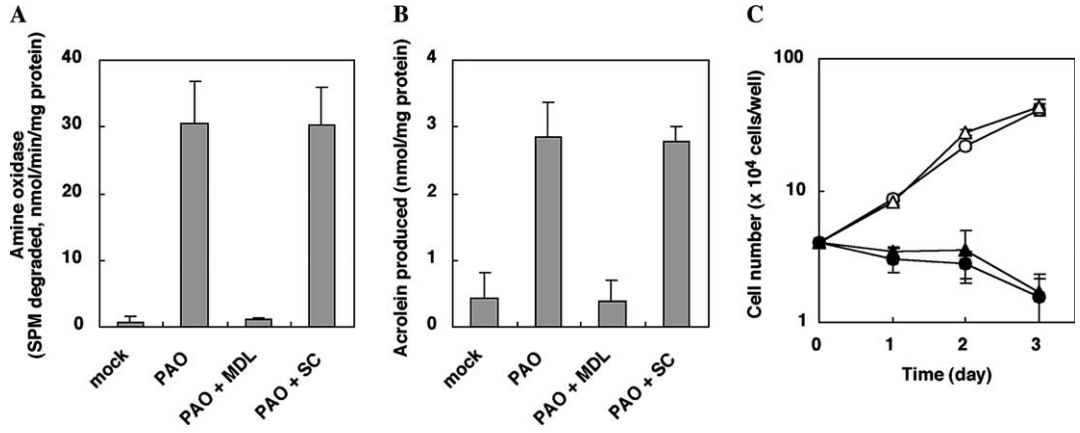

Fig. 7. Production of free acrolein from spermine by polyamine oxidase in NIH3T3-PAO cell lysate and its cytotoxicity. (A) Mock and pcDNA3.1(-)-hPAO1 transfected NIH3T3 cells were cultured for 24 h, and the activity of amine oxidase in the cell lysate was measured in the presence and absence of 0.25 mM MDL72527 (MDL) or 0.1 mM semicarbazide (SC). Values are means ± SE of triplicate determinations. (B) Free acrolein produced from 0.25 mM spermine by the cell lysate of NIH3T3-PAO cells. Free acrolein was measured as described in Materials and methods. Values are means ± SE of triplicate determinations. (C) NIH3T3 cells (5×10^4 cells/ml) were cultured in the presence and absence of spermine and cell lysate (25 μg/ml protein) of NIH3T3-PAO cells, and the viable cell number was counted at the designated times. The following were added to the medium containing 10% horse serum. ○, cell lysate of NIH3T3 cells and 50 μM spermine; ●, cell lysate of NIH3T3-PAO cells and 50 μM spermine; △, cell lysate of NIH3T3-PAO cells, 50 μM spermine, and 0.1 mM MDL72527; ▲, cell lysate of NIH3T3-PAO cells, 50 μM spermine, and 0.1 mM semicarbazide. Values are means ± SE of triplicate determinations.

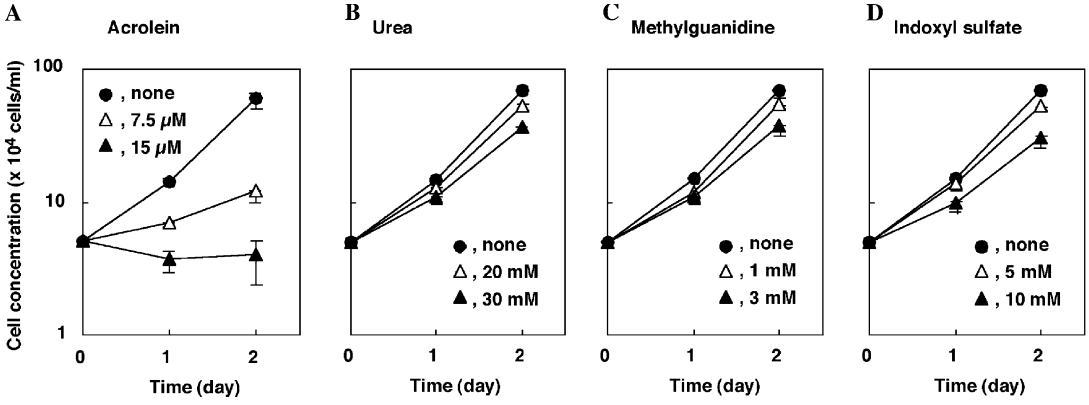

Fig. 8. Effect of acrolein, urea, methylguanidine, and indoxylsulfate on cell growth of FM3A cells in the presence of 2% FBS. Cells were cultured under standard conditions except that chemicals shown in the figure were added to the medium together with 2% FBS. Values are means ± SE of triplicate determinations.

