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Desmosines in human urine

Amounts in early development and in Marfan's syndrome

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Desmosines from 24h human urine samples were isolated, characterized and quantified. The desmosines are in peptidyl form (1000–1500 molecular weight), and their amount is decreased by two-thirds between 7 and 25 years of age. Patients with Marfan's syndrome have significantly lower urinary amounts of desmosines than do comparable controls during the early developmental period.

Elastin, a structural protein of many body tissues, contains the distinctive lysyl-derived cross-links desmosine and isodesmosine (Thomas *et al.*, 1963). Once these cross-links form, elastin becomes relatively insoluble and resistant to most proteinases. In laboratory animals, elastin synthesis is most active during the late foetal and early developmental periods, when elastin-containing membranes form (Looker & Berry, 1972; Lee *et al.*, 1976). A breakdown of elastin-rich membranes occurs in arterial tissue during the developmental period (Ehrich *et al.*, 1931; Gross *et al.*, 1934; Levene, 1956; Velican & Velican, 1976), and this phenomenon is most remarkable in the Marfan's syndrome (Takebayashi *et al.*, 1973).

In a brief note, Weiss & Steven (1968) reported peptides in human urine derived from connective-tissue protein and suggested that some of the peptides were cross-linked by isodesmosine and desmosine and represented breakdown products of elastin; however, further characterization of human urinary desmosines has not appeared in the literature. Goldstein & Starcher (1978) reported an increase in urinary desmosines in hamsters after the destruction of lung tissue by elastase instillation. We set out to isolate and characterize urinary desmosines as biochemical markers of elastin degradation in man.

Desmosine and isodesmosine (desmosines), determined as borohydride-reduced compounds, were isolated by a multi-step procedure from human urine. The amounts of desmosines were determined at different age periods and in patients with clinically established Marfan's syndrome. The highest amounts of urinary desmosines are found in the early developmental period (8–11 years), and the amount declines during the pubertal and the young adult period. Patients with Marfan's syndrome excrete consistently smaller amounts of desmosines during these pre-adult periods.

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Materials and methods

Materials

NaB^3H_4 (350mCi/mmol–10Ci/mmol) was purchased from ICN, Irvine, CA, U.S.A. Authentic desmosines were kindly provided by Dr. Barry Starcher (Washington University Medical Center, Department of Internal Medicine, Pulmonary Division, St. Louis, MO, U.S.A.).

Procedure for isolation of urinary desmosines in human urine

Volunteers were placed on an elastin- and collagen-free diet 24h before and during the urine collection period. Then 24h urine samples (200–2000ml, 25–50g of solids) were precipitated with acetone (acetone/urine ratio 4:1, v/v) in the presence of NaCl (1.1%) at 4°C, were allowed to settle overnight (4°C), and the supernatant was removed and discarded. The precipitate was dissolved in 1.1% NaCl (100–300ml) and re-precipitated with acetone as described above. The second acetone precipitate (4–10g) was triturated in 0.1M-pyridine/acetate buffer, pH5.5, and the supernatant was fractionated on a pre-calibrated Sephadex G-15 column (described below) with 0.1M-pyridine/acetate buffer, pH5.5. The eluates (1.2ml/fraction) were divided into a void-volume fraction A and a peptide-containing fraction B. Fraction A contained material of >1500mol.wt., whereas fraction B contained material of 400–1400mol.wt.

Fractions A and B were reduced either with

$NaBH_4$ (80mg/ml) or with NaB^3H_4 (100mCi) in 0.2M-sodium phosphate buffer, pH7.4, for 2h15min. The reduced solutions were desalted on Dowex 50W (H^+ form) columns (10cm × 0.9cm), freeze-dried and hydrolysed in 6M-HCl for 22–24h at 105°C, under vacuum, after flushing with N_2.

