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Fibroblast changes in cutaneous ageing

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Summary. With ageing there are progressive modifications in the connective tissue of the dermis. In ten young subjects the collagenous bundles are thick and the fibroblasts are active cells in close contact with collagen fascicles. In ten elderly subjects collagen fibres are fragmented and the fibroblasts are quiescent, without any contact with collagen.

In ageing the most important lesion is the destruction of the relationship between fibroblasts and interstitial matrix. A role for fibronectin in this adhesion is suggested: in old subjects the papillary network of fibronectin is poorly developed. Furthermore, in the fibroblast we can see architectural changes in the cytoskeleton; this modification breaks up the cytoskeleton – plasma membrane – fibronectin unit and explains the secretory and metabolic changes observed in ageing, the dysfunction of the cell-interstitial matrix unit, and also the structural changes.

Key words: Fibroblast – Ageing – Dermis

With ageing, major changes can be seen within connective tissue components of the dermis. Collagen loses its regular and fascicular appearance, ground substance increases, elastic material decreases and fibroblast populations become "at rest" (Bouissou et al. 1970, 1971 and 1973). The resulting dermal ageing is different in different individuals and is related to genetic background and exposure to multiple insults (Golstein 1979; Hayflick 1974; Robert and Robert 1974).

As a part of previous investigations on dermal ageing, we have studied the morphological changes of dermal fibroblasts in an attempt to determine the role and the relevance of these cells in the "fibroblast interstitial matrix" adhesiveness and consequently in the conservation of connective tissue integrity. The techniques used included light microscopy, transmission and scanning electron microscopy, and immunohistochemistry, the latter utilizing an anti-fibronectin antibody.

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Material and methods

Excisional skin biopsy specimens were obtained from a total of 20 subjects from two groups: group 1, ten subjects aged 10–25; group 2, ten subjects aged 60–75. Biopsies were performed on autopsy material in order to avoid alterations or modifications of the dermis that might have been related to anaesthesic procedures. The biopsy specimens were taken in the autopsy room not later than 2 h post mortem. Immediately after excision (internal part of the arm) each specimen was prepared for light microscopy, transmission and scanning electron microscopy. Six subjects (three in each group) were examined by immunofluorescence.

Light microscopy. Tissue was fixed in Bouin's solution, embedded in paraffin, sectioned and stained with haematoxylin-eosin-saffran and Masson's trichrome. Sections were also stained using the Sirius red technique (Junqueira et al. 1978 and 1979) and examined with polarized light in order to evaluate the general distribution of type I and type III collagens. Verhoeff's technique for the demonstration of elastic fibres was also used.

Immunofluorescence. The anti-fibronectin antibody used in this study was supplied by l'Institut Pasteur de Lyon, France (Dr. J.A. Grimaud). This antibody was produced in rabbits with purified human fibronectin according to the technique described by Dessau et al. (1978).

Specimens were fixed immediately in liquid nitrogen and stored in a deep-freeze at −80° C. To establish a correlation between histological and immunofluorescence results, frozen sections were also stained with toluidine blue. Frozen sections, about 8 μ thick, were treated for 30 min with fibronectin antibody, washed with PBS and then treated with anti-rabbit gamma-globulin antibodies conjugated with fluorescein-isothiocyanate (Institut Pasteur, France). All specimens were washed in PBS, mounted in buffered glycerol and examined with a Leitz microscope equipped for immunofluorescence.

Electron microscopy. Each biopsy specimen was sliced into small fragments and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. The tissue was postfixed in 1% osmium tetroxyde, dehydrated with graded alcohols and embedded in Epon 812. Sections 0.5 μ thick were stained with toluidine blue. From appropriate areas papillary dermal ultrathin sections were cut with a Reichert O M U 2 microtome, stained with uranyl acetate and lead citrate, and examined using with the Hitachi 300 transmission electron microscope. Five different fragments were examined in each biopsy, and a mean of fifteen fibroblasts were observed in each subject.