bazide, together with polyamine oxidase disturbed the cytotoxicity. Addition of a cell lysate prepared from NIH3T3 cells to the medium did not cause cytotoxicity to NIH3T3 cells in the presence of 50 μM spermine.

Effects of other metabolites accumulated in blood of renal failure patients on cell growth

It has been reported that several metabolites (urea, methylguanidine, and indoxylsulfate) are accumulated in blood of renal failure patients and considered as candidates of uremic "toxin" [21–23]. Thus, effects of these metabolites on cell growth were examined. As shown in Fig. 8, each of metabolite was only slightly inhibitory on cell growth of FM3A cells, although acrolein was very toxic for cell growth. The results indicate that acrolein is the only toxic compound accumulated in blood of renal failure patients among the metabolites tested.

Discussion

In this study we have found a decrease in spermine and an increase in putrescine, amine oxidase, and acrolein in the plasma of patients with chronic renal failure. Since spermine is degraded by polyamine oxidase to putrescine via spermidine, the level of spermidine was only slightly lower in plasma of renal failure patients than normal subjects. The level of acrolein produced from spermine was well correlated with the degree of seriousness of chronic renal failure. Thus, it is thought that both polyamines and polyamine oxidase are first released from damaged kidney and subsequently from damaged cells such as erythrocytes. Our results also indicate that acrolein is the only toxic compound among the tested compounds, which accumulate, in blood of patients with renal failure. Accordingly, acrolein may function as the uremic "toxin," which accelerates the progression of uremia.

According to our results [8], approximately 5 μM acrolein inhibits cell growth by 50% under the condition where cell density is 10^4–10^5 cells/ml. The concentration of free acrolein in blood of renal failure patients was 1.3 μM (Fig. 2). The recovery of free acrolein under our experimental conditions was about 30% (unpublished results), suggesting that the concentration of free acrolein is equivalent to 4.3 μM. Such a concentration of acrolein may be enough to damage the functions of cells and proteins even if cell density or protein concentration is high in blood.

It has been reported that acrolein can be produced from membrane phospholipids [16], although the major aldehydes produced during lipid peroxidation are 4-hydroxy-2-nonenal and malondialdehyde [24]. These two aldehydes were much less toxic compared with acrolein (unpublished results). We measured free acrolein by the method described in Materials and methods under the same conditions in which acrolein has been reported to be produced from membrane phospholipids [16]. However, acrolein production was very low. Thus, our results suggest that acrolein is mainly produced from spermine, but not from membrane phospholipids. There are also reports that 3-aminopropanal produced from spermine is strongly involved in cell damage during ischemia [25,26]. T cell proliferation is also repressed by polyamine oxidation in peripheral blood mononuclear cells [27]. In this case, it has been reported that both acrolein and H_2O_2 produced from spermine are involved in cell damage. Acrolein has been reported to cause lipid peroxidation of erythrocytes and then hemolysis [28]. Thus, acrolein may be involved in various kinds of cell damage.

Our results suggest that an inhibitor of polyamine oxidase may be helpful for improving the symptoms of chronic renal failure. The inhibitor may delay the commencement of hemodialysis. Since MDL72527 is a potent inhibitor of polyamine oxidase, it may be possible to develop a therapeutically useful drug using MDL72527 as a lead compound.

In previous reports [10,29], the levels of polyamines in serum were higher in renal failure patients than in ambulatory uremic children or in normal subjects. However, the results in this study show that the level of putrescine was increased, whereas the level of spermine was decreased in the plasma of renal failure patients. Such a discrepancy remains to be clarified.

Acknowledgments

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