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A UV-spectroscopic study of the *in vitro* association of type I+III collagens with elastin solubilized peptides

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The recently described *in vitro* association between elastin or elastin solubilized peptides (ESP) and type I + III collagens has been previously proved by scanning and transmission electron microscopy and by histological studies. The use of a UV-spectroscopic method corroborates the identity of the reaction and enable us to be more precise about its mechanism. The collagen-ESP association is characterized by a 224 nm peak absorbance; the kinetic at this wavelength shows the same three characteristic phases as observed by turbidimetry curves for collagen fibrillogenesis. Spectroscopic studies concerning the effect of Ca^{2+} on the reaction reveal that its presence decreases the lag phase and increases the speed of the growth phase; without Ca^{2+}, collagen can self-react but with a smaller amplitude. Our investigations confirm that, when present, ESP plays a conclusive role in collagen organization.

1. Introduction

Artificial connective matrices such as collagen membranes are currently used in a broad range of applications. These reconstituted matrices are increasingly applied as support for cell culture in-so-far-as connective tissue components influence cell adhesion, cell morphology and differentiation [1]. They are also developed for biomedical practice as wound healing inducing materials [2], skin substitutes [3], vascular prosthesis [4], etc.

However, their reconstitution *in vitro* also provides a useful method to study the physiological interactions between their components: elastin, collagens, proteoglycans, etc. For this reason the conditions for collagen fibril formation have been investigated by turbidimetric measurements and electron microscopic studies [5].

We have previously described a new reaction between elastin or elastin solubilized peptides (ESP) and type I + III collagens [6]. This (probably) polar association yields a sort of connective matrix which can be markedly improved by the addition of fibronectin (FN), laminin (LN), and type IV collagen (C IV). After the addition of heparan sulfate (HS), the material has a composition and structure very close to that of natural arterial subendothelium (Fig. 1).

With scanning electron microscopy, we observed the tight association between both components [6]. Transmission electron microscopy corroborated these observations : type I + III collagens were aligned and oriented along the elastin fibres (Fig. 2). Histological studies with trichrome staining showed the same arrangement of collagen fibres with ESP [7].

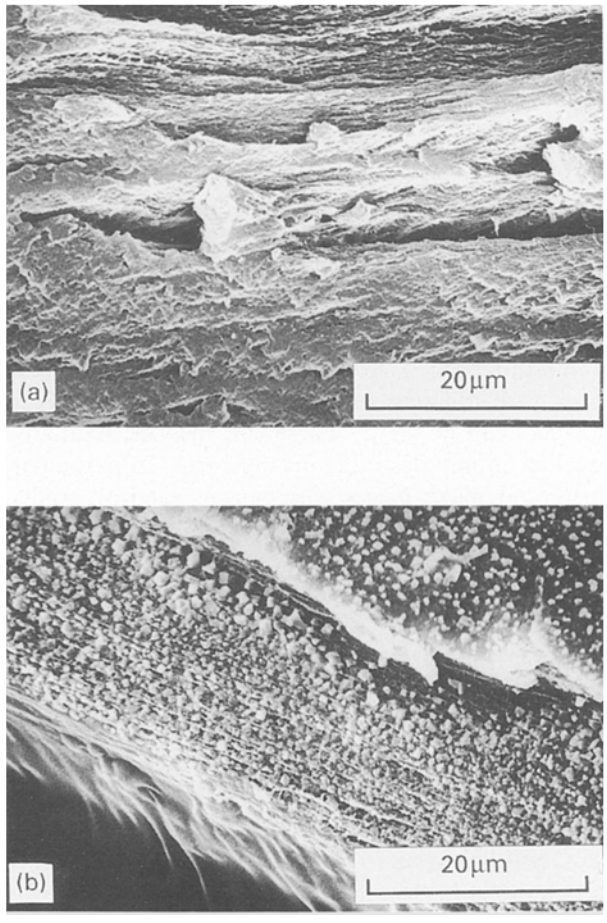

Figure 1 Scanning electron micrographs of the ESP–collagen material in the presence of fibronectin, laminin, type IV collagen: (a) without; (b) with proteoglycans. Note the more regular lamellar structure in the presence of proteoglycans.