Samples for observation by scanning electron microscope were dehydrated in graded acetone solutions and dried with liquid CO_2 by the critical point method. The specimens were coated with a 20-nm layer of gold and examined with a Jeol T 200 scanning electron microscope.

Results

Our study focused on changes in the papillary dermis, since the most striking changes associated with ageing are observed at this level. In fact, our previous studies have shown that mid- and deep dermis alterations are milder and less consistent. Thus, they will not be discussed in this study (Bouissou et al. 1970, 1971 and 1973).

Group 1: Dermis of young adults (aged 10–25)

By light microscopy, the papillary dermis of young adults showed the presence of well-developed elastic fibres arranged in a network, giving rise to numerous fibrils, which tended to attach to the basement membrane of the epidermis (Fig. 1 a). Collagen fibres were grouped in thick and compact fascicles which are typical of the so-called "skin type 0". In all instances,

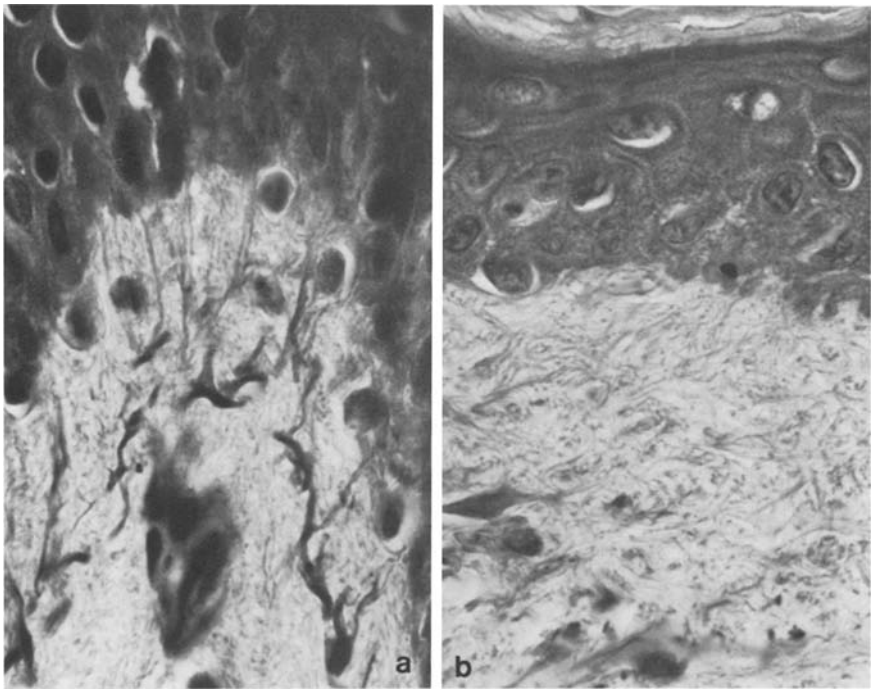

Fig. 1 a, b. Light microscopy. **a** Papillary dermis of *young adult*: "skin type 0". Elastic fibres arranged in a network Verhoeff stain. × 100. **b** Papillary dermis of *ageing adult*: "skin type II". Disappearance of the papillary elastic network Verhoeff stain. × 100

Sirius red staining showed yellow-orange or red fibres and a few thin green fibres. These observations visualized the two kinds of cutaneous collagen (type I stained in yellow – the most abundant – and type III in green).

By immunofluorescence, fibronectin was a predominant component of the basement membrane regions, around capillaries, and in the dermal epidermal junction, where some clusters are seen. In papillary dermis, fibronectin was shown to form a thin irregular network, associated with collagen bundles (Fig. 2a).

By scanning electron microscopy, groups of thick, regular and well-oriented collagen fibres were seen. They were lightly wavy and generally assembled in a parallel fashion. However, in the papillary dermis some bundles were thinner and more flattened (Fig. 3a).