After removal of the HCl, the hydrolysate was streaked on Whatman 3MM paper for chromatography [butan-1-ol/acetic acid/water (4:1:1, by vol.); 66h (Starcher, 1977)]. The origin (12.5mm on either side) was cut out, and eluted with water and analysed for reduced desmosines on the Beckman Amino Acid Analyzer specially programmed with a five-buffer system to ensure the separation of reduced desmosine from contaminants present in hydrolysates of elastin-containing tissues (Boucek *et al.*, 1979).

Alkali treatment (10M-NaOH) of authentic and urinary desmosines was performed as described by Anwar & Oda (1966). Free lysine produced by the reaction was analysed on the Beckman Amino Acid Analyzer.

Calibration of the Sephadex column

A Sephadex G-15 column (100cm × 1.6cm), equilibrated with 0.1M-pyridine/acetate buffer, pH5.5, was calibrated for void volume (cytochrome *c*), total volume (dinitrophenyl-lysine) and the elution position for free desmosine and isodesmosine. Fraction B contained fractions immediately after the void volume plus ten fractions after the elution position of authentic desmosines (total number of fractions for B approx. 30; 1.2ml/fraction).

Patient selection

Strict criteria were used for the diagnosis of Marfan's syndrome and were in accordance with those reported by Bowers (1969). The major criteria included a positive family history and dislocated ocular lenses (ectopia lenti); the minor criteria were the skeletal abnormalities arachnodactyly, dolichocephaly, pectus excavatum and kyphoscoliosis, and cardiovascular involvement, namely aortic dilatation or aneurysm, and aortic or mitral valvular incompetence (echocardiographically determined). All of the patients with Marfan's syndrome were required to have one major and two minor criteria before the diagnosis was accepted and urinary collections were made.

Results and discussion

Efforts to isolate the desmosines from human urine began by testing several methods of decreasing the volume and the solid weight of 24h urine samples. Freeze-drying proved to be unsatisfactory because of foaming and because of the extremely viscous solutions produced in some urines. Acetone precipitation, previously used to isolate and concentrate hydroxylysine-containing peptides from human urine (Cunningham *et al.*, 1967), precipitates the urine solids, and a second acetone precipitation further decreases the solid weight from a 24h urine sample to one-fifth (Table 1).

Characterization of desmosines in human urine

After gel filtration, borohydride reduction, hydrolysis and paper-chromatographic separation of fraction B, the eluate from the origin was applied to the Amino Acid Analyzer and a ninhydrin-positive material (peak X) was eluted in the region of the borohydride-reduced authentic desmosines (Fig. 1). Peak X was collected, desalted and applied to two cation-exchange columns (60cm × 0.9cm and 30cm × 0.9cm; resin from Mark Instrument Co., Villanova, PA, U.S.A.); with 0.25M-sodium citrate buffer, pH3.25 and pH4.2, and 0.35M-citrate buffer, pH5.25, peak X was co-eluted in each case with authentic desmosines. Additionally, peak X and authentic reduced desmosines had identical electrophoretic mobilities in high-voltage paper electrophoresis with pyridine/acetic acid/water mixture (1:10:90, by vol.), pH3.5. Treatment of peak X and reduced authentic desmosines with alkali (10M-NaOH) produced free lysine, which was identified by its elution pattern on the Amino Acid Analyzer. U.v.-absorption spectral characteristics of peak X and authentic desmosines are lost after borohydride reduction. When urinary fraction B was reduced with NaB^3H_4, peak X was rendered radioactive. Similarly to authentic reduced desmosines, peak X was insoluble in butan-1-ol/acetic acid/water (4:1:1, by vol.) and remained at the origin of the paper chromatogram. These characterizations strongly support the identity of peak X as urinary desmosines.

The isolation of the desmosines by acetone precipitation procedures was reproducible within the experimental error of ±10% when duplicate urine samples (50% of the same 24h collection) were processed through the entire isolation procedure

Table 1. *Isolation of desmosines from human urine*
For full experimental details see the text.