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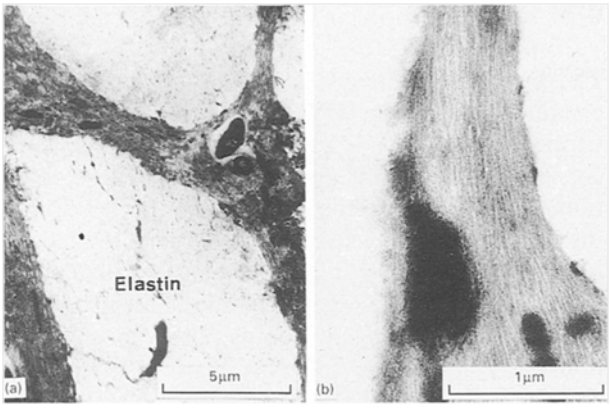

Figure 2 Transmission electron micrographs of the gel made of bovine elastin and human type I + III collagens. Collagen fibres are aligned and oriented along elastin (opaque to electron)

In the present study, we report on the use of a UV-spectroscopic method to investigate the *in vitro* behavior of type I + III collagens with ESP and to determine the biochemical conditions of their association.

2. Materials and methods

2.1. Bovine ligamentum nuchae elastin

This was obtained from Sigma (St Louis, MO, USA).

Elastin solubilized peptides (ESP) were prepared by alkaline hydrolysis according to a modification of the M.P. Jacob and W. Hornebeck method [8]. Briefly, 1 g elastin was suspended in 50 ml t-butanol (Merck) and gently stirred; 50 ml of 1 M KOH aq. solution were added and the suspension was stirred until dissolution (48 h at 25 °C); 50 ml of water was then added and the clear solution was neutralized with acetic acid. The resulting solution was dialysed overnight. Elastin peptides were lyophilized (Rdt = 80–90%). When used, ESP were dissolved in PBS, 10 mg ml^{-1}.

2.2. Collagens

Type I + III collagens (10 to 30% of type III) were prepared by Institut Mérieux (Marcy l'Etoile, France), from human placenta or by Coletica (Lyon, France) from placenta or young bovine skin, using the classic pepsic digestion process followed by saline precipitation.

2.3. Fibronectin, type IV collagen, heparan sulfate

These were obtained from Institut J. Boy (Reims, France).

2.4. Laminin

Isolated from basement membrane of Englebreth-Holm-Swarm mouse sarcoma; this was obtained from Sigma (St Louis, MO, USA).

2.5. Buffers

PBS: 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH = 7.4.

MTPBS: 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH = 7.4.

2.6. UV-absorbance spectra

UV-absorbance spectra were recorded with a Beckman, model DU 8, spectrophotometer. Measurements were made at 22 °C in a quartz cell with a light patch of 1 cm at a speed of 20 nm min^{-1} and between 200 nm and 300 nm. The UV-absorbance spectra of a mixture of equal concentrations of collagen and ESP ranging from 0.1 to 1 mg ml^{-1} were carried out in PBS with or without incubation at 37 °C.

2.7. Spectroscopic analysis and kinetics of collagen/ESP interactions

Measurements were taken at 224 nm, at 1 min intervals with a Kontron, model Uvikon 45, spectrophotometer equipped with thermostated cell. Optical density was plotted as a function of time. The optimal collagen/ESP ratio, temperature, pH and Ca^{2+} effects and kinetic of the reaction were analysed.

2.8. Preparation of ESP-collagen material for histological study

To a solution of 1 ml ESP, 10 mg ml^{-1}, 0.6 ml of PBS and 0.4 ml of type I + III collagen solution, 10 mg ml^{-1}, were added successively. The mixture was homogenized with a Vortex, then the reaction was carried out at 37 °C and stopped after 30 min by centrifugation at 6000 rpm for 15 min. The pellets were washed in 2 ml PBS, air dried and processed for optical microscopy studies.

3. Results

3.1. UV-absorbance spectra

The UV-absorbance spectrum of the collagen shown in Fig. 3a, reflects the low frequency of aryl aromatic amino acids. For the ESP spectrum (Fig. 3b) the multiple peaks are characteristic of absorbance bands of phenylalanine, desmosine (256–268 nm) and tyrosine, isodesmosine (275 nm) [9, 10]. They are reproducible but too weak to be used to follow the interaction between ESP and collagen,

Through our experiments, we showed that the UV-absorbance spectrum of collagen and ESP mixture without incubation is the result of addition of spectra of both components. Fig. 4 shows the difference between the UV-absorbance spectra of the mixture with or without 1 h incubation at 37 °C.