Transmission electron microscopy revealed dense thick and voluminous collagen fascicles. Collagen fibres featuring a regular diameter with a characteristic periodicity of about 64 nm (Fig. 4a) and variable amounts of mature elastic fibres and cell extensions were interspersed between collagen fascicles. Fibroblasts were elongated and displayed a close association with collagen fibres. They were often enlarged and stellate, showing central nuclei with a prominent nucleolus (Fig. 5a). Their most notable characteristic was the presence of a well-developed rough endoplasmic reticulum and a prominent

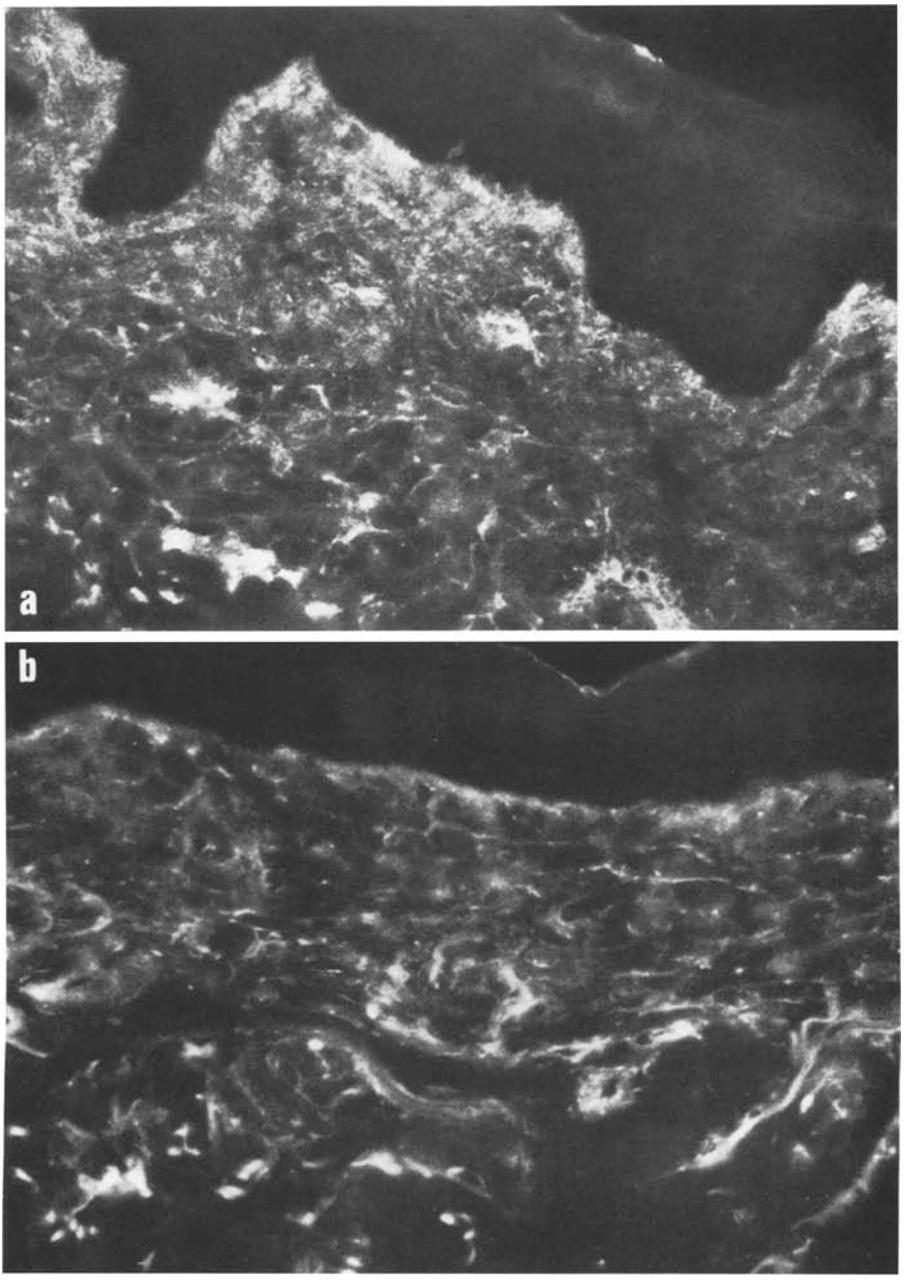

Fig. 2a, b. Indirect immunofluorescent labelling of the fibronectin. **a** *Young adult:* the fibronectin is abundant in the superficial dermis. × 40. **b** *Ageing adult:* the fibronectin is less abundant in the superficial dermis. × 40

