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ADHESION OF MOUSE HEPATOCYTES TO TYPE I COLLAGEN: ROLE OF SUPRAMOLECULAR FORMS AND EFFECT OF PROTEOLYTIC DEGRADATION

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SUMMARY. Mouse hepatocytes were found to adhere much stronger to intact native type I collagen than to native, telopeptide-devoid collagen. Removal of telopeptides (by pepsin treatment) caused disintegration of supramolecular collagen aggregates. Similar differences in hepatocyte adhesion were observed between oligomeric and monomeric collagens separated from intact native preparations. It was concluded that the more active cell binding to intact collagen is due to a higher binding affinity of hepatocytes to supramolecular (oligomeric) forms of type I collagen. Heat denturation did not affect cell adhesion to intact collagen but restored adhesion to telopeptide-devoid substrates. Cell adhesion to denatured collagen was RGD-dependent whereas adhesion to native substrates proved RGD-independent. These data provide the first evidence of the role of supramolecular arrangement and the effect of proteolytic degradation of type I collagen on its adhesive behavior.

Collagens, the major components of extracellular matrix (ECM), belong to a family of cell adhesive proteins that play important roles in development, differentiation, and tumorogenesis [1-4]. Cell-matrix interactions are mediated by a variety of cell surface receptors having specific binding affinities to particular sites on ECM proteins [1, 5]. Molecules of type I collagen are monomers composed of a long central triple-helical domain and short N- and C- terminal nonhelical sequences, named telopeptides. The latter contain the sites of intermolecular cross-linking, and therefore markedly contribute to the formation of fibrils-supramolecular collagen aggregates composed of the orderly arranged monomers [6]. Various proteolytic events occur in vivo (in particular, those accompanying tissue remodeling or tumor invasion) that may lead to a cleavage ot telopeptides, thus inducing disaggregation of fibrils and facilitating unfolding (denaturation) of the triple helix. All these structural modifications can alter the cell-binding affinity of collagen.

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Little is known about the effects of proteolytic and conformational modifications and supramolecular arrangement on the adhesive properties of ECM proteins [7-9]. Here we report that proteolytic cleavage of telopeptides of native type I collagen markedly decreased its ability to promote adhesion of mouse hepatocytes. The data indicate that telopeptides are not involved in hepatocyte binding to type I collagen. Experiments with oligomeric and monomeric collagens, isolated from the intact native preparations, showed that hepatocytes attached more efficiently to collagen in supramolecular assemblies than to collagen preparations consisting of monomers. This observation provides direct evidence that disassembly of supramolecular forms is responsible for the diminished hepatocyte adhesion to the telopeptide-devoid collagen. Heat denaturation had no effect on hepatocyte adhesion to intact collagen but, in fact, restored the hepatocyte-binding capacity of telopeptide-devoid substrates. This finding is dicussed in the light of the observation that denaturation leads to unmasking of integrin-specific, RGD-containing cell-binding sites, hidden in the triple helix and not normally accessible for hepatocyte binding to native substrates.

MATERIALS AND METHODS

Reagents. Acid-soluble type I collagen was purified from rat tail tendon by two cycles of solubilization-salting-out followed by DEAE-chromatography as described [10]. The preparations did not contain detectable amounts of noncollageneous impurities, as demonstrated by SDS-PAGE (Figure 1) and the fact that about 98% of the protein was degraded by highly purified bacterial collagenase. Cleavage of telopeptides was accomplished by treatment of the protein, dissolved in 0.5 M acetic acid, pH 2.5, with pepsin (Calbiochem) at an enzyme-substrate ratio of 1:10 (w/w) at 10 °C for 15 hours. Denaturation of collagen was performed by heating the protein solutions (100 μg/ml) in phosphate buffered saline (PBS; 60 °C, 20 min.) followed by rapid cooling. Separation of the intact pepsin-untreated collagen (further referred to as "crude collagen" [11]) into oligomeric and monomeric fractions was achieved by partial fibril assembly as described [11]. Both fractions, as well as the crude and pepsin-treated collagens, retained their triple-helical conformation, as seen from their thermal hyperchromicity profiles (not shown). Anti-collagen antisera were raised in rabbits by immunization with crude collagen, and anti-collagen IgG was isolated by Protein A-Sepharose chromatography. An ELISA assay, using crude and telopeptide-devoid collagens as antigens, showed that the IgG was mainly anti-telopeptide antibodies, corroborating the known immunogenic characteristics of type I collagen [12]. GRGDTP- and GRGESP- peptides were gifts from Dr. E. Ruoslahti. Cl. Histolyticum collagenase for hepatocyte isolation (Cat N 17449) and OGGR-peptide were from Serva Fine Biochemicals. Highly purified preparations of bacterial collagenase free from detectable amounts of non-specific proteases, were provided by Dr. N. Solovyeva.