The maximum absorbance observed at 224 nm in the difference spectra characterized the change in the surrounding of the peptide bonds revealed by the reorganization of the collagen and the ESP in the presence of each other [11]. However, a contribution of diffusion phenomenon can be observed. No modification was observed in the UV-absorbance spectra of collagen and ESP after 3 h incubation at 37 °C.

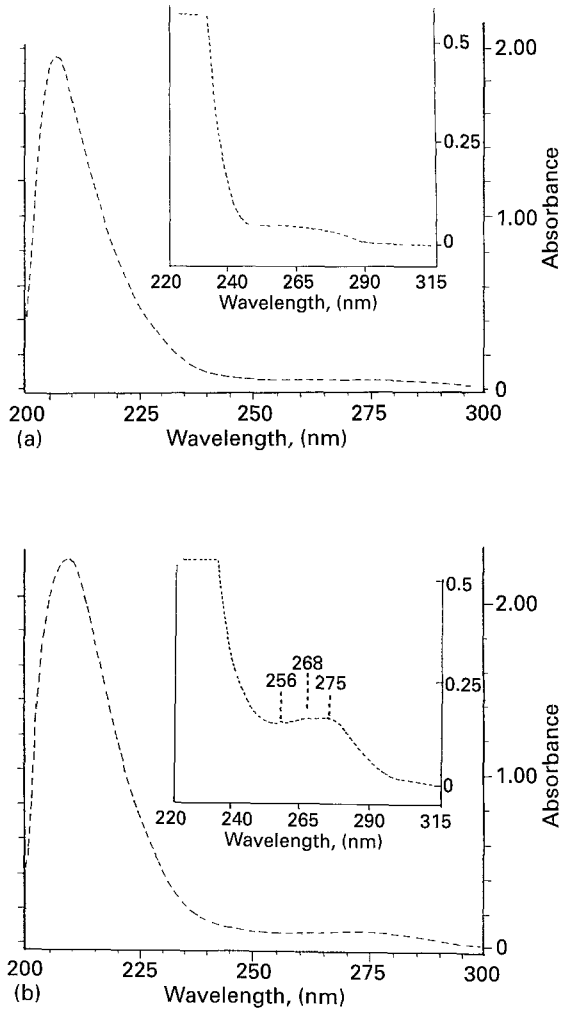

Figure 3 UV-absorbance spectrum of collagen and ESP: (a) Absorbance spectra were obtained for the collagen solution at a concentration of 200 $\mu g\,ml^{-1}$ in PBS without incubation at 37 °C. The insert shows the UV-absorbance spectrum of collagen for a fixed span (0.5 units of optical density); (b) absorbance spectra were obtained for ESP solution at a concentration of 200 $\mu g\,ml^{-1}$ in PBS without incubation at 37 °C. The insert shows the UV-absorbance spectrum of ESP for fixed span (0.5 units of optical density). Vertical dashed lines indicate the wavelength values of the peak corresponding to the aryl aromatic aminoacids.

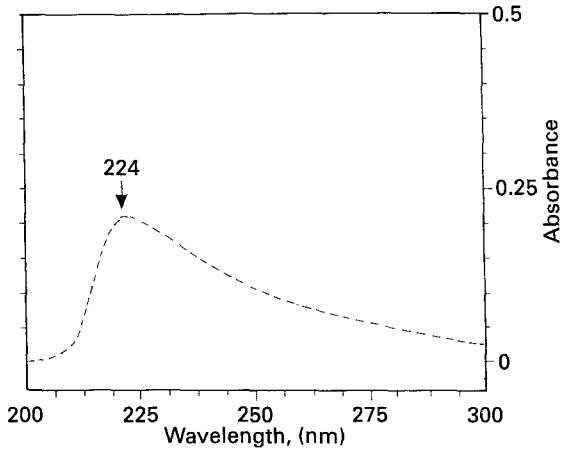

Figure 4 Effect of incubation at 37 °C on the absorbance spectra of the ESP and collagen mixture. Difference UV-absorbance spectrum of the ESP (100 $\mu g\,ml^{-1}$) and collagen (100 $\mu g\,ml^{-1}$) mixture obtained by subtraction of the absorbance spectrum of the ESP, incubated for 1 h and collagen mixture minus the absorbance spectrum of the same mixture without incubation.