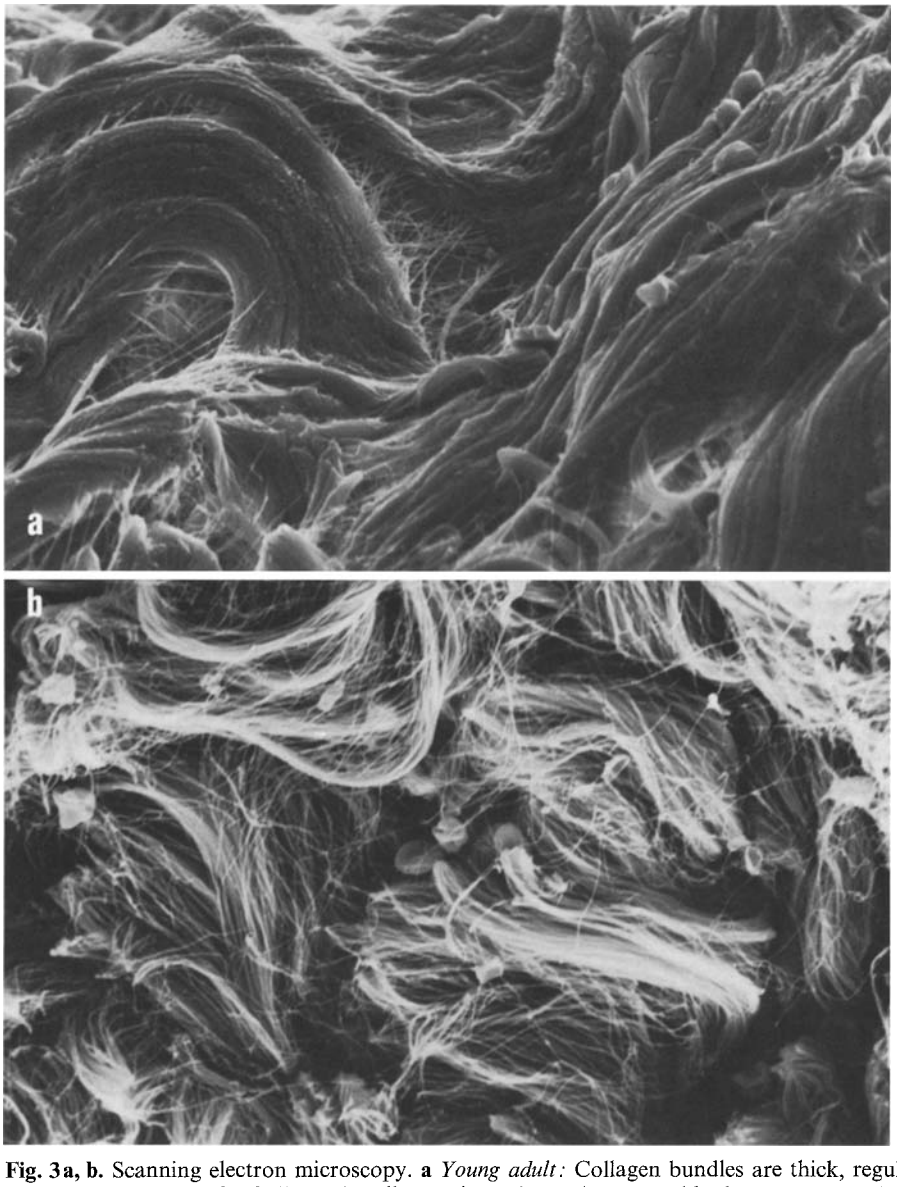

Fig. 3a, b. Scanning electron microscopy. **a** *Young adult:* Collagen bundles are thick, regular. ×1,000. **b** *Ageing adult:* Collagen bundles are irregular and separated by large spaces. ×1,000

Golgi complex. Variable numbers of mitochondria, ribosomes and polyribosomes were also seen. The amount of ergastoplasm appeared to parallel the secretory activity of the cell. The cytoskeleton of these cells was made of a fine network of microfilaments visible throughout the cytoplasm (Fig. 6a). They were grouped in small bundles at the periphery of nuclei, but were predominant in the vicinity of secretory areas. The main morpho-