Hepatocyte preparation. Mouse liver was washed free of blood by in situ pumping 15 ml PBS, containing 0.5 mM EGTA, through the lower caval vein, with the portal vein being cut, at a rate of 5 ml/min. Then 50 ml of the culture medium (medium 199) supplemented with 20 mM HEPES, pH 7.4, 5 mM $CaCl_2$ and 200 μg/ml of bacterial collagenase was perfused at a rate of 7 ml.min. The rest of the procedure was as described [13]. Preparations containing about 90% of viable cells, as judged by the trypan blue exclusion test, were used for adhesion experiments.

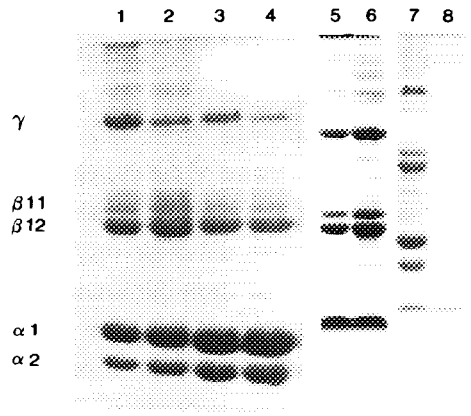

Figure 1. SDS-PAGE of the different type I collagen preparations. Slab gels with 4% (1- 6) and 7.5 % (7, 8) polyacrilamide were used. Approximately 70-100 μg of collagen was applied to each lane. The gels were stained with Coomassie Brilliant Blue R-250. Collagens: 1, intact (crude); 2, heat-denatured; 3, telopeptide-devoid; 4, telopeptide-devoid and heat-denatured; 5, monomeric; 6, oligomeric; 7, crude incubated at 37°C for 90 min without collagenase; 8, the same as in 7 incubated with 10 μg of *Cl. Histolyticum* collagenase free from nonspecific proteases. Lanes (1-4), (5,6), and (7,8) represent different slab gels.

Cell attachment assay. Ninety six-well microtiter plates were coated with varying concentrations of different collagen preparations in PBS. After protein coating, the wells were blocked with bovine serum albumin (BSA, 2.5 mg/ml) in medium 199. Hepatocytes were seeded in this medium at 20,000 cells per well. After 1 hour at 37 °C the wells were washed, and the attached cells were lysed by 0.25% Triton X-100. Tthe hexosaminidase activity in the lysates, as determined by a fluorogenic method [14], was taken to be a measure of cell attachment.

Results And Discussion

In our first studies on hepatocyte adhesion to the type I collagen substrates, we found that removal of telopeptides from native collagen, accomplished by pepsin treatment, markedly decreased the protein cell-binding capacity (Figure 2). One could suppose that the diminished cell adhesion to the telopeptide-devoid collagen reflects the presence on hepatocytes of a receptor(s) with specific affinity to the telopeptides. To test this hypothesis, adhesion assays were performed in the presence of anti-collagen IgG, mainly represented by anti-telopeptide antibodies. This IgG had only a slight inhibitory effect on hepatocyte attachment to both crude and pepsin-treated collagens (data not shown). Therefore, telopeptides are unlikely to be involved in hepatocyte binding to type I collagen. To date, only limited information is available concerning the role of triple helical and nonhelical domains in cell interactions with different collagens. The major, pepsin-generated, triple-helical domain of type IV collagen is twice as active in supporting endothelial and melanoma cell adhesion as the nonhelical NC1 domain [15, 16]. Other studies show the NC1 domain has no cell binding capacity [17].