3.2. Kinetic properties of the collagen–ESP interactions

The kinetics of collagen and ESP interaction were examined directly in UV-absorbance at a constant temperature of 37 °C (Fig. 5). The characteristic curve consists of a lag phase which is defined by the projection of the tangent to the point of greatest $dO.D/dt$ to the baseline and which corresponds to a low absorbance change phase; a growth phase with a rapid increase in the optical density; and a plateau with no absorbance change [12]. The amplitude of the reaction was given by the difference between the plateau and the baseline values.

This characteristic curve may be observed by using collagen alone in MTPBS [13–15] but the amplitude of the reaction was very low compared to the amplitude of the reaction with the collagen and ESP mix (Figs 5 and 6). The addition of ESP during the plateau of the collagen kinetic was followed immediately by a growth phase and a plateau. The total amplitude of this reaction was the same as the amplitude of the collagen and ESP mix reaction. No change was observed in the collagen control when PBS was added in place of the ESP to the same concentration as

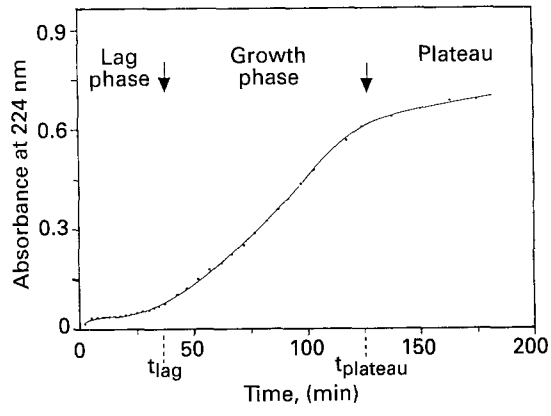

Figure 5 Time course of collagen–ESP reaction in PBS at 37 °C. The reaction was monitored at 224 nm. The vertical arrows indicate the lag phase, the growth phase and the plateau. The baseline was arbitrarily set at 0.

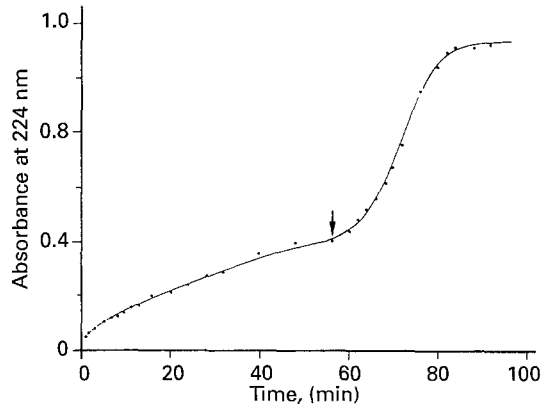

Figure 6 Effect of the addition of ESP on the time course of the collagen fibril formation. The ESP (100 $\mu g\,ml^{-1}$) were added during the plateau. as indicated by a vertical arrow. The reaction was carried out in MTPBS at 37 °C, with 100 $\mu g\,ml^{-1}$ of collagen.

previously described. Similarly, ESP were unable to self-react.

3.3. Effect of the Ca^{2+} on the kinetic of collagen–ESP interactions

The binding of Ca^{2+} by elastin has been previously reported [16]. The presence of 2 mM of Ca^{2+} resulted in a lag of the collagen and ESP interaction (Fig. 7a). Removal of Ca^{2+} by using MTPBS greatly reduced the reaction lag (Fig. 7b). Addition of the chelator in MTPBS gave rise to a similar kinetic as MTPBS alone (data not shown). Moreover, the absence of Ca^{2+} in MTPBS was able to induce collagen interactions without ESP, but with small amplitude (Fig. 7c).

3.4. Structural studies

We have previously described collagen as been able to react without ESP, in the absence of Ca^{2+}, and we have already shown that histological studies with trichrome staining exhibit the orientation of type I + III collagens along elastin fibres. Therefore, in order to investigate the reality of the reaction between collagen and ESP, we analysed the following products by histological techniques: the ESP–collagen membrane and the collagen product in the absence of Ca^{2+}.

