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COVALENT BINDING OF ACETALDEHYDE TO TYPE III COLLAGEN

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SUMMARY. Incubation of neutral salt soluble type III pN-collagen with [^{14}C]acetaldehyde *in vitro* resulted in the formation of spontaneously stable acetaldehyde-protein adducts. This reaction occurred primarily at lysine residues and it was not affected by 0.2-2 mM concentrations of ascorbate but addition of sodiumcyanoborohydride increased the stable adducts by 3-5-fold. When confluent cultures of human skin fibroblasts were incubated with physiologically relevant concentrations of acetaldehyde, it became covalently bound to type III procollagen secreted into the medium. We propose that acetaldehyde binding to collagen fibrils occurs *in vivo* following chronic alcohol consumption.

The oxidation of ethanol in the liver yields acetaldehyde, which is a highly reactive metabolite. Recent *in vitro* studies have demonstrated that acetaldehyde reacts with plasma (1), red cell (2, 3), and hepatic proteins (4) forming both stable and unstable condensation products. Should such binding occur *in vivo* following alcohol consumption it may lead to a number of functional consequenses, including inhibition of enzyme activities (5), modification of the metabolism of circulating proteins (6, 7), and generation of immunological responses (8, 9). Furthermore, acetaldehyde modified proteins could serve as a possible biochemical marker of cumulative alcohol consumption (2, 8, 10). The concentrations of acetaldehyde which exist in the blood of alcoholics are usually below 200 μM (11) and unfortunately, the *in vitro* binding studies have often involved supraphysiological concentrations of acetaldehyde together with artificial reducing agents. Thus the relevance of the above findings to *in vivo* situations has remained as a matter of controversy.

In the present study we examined the nature and extent of acetaldehyde binding to type III collagen, a major component of connective tissues. During the development of fibrosis in internal organs when there is a net increase in connective tissue components, it is

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usually the first collagen deposited (12). Thus monitoring the metabolism of type III collagen has been widely used as a diagnostic method for the development of fibrosis (13). This collagen type is synthesized as soluble procollagen, which is processed first from the carboxyterminal end only, such that many tissues contain significant amounts of partially processed collagen retaining the aminoterminal end (pN-collagen) (14). Acetaldehyde binding to type III collagen was demonstrated under physiologically relevant conditions both in vitro and in cell culture.

MATERIALS AND METHODS

Materials. 1,2-[^{14}C]acetaldehyde (9.5 mCi/mmol) and L-2,3-[^{3}H]proline (40 Ci/mmol) were purchased from New England Nuclear (Boston, Mass.). L-ascorbate was from Merck (Darmstadt, FRG) and sodiumcyanoborohydride was a product of Sigma (St. Louis, Mo). The cell culture reagents were obtained from Nunc Products (Roskilde, Denmark) and Gibco Biocult (Paisley, U.K). DEAE-Sephacel, Sephacryl S-500 and Protein A-Sepharose Cl-4B were obtained from Pharmacia (Uppsala, Sweden). All other reagents were of analytical grade.

Preparation of type III pN-collagen. Collagen was extracted from foetal calf skin in 0.05 M Tris-HCl buffer, pH 7.4 containing 0.2 M NaCl, 2 mM p-phenylmethanesulfonyl-fluoride, 2 mM p-hydroxymercuribenzoate, 1 mM N-ethylmaleimide and 10 mM EDTA. Type III pN-collagen was subsequently purified by fractional salt precipitation and DEAE-cellulose chromatography as previously described (15). Final purification was achieved by Sephacryl S-500 gel filtration. The pooled fractions were tested for purity by bacterial collagenase (grade CLSPA, Worthington) digestion, electrophoresis on 6.5% SDS-polyacrylamide gels and amino acid analysis.

Acetaldehyde binding assays. Acetaldehyde-protein binding was studied essentially according to the procedures described elsewhere (1, 8). Neutral salt soluble type III pN-collagen (0.5 mg/ml in phosphate buffered saline, PBS), bovine serum albumin, BSA (fraction V, from Sigma), human hemoglobin prepared as described previously (2), and low density lipoprotein (from Sigma) were incubated in tightly sealed vials at 25°C with acetaldehyde in the presence or absence of ascorbate. All mixtures were chilled on ice at various time intervals and aliquots were removed for radioactivity and protein determinations. The total adducts were determined following reduction of non-stable acetaldehyde-protein linkages with sodiumcyanoborohydride as described previously (1). Radioactivity bound to proteins was determined either after precipitation and extensive washing with 10% TCA or after dialysis.