Figure 1 demonstrates that removal of telopeptides led to the virtually complete disapperance of high molecular weight collagen associates (i.e. with Mr higher than that of gamma-bands), which comprise supramolecular aggregates, cross-linked by the intermolecular covalent bonds located in telopeptides [6, 11]. These data suggested that dissociation of the collagen aggregates may be the principal cause of the decreased hepatocyte-binding activity of telopeptide-devoid collagen. To test this idea directly, hepatocyte adhesion was assessed using two native collagen substrates isolated from the crude preparations: (i) oligomeric collagen, consisting of those monomers involved in fibril formation; and (ii) monomeric collagen, comprising the monomers not involved in fibril formation [11]. Figure 1 demonstrates that oligomeric and monomeric collagens have virtually the same patterns of electrophoretic bands as the crude and telopeptide-devoid collagen, respectively. Importantly, crude and oligomeric collagens had roughly equal proportions of high molecular weight bands, while telopeptide-devoid and monomeric collagens were completely devoid of them. In order to rule out the possibility that monomeric collagen represents a fraction of truncated monomers with telopeptides cleaved off during the purification procedure, the integrity of the telopeptides was verified by near-UV spectroscopy [18]. The data (not shown) indicate that crude, oligomeric, and monomeric collagens all contained intact telopeptides, while the pepsin-treated preparations were devoid of them, as expected.

Figure 2 shows that the hepatocyte binding capacity of both crude and telopeptide-devoid collagens did not differ markedly from that of oligomeric and monomeric collagens, respectively. The absence of significant differences between crude collagen and oligomeric collagen is explained by the fact that the oligomeric fraction constitutes the major part (approx. 90%) of the crude preparations. In addition, the weaker cell adhesion to monomeric versus oligomeric substrates, both of which contain intact telopeptides, confirms the above conclusion that telopeptides themselves do not contribute to the binding of hepatocytes to type I collagen.

Figure 2 demonstrates that thermal denaturation of crude collagen did not substantially affect its hepatocyte-binding capacity, corroborating the previously published data [19, 20]. In contrast, denaturation of telopeptide-devoid collagen increased its cell-binding activity to the level characteristic of the crude substrate. Similar results were obtained with oligomeric and monomeric collagens (not shown). Recently it was shown that thermal-induced unfolding of the triple helix leads to the unmasking of the integrin-specific RGD-containing adhesive sites in type I collagen [7, 8, 20]. The involvement of integrins in attachment of various cell types to type I collagen has been demonstrated in a number of studies [8, 17, 21, 22]. It may be suggested, therefore, that the enhanced binding of hepatocytes to denatured telopeptide-devoid (monomeric) collagen is due to the unmasking of cryptic RGD sties.

Figure 3 demonstrates that the GRGDTP hexapeptide strongly inhibited hepatocyte adhesion to denatured substrates, but had no effect on native substrates. The control hexapeptide (GRGESP) and the unrelated OGGR tetrapeptide did not inhibit binding to any