Our results showed alignment and orientation of the collagen fibres in the presence of ESP. In the absence of ESP, the arrangement of the fibres appears to be more irregular (Fig. 8a, b).

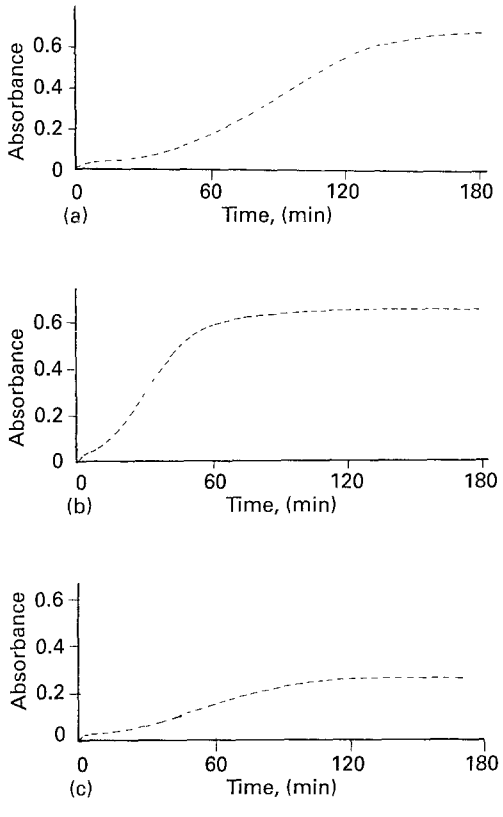

Figure 7 Kinetics of collagen fibril formation in different conditions: (a) presence of 2 mM Ca^{2+} and 100 $\mu g\,ml^{-1}$ ESP, (b) absence of Ca^{2+}, presence of 100 $\mu g\,ml^{-1}$ ESP; (c) absence of Ca^{2+} and ESP.

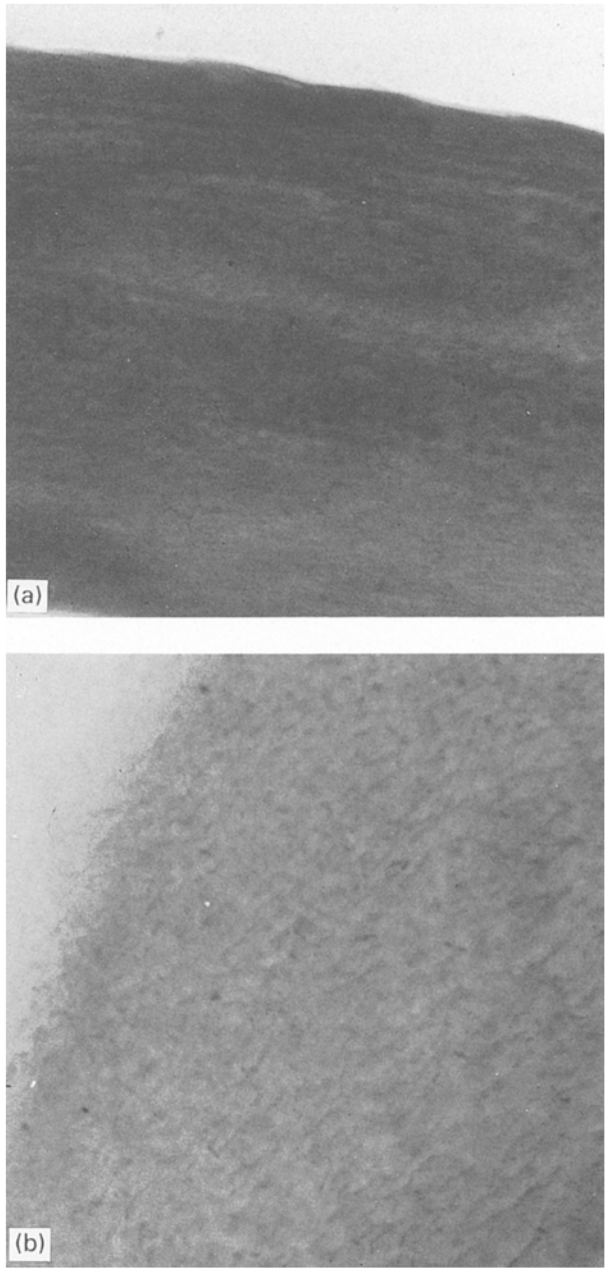

Figure 8 Histology of: (a) the material made of 10 mg ESP and 6 mg type I + III collagens; (b) the product obtained with 10 mg type I + III collagen in the absence of Ca^{2+}. In the presence of ESP, collagen fibres are aligned; in their absence, collagen fibres do not exhibit this parallel orientation.