Isolation step	Weight of solids (g)
24h urine collection (200–2000ml)	25–50
Second acetone precipitate	4–10
Sephadex G-15 fraction B (400–1400mol.wt.)	0.5–1.5
Reduced products from Dowex column	0.010–0.020
Paper chromatography (origin area)	0.001–0.0025
Peak X	10–77 ($\times 10^{-6}$)

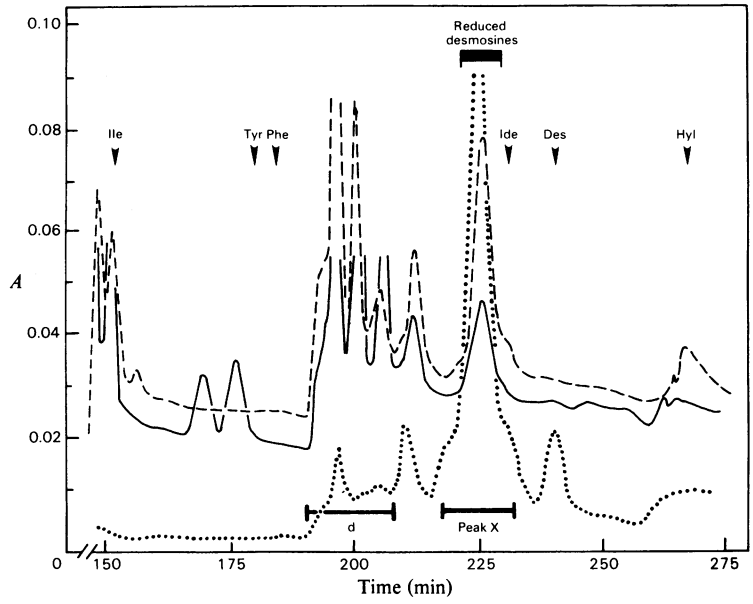

Fig. 1. *Elution patterns of reduced authentic and human urinary desmosines*
For full experimental details see the text. ----, 16% 24h urine collection (control, 11.5 years old); ——, 50% 24h urine collection (Marfan's-syndrome patient, 8 years old); ·····, authentic reduced isodesmosine (Ide) and desmosine (Des). d, Elution position of glucosaminitol and galactosaminitol, β-aminoisobutyric acid, β-alanine and γ-aminobutyric acid.

independently. No desmosines were found in fraction A, and all of the urinary desmosines were found in the peptide-containing fraction B.

Characterization of peptidyl-desmosine in human urine

To ensure that precipitation of desmosines by acetone was quantitative, and to estimate the molecular weight of peptidyl-desmosines, half of the 24h sample was successively passed through an Amicon ultrafiltration cell with PM-30, PM-10, UM-2 (approx. 1000mol.wt.) and UM-0.5 (approx. 500mol.wt.) membranes, and the other half was fractionated in accordance with the isolation procedure reported in the Materials and methods section. Each of the membrane-retained fractions was freeze-dried and passed through the pre-calibrated Sephadex G-15 column with pyridine/acetate buffer. The eluates were then processed as described in the Materials and methods section.

Desmosine-containing material isolated by membrane filtration and its quantification was within the experimental error of the acetone precipitation procedure (±12%, as determined by simultaneous processing of acetone precipitates of 50% samples of a 24h urine). All of the urinary desmosines were found in the UM-2-membrane-retained fraction, suggesting a peptidyl-desmosine of molecular weight greater than 1000. Additional refinement of the molecular weight of the peptidyl-desmosine fraction is provided by its inclusion in the post-void-volume fraction of the Sephadex G-15 fractionation (fraction B), suggesting a molecular weight of 900–1400. Since the molecular weight of desmosines is 540, the urine must contain only peptidyl forms of desmosines.