For metabolic labelling, confluent cultures of human skin fibroblasts were incubated overnight in fresh Dulbecco's modified Eagle's medium supplemented with 0.2 mM ascorbic acid, 100 U/ml penicillin, 100 U/ml streptomycin and either various concentrations of [^{14}C]-acetaldehyde or L-2,3-[^{3}H]proline (5 µCi/ml). The mediums containing the newly synthesized proteins were collected after centrifugation and dialyzed extensively against either 0.2 M ammonium bicarbonate or 0.4 M NaCl, 5 mM EDTA, 50 mM Tris/HCl, pH 8.0, 1% (v/v) Nonidet P-40, 0.02% (w/v) NaN_3. Subsequent immunoprecipitations for the determination of type III pro-collagen related materials were carried out as described by us earlier (16).

Gel electrophoresis and fluorography. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS/PAGEs) on 6.5% gels according to the method of Laemmli (17). The incorporation of acetaldehyde in proteins was detected by fluorography after impregnating the gels with 2,5-diphenyloxazole (Sigma) in dimethylsulfoxide.

Other methods. For the determinations of the amino acids proteins were hydrolyzed with 6 M HCl for 24 h at 110 °C and the analysis was performed on a Kontron Liquimat 111 amino acid analyzer. Protein concentrations were determined by the method of Lowry et al. (18). The [^{14}C]-radioactivity was measured using a Wallac liquid scintillation spectrometer.

RESULTS

Incubation of the isolated neutral salt soluble type III pN-collagen with [^{14}C]acetaldehyde resulted in the formation of adducts, which were stable to conditions such as TCA-precipitation or dialysis (Fig. 1). Acetaldehyde binding to type III pN-collagen was 5-, 10- or 2-fold more per mg of protein than the binding under similar conditions to bovine serum albumin, human hemoglobin or low density lipoprotein, respectively. The presence of ascorbate in the incubation medium did not affect the formation of stable adducts with type III pN-collagen, although it increased the formation of stable adducts with acetaldehyde and bovine serum albumin. The spontaneously stable acetaldehyde-collagen adducts, as determined following stabilization with sodiumcyanoborohydride, comprised 20-40 % of the total adducts. Incorporation of [^{14}C]acetaldehyde in the isolated type III pN-collagen was further tested by SDS-PAGEs on 6.5% gels followed by fluorography. Upon reduction acetaldehyde was visualized in type III collagen and type III pN-chains (carrying the aminoterminal extension peptides) (Fig. 2). Following incubations of the neutral salt soluble type III pN-collagen with physiologically relevant acetaldehyde concentrations no collagen crosslinking by acetaldehyde per se was detected, as analysed

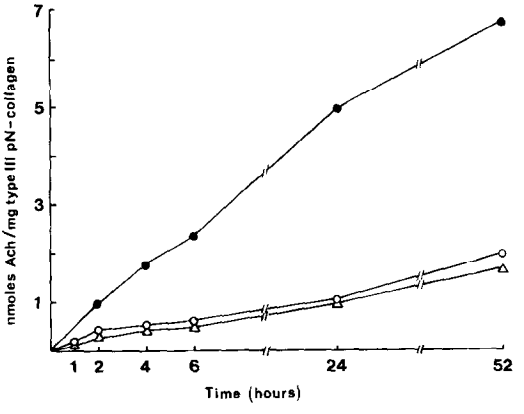

Figure 1. Formation of adducts with neutral salt soluble type III pN-collagen and acetaldehyde (Ach) in vitro. The reaction mixture consisted of 100 µM [^{14}C]acetaldehyde and type III pN-collagen (0.5 mg/ml in PBS, pH 7.4) without reducing agents (open circles), in the presence of 0.5 mM l-ascorbate (open triangles) or 10 mM sodiumcyanoborohydride (closed circles).

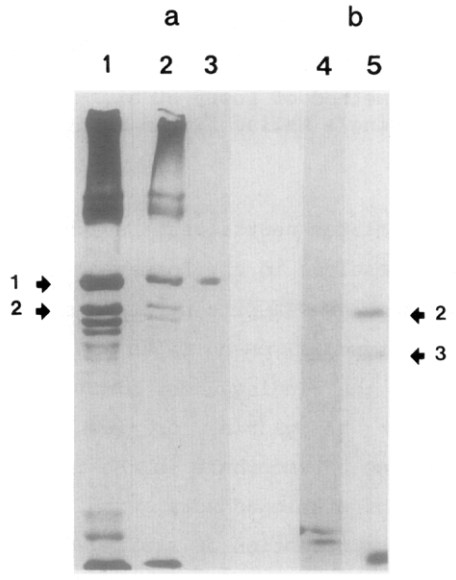

Figure 2. SDS/PAGE analysis of the acetaldehyde containing macromolecules in human skin fibroblast medium (Fig. 2a) and of the isolated neutral salt soluble type III pN-collagen (Fig. 2b) The molecular mass markers, indicated by arrows, were human type III procollagen (1), human type III pN-collagen (2) and type III collagen (3). The sample (reduced) in the lane 1 is [^{3}H]proline labelled material from human skin fibroblast medium, (2) [^{14}C]acetaldehyde containing medium macromolecules, (3) acetaldehyde labelled proteins immunoprecipitated with the antibodies against the aminoterminal propeptide of type III procollagen, (4) [^{14}C]acetaldehyde containing bovine type III pN-collagen and type III collagen, (5) [^{125}I]-labelled bovine type III pN-collagen and type III collagen.