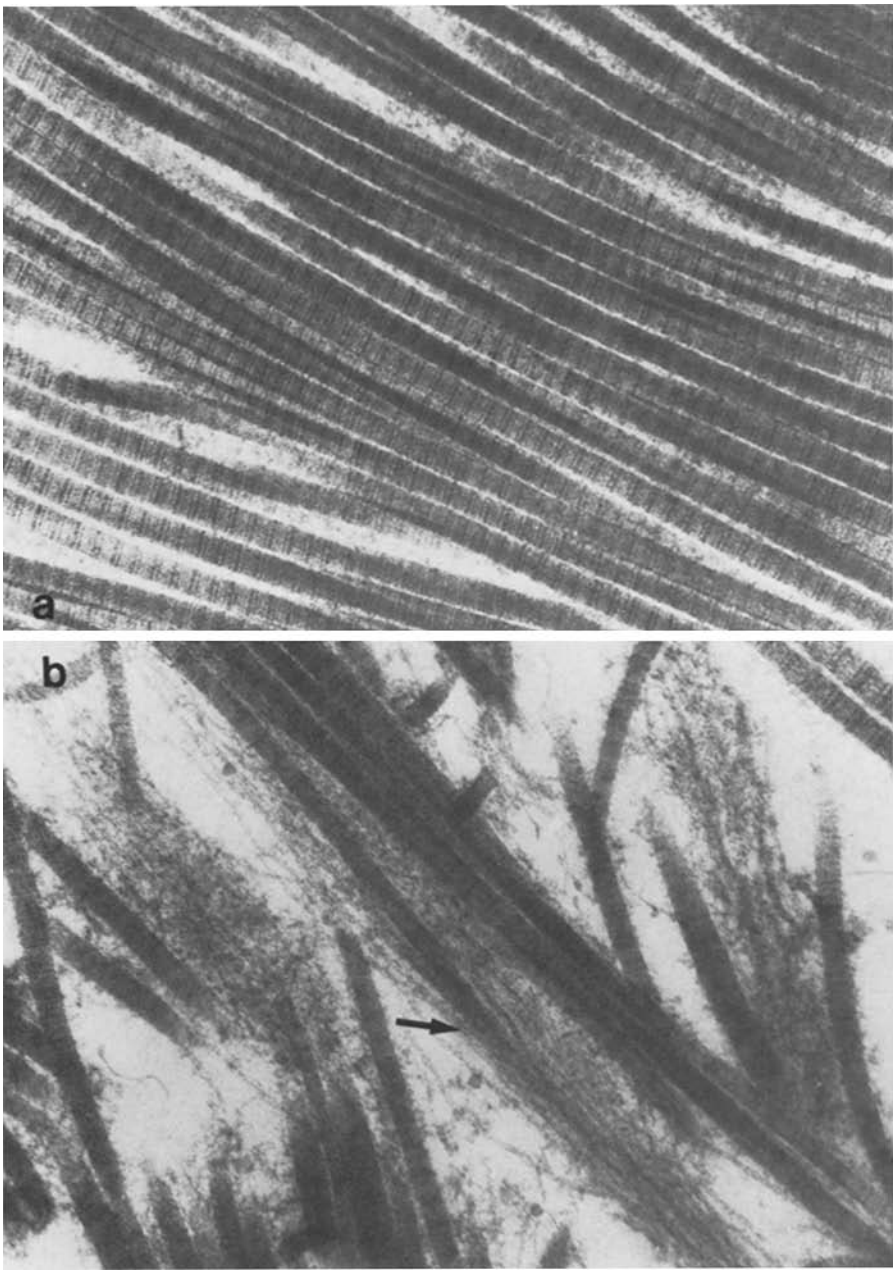

Fig. 4a, b. Transmission electron microscopy. **a** *Young adult:* Voluminous collagen fascicle, regular fibres with characteristic periodicity. Uranyl acetate-lead citrate stain. × 30,000. **b** *Ageing adult:* Thin collagen fascicle, one fibre with "bruched" aspect (arrow). Uranyl acetate – lead citrate stain. × 30,000