If the molecular location of desmosines is similar in human elastin to that reported by Shimada *et al.* (1969) and Mecham & Foster (1978) for bovine ligamentum nuchae, then a urinary peptidyl-desmosine of molecular weight 900–1400 suggests degradation of the elastin polypeptide to the level of a desmosine that cross-links five alanine residues alone or including four or five adjacent residues.

Urinary peptidyl-desmosine excretion during early developmental periods

During the early developmental period, the amounts of peptidyl-desmosines from 13 healthy human volunteers decreases, suggesting a declining elastin degradation during this period (Fig. 2). High amounts of urinary peptidyl-desmosines in pre-pubertal-age children (4–8 years), which decrease during the pubertal and young adult periods, suggest hormonal modulation of elastin degradation in man.

Urinary peptidyl-desmosine excretion in the Marfan's syndrome

Marfan's syndrome, a heritable defect in connective tissues in man, is characterized by extensive

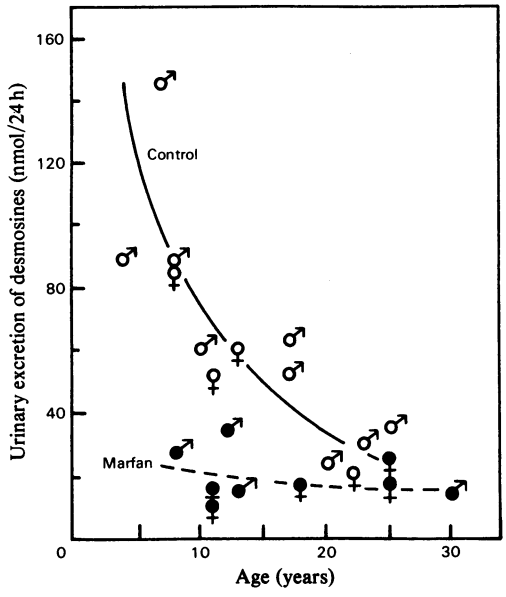

Fig. 2. *Urinary desmosines in healthy controls and in patient with Marfan's syndrome*
For full experimental details see the text. ○, Healthy controls; ●, patients with Marfan's syndrome.

loss of elastin-rich arterial membranes and by aneurysm formation, implying excessive elastin degradation in this disease.

Urinary peptidyl-desmosines from pre-adult (10–18 years) Marfan's-syndrome patients, however, were consistently lower than those from comparable healthy control volunteers (Fig. 2). After the rapid growth period, the excretions of urinary peptidyl-desmosines are similar in Marfan's-syndrome patients and controls.

Relatively low amounts of urinary peptidyl-desmosines in the pre-adult Marfan-syndrome patients with clinical evidence of loss of elastin-rich membranes (aortic dilatation or aneurysm formation) could be due to a limited desmosine formation because of low lysyl oxidase activity [as in X-linked cutis laxa (Byers *et al.*, 1980)] or secondary to an attenuated conversion of precursor aldehydes and lysyl cross-links into the desmosines. Normal lysyl oxidase activities are reported by Layman *et al.* (1972) for fibroblasts cultured from Marfan's-syndrome patients, and recent findings in our laboratories of a high ratio between precursor (lysinonorleucine) cross-links and the desmosines in aortic tissues from a Marfan's-syndrome patient (R. J. Boucek, N. L. Noble, Z. Gunja-Smith & W. T. Butler, unpublished work) suggest an attenuated precursor–product relationship in some fraction of the total elastin synthesized during the rapid growth and developmental period. The low amounts of urinary desmosines in the Marfan's-syndrome patient would therefore indicate not a diminished elastin turnover but a normal or even an accelerated turnover that escapes detection because of the attenuated conversion of lysyl-derived precursor material into the desmosines.

A diminished excretion of urinary peptidyl-desmosine during the pre-adult growth period in Marfan's syndrome is of diagnostic importance in clinical medicine and is the first direct evidence of altered elastin cross-linking in a heritable connective-tissue disease in man.

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