4. Discussion

A UV-spectroscopic approach to *in vitro* collagen fibrillogenesis using matrix components (collagen and ESP) derived from two connective tissues was studied in this work. We also studied the effect of different parameters including ESP concentration, pH and Ca^{2+}, on the collagen–ESP reaction.

We aimed to determine the optimal conditions for fibril and matrix formation *in vitro* and the qualities of the resulting synthetic biomaterials for clinical applications.

Numerous reports based on turbidimetric studies have been published on *in vitro* collagen self-assembly and fibril formation. However, in this analysis, we have shown that collagen–ESP association was

characterized by a UV-absorbance peak at 224 nm corresponding to surrounding changes in peptidic bonds during the rearrangement of the collagen. The kinetic of the collagen-ESP interaction in different experimental conditions was investigated at this wavelength.

Turbidity curves were shown to consist of three phases: an initial lag phase with no change in absorbance, a growth phase in which the turbidity rapidly increases and a plateau where there is no further change in optical density. We observed that the kinetic curves obtained from collagen–ESP reaction at 224 nm show the three characteristic phases as described above but significant differences may also be observed: (1) a decrease in the duration of the lag phase; (2) a decrease in the rate of the growth phase; (3) an increase in amplitude of the optical density.

The UV-spectrophotometric analysis at 224 nm is more sensitive than turbidimetric measurement. Whereas an increase in turbidity corresponds to an aggregation of molecules in a non–homogenous medium, an increase of optical density at 224 nm reveals a reorganization of the collagen at the molecular level.

We also studied the effect of calcium on the rate of collagen–ESP reaction. The curves obtained in the absence of calcium cations exhibit a decrease in the lag phase, followed by an increase of the speed of the growth phase; no change in the amplitude of the reaction was observed. From our observations concerning the effects of calcium on the kinetic curves, we conclude that only free Ca^{2+} cations may regulate the rate of collagen–ESP association.

We described collagen as being able to self-react in the absence of calcium, to a weaker extent than the ESP and collagen mix did, and we showed that this collagen could still react with the same total amplitude after addition of ESP.

Histological studies also revealed that the presence of ESP is necessary for collagen fibril organization. Electron microscopy observations of samples taken during the plateau showed the same regular organization. Therefore, this led us to believe that, in the first phase of the reaction, collagen underwent a partial reorganization with removal of Ca^{2+} by binding with ESP, by charge exclusion or neutralization. In a second step, ESP may play a more important role in collagen association leading to the parallel organization of collagen fibres.

Consequently, histological studies, investigations using labelled ESP or collagen, electron micrographs and also UV-spectroscopic studies, demonstrated the tight association between type I + III collagens and elastin, or better still solubilized elastin.

The yielded product was improved by the addition of connective proteins and proteoglycans which produced a connective matrix whose structural appearance and composition is very close to that of natural subendothelium. Moreover, this matrix exhibited non-thrombogenic properties and the capacity to promote endothelial cell cultures, maintaining their phenotypic expression [13].

These results prompted us to investigate the ability of ESP to be covalently linked to synthetic lattices (Dacron®, Vicryl®,...), the yielded product retaining the capacity to bind collagen. Thus, this connective biocomposite tissue enables us to elaborate original vascular prostheses, now in progress in our laboratory [17].

This UV-spectroscopic approach used to study *in vitro* association of collagen fibres illustrated the interaction between the two main components of the connective tissues: elastin and collagens. The histological studies revealed an interaction which is markedly influenced by addition of connective proteins and glycosaminoglycans. Now, investigations are in progress in our laboratory using this UV-spectroscopic method. with a view to determining the role and the importance of each component: fibronectin, laminin, type IV collagen, heparan sulfate, especially their order of addition.

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