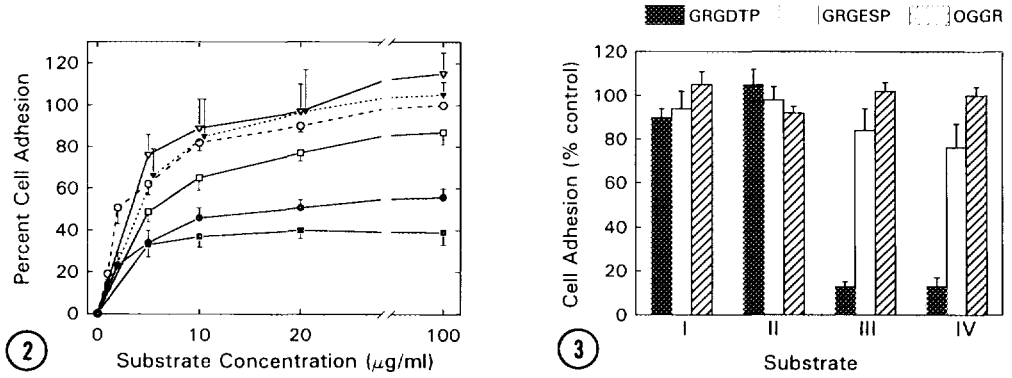

Figure 2. Mouse hepatocyte adhesion to different type I collagen substrates. Twenty to thirty thousand cells in medium 199 containing 2.5 mg/ml BSA were seeded in 96-well plates coated with collagens: (○) crude; (●) telopeptide-devoid; (▽) heat-denatured; (▼) telopeptide-devoid and heat-denatured; (□) oligomeric; (■) monomeric. The plates were incubated at 37°C for 60 min. Cell adhesion is given as percent of the amount of cells that attached to wells coated with crude collagen at a concentration of 100 μg/ml. Each point represents the mean (± SE) of 5-7 independent experiments with 6 replicates for each substrate concentration point in every experimental series.

Figure 3. Effect of synthetic peptides on hepatocyte adhesion to different type I collagen substrates. Hepatocytes ($2 \cdot 10^4$) were plated in wells coated with 100 μg/ml of the collagen substrates. Five hundred μg/ml of a peptide, dissolved in medium 199 containing 2.5 mg/ml BSA, was added to the respective wells. The plates were incubated at 37°C for 60 min. Cell attachment without peptides was taken as a control. The data are the means (± SE) of 4-6 independent experiments with 6 replicates for each data point in every experimental series. Substrates: I, crude collagen; II, telopeptide-devoid collagen; III, heat-denatured collagen; IV, telopeptide-devoid and heat-denatured collagen.

substrate. Thermal denaturation did not affect the integrity of telopeptides, as demonstrated by the finding that the native and denatured preparations had the same proportions of high molecular weight bands (Figure 1). Thus, the diminished cell-binding capacity of telopeptide-devoid substrates, resulting from dissociation of the supramolecular aggregates, was restored due to the exposure of previously hidden binding sites. However, the question arises why the denaturation of crude collagen did not increase its adhesive efficiency. We speculate that the supramolecular assemblies of type I collagen promote hepatocyte attachment much more actively if they retain their helical conformation, which is necessary for maintaining fibril integrity. Thus, denaturation of crude (oligomeric) collagen may result in decreased cell binding, but this decrease is compensated by unmasking hidden binding sites. The net effect would be unchanged binding.

In summary, our results indicate that: (1) hepatocytes possess a much higher affinity to the supramolecular assemblies of type I collagen than its monomeric forms; and (2) even slight degradation or conformational transitions of collagen can cause considerable modifications of its ligand behavior. An important problem of ECM studies is the role of cell-binding

alterations of its proteins in tumor invasion and metastases [4, 23]. In our preliminary experiments (unpublished), no differences have been found in attachment of rat hepatoma cells to the crude and telopeptide-devoid preparations of type I collagen. An intriguing question is whether the adhesion of hepatocytes and hepatoma cells to distinct collagen forms is mediated by the same or different cell surface receptors. Studies aimed at clarifying this question are in progress.

Another aspect concerns the role of ECM in modulation of cellular differentiation. A number of cell types, including isolated hepatocytes, exhibit a differentiated phenotype when grown in a 3-dimensional gel of type I collagen fibrils [24, 25]. The precise mechanisms underlying this phenomenon are still poorly understood. A higher cellular adhesion to fibrillar collagen may be an initial step in the chain reactions leading to cellular differentiation.

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