on SDS/PAGEs or Sephacryl S-500 chromatography. While the amino acid chromatography of the acetaldehyde modified type III pN-collagen revealed multiple sites of modification, the main peak (60%) of the bound radioactivity corresponded to the derivatization of lysine.

When confluent cultures of human skin fibroblasts were labelled overnight with [^{14}C]acetaldehyde, it became covalently bound to the macromolecules secreted into the medium (Fig. 2). The incorporation of acetaldehyde to type III pro- and pN- collagens represented 10-15 % of the total radioactivity present in the medium macromolecules, as determined either by densitometric scanning or immunoprecipitation with antibodies against the aminoterminal propeptide of type III procollagen. In these experiments as well as parallel control experiments (incubations without acetaldehyde), the main type III collagen related material in the incubation medium was consistently the unprocessed procollagen.

DISCUSSION

In this study we report the first demonstration of acetaldehyde, the first metabolite of ethanol, binding to a component of connective tissues. Spontaneously stable acetaldehyde-protein linkages were readily formed with type III collagen at concentrations of acetaldehyde, which exist in the blood of alcoholics during ethanol oxidation (11), indicating that this reaction should occur in vivo.

Acetaldehyde was found to locate predominantly at lysine residues which is in agreement with previous findings of acetaldehyde binding to other proteins (2) The adduct formation with the epsilon-amino-groups of lysines, which proceeds via Sciff base intermediates, is, however, a slowly reversible unless a reduction is performed (3). The mechanisms of spontaneous stabilization in vivo have not yet been fully elucidated. Although ascorbate, a naturally occurring reducing agent, increased the formation of stable adducts with albumin as reported previously (19, 20) we found no effect for ascorbate in stabilizing the acetaldehyde-protein linkages with collagen, suggesting that other mechanisms must also be involved.

Previous studies have suggested a role for acetaldehyde in stimulating the synthesis of collagens and collagen mRNAs in cultured fibroblasts (21,22,23). It appears that binding of acetaldehyde to lysine-rich nucleic acid binding proteins could account for the increase in collagen gene transcription. While the collagen synthesis in the liver of alcoholics is induced, the present findings indicate that it would simultaneously also become modified with acetaldehyde. Such a modification could perhaps interfere with the normal assembly and metabolism in a manner analogous to other chemical modifications of collagen, such as nonenzymatic glycosylation of lysines (24).

In the present study we chose a neutral salt soluble collagen, which unlike the insoluble collagens is not crosslinked, for experimentation in order to investigate the possibility whether acetaldehyde induces crosslinks in collagen. Crosslinking by acetaldehyde has previously been reported for erythrocyte membrane proteins (25). Such was, however, not observed for collagen at acetaldehyde concentrations which exist in the blood of alcoholics. Nevertheless, acetaldehyde located at lysine residues could be interfering at sites of intermolecular crosslinking (26). It should be noted that there is in fact an increase in collagen solubility in alcoholic livers which may be due to a decrease in mature type crosslinks (27). Future studies should address the possibility whether acetaldehyde binding to collagen could affect its

ability to resist degradation and lead to an increased turnover of the connective tissue in alcoholic liver disease.

The present findings open the possibility of developing methods for the detection of acetaldehyde modified collagens, which would be valuable in the differential diagnosis of alcoholic liver fibrosis. This would be analogous to the methods for hemoglobin- and albumin- acetaldehyde adducts, which are currently under extensive investigations (8,10,28). The present data indicate that acetaldehyde incorporation to collagen is more efficient than that to albumin or hemoglobin. Considering the above plus the fact that collagens have long half lives, attempts should be made to detect acetaldehyde modified collagens from liver biopsies of alcoholics to analyze cumulative exposure to ethanol and acetaldehyde from the liver. Applying recent findings that acetaldehyde-protein adducts are immunogenic (8), our preliminary studies indicate that collagens modified with low concentrations of acetaldehyde react with antibodies raised against acetaldehyde modified epitopes in proteins, which should allow the development of sensitive immunoassays for such condensates.

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