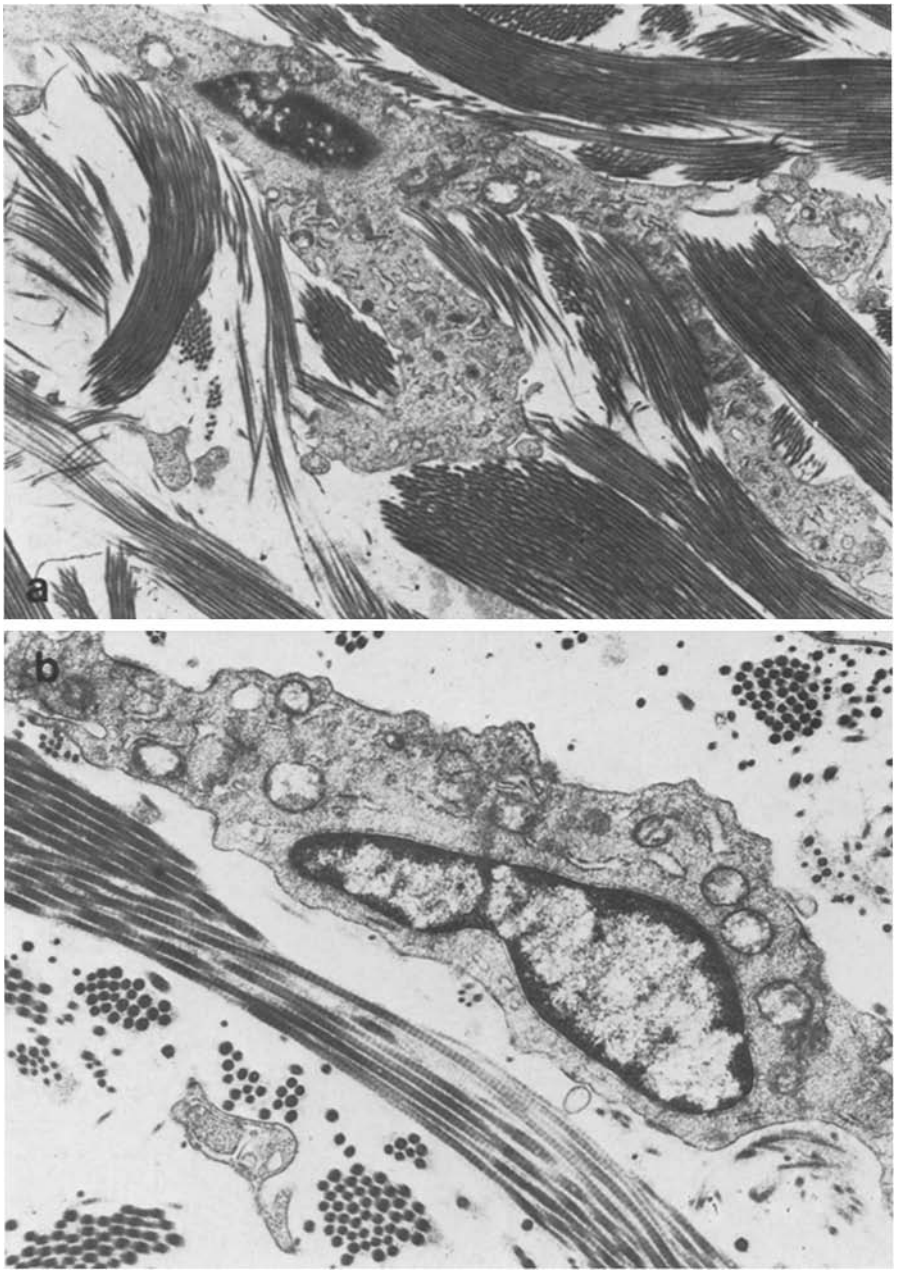

Fig. 5a, b. Transmission electron microscopy. **a** *Young adult:* Elongated, stellated fibroblast with close connection with collagen fibres. Uranyl acetate – lead citrate stain. × 5,000. **b** *Ageing adult:* Fibroblast is flattened and quiescent, without direct contact with collagen. Uranyl acetate – lead citrate stain. × 10,000

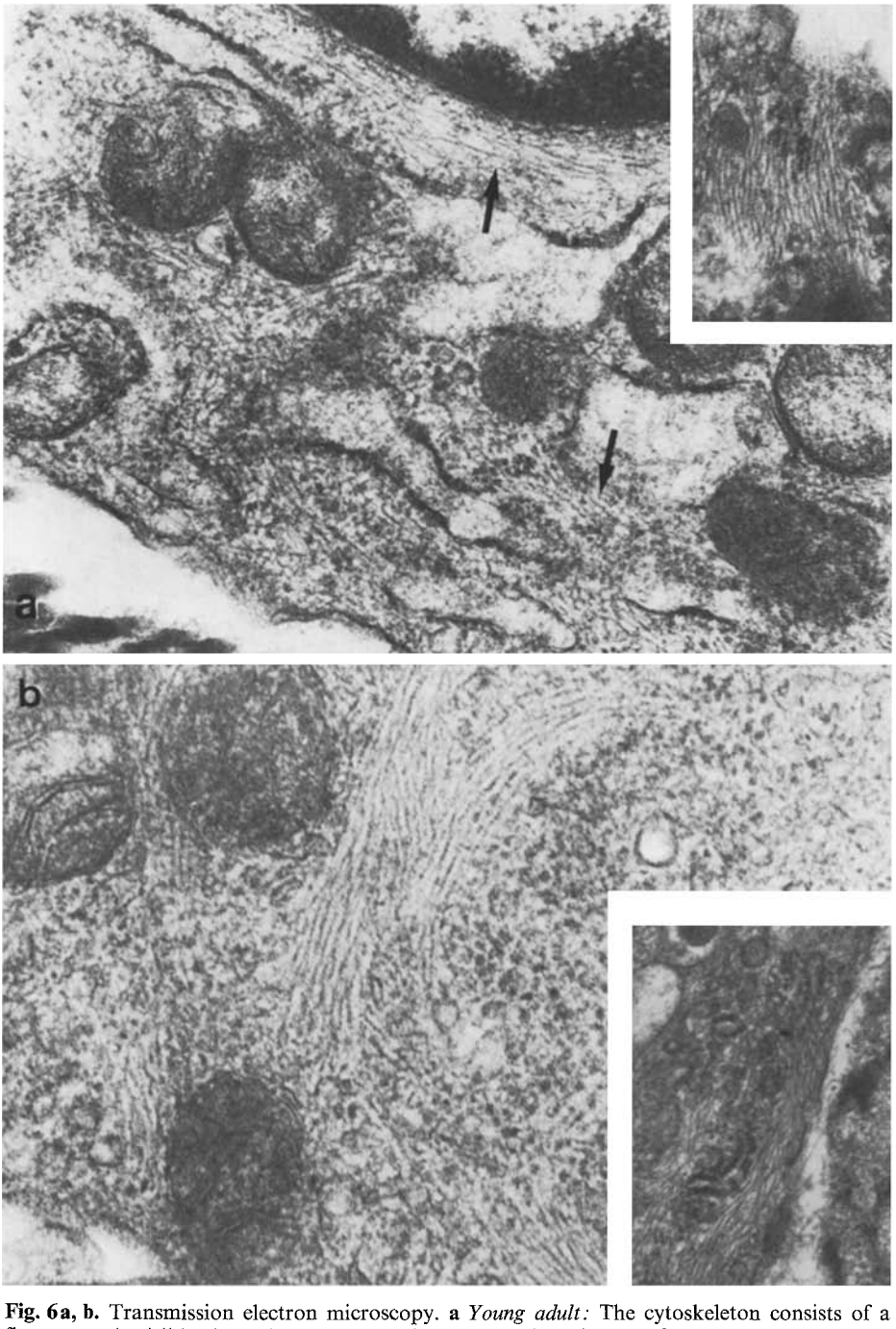

Fig. 6a, b. Transmission electron microscopy. **a** *Young adult:* The cytoskeleton consists of a fine network visible throughout the cytoplasm (*arrows*) and near of secretory areas (*inset*). Uranyl acetate – lead citrate stain. × 30,000. **b** *Ageing adult:* The cytoskeleton is characterized by voluminous bundles of filaments. Uranyl acetate – lead citrate stain. × 40,000. *Inset:* Fascicles without any contact with the plasma membrane. Uranyl acetate – lead citrate. × 20,000

Table 1. Cytoplasmic characteristics of fibroblasts in young and elderly subjects

Subjects	RE R	Cytoskeleton			Golgi	Dense bodies (lysosome)
		N	B	S		
Young (10)	+++	++	0	++	>1	<2
Elderly (10)	+	+	+++	0	1	>2

Abbreviations: RE R = Rough endoplasmic reticulum; N = Network of microfilaments through the cytoplasm; B = Fascicles or bundles of microfilaments through the cytoplasm; S = Short and small bundles of microfilaments in relation to secretory areas; In parentheses, the number of subjects; For Golgi and dense bodies the mean was calculated after enumeration of these organelles in all examined cells (10 in each biopsy)

logical characteristics of young fibroblasts and their semi-quantitative valuation are summarized in Table 1.

The abundance of compact collagen bundles and the presence of active secreting fibroblasts in close contact with collagen fascicles were the hallmark of the dermis of young individuals.

Group 2: Dermis of ageing adults (aged 60–75)

By light microscopy, the dermis of ageing subjects was characterized by the disappearance of the papillary elastic network and modifications of collagen. The collagen lost its typical fascicular pattern and became granular. These morphological changes corresponded to the so-called "skin type II" (Fig. 1b) and each specimen studied within this group showed a similar picture. The intense yellow-orange staining of the dermis by the Sirius red technique confirmed the predominance of collagen type I. Scanty amounts of type III collagen were also demonstrated as small fascicles in the upper part of the dermis. This is explained, in fact, by the looser consistency of papillary dermis (since it has been shown that there is no quantitative variation in the amount of collagen during the life-span (Gay et al. 1976; Meigel et al. 1977; Fleishmajer et al. 1980).

By immunofluorescence, the papillary network of fibronectin was less well developed, as revealed by immunofluorescence investigation using the antifibronectin antibody (Fig. 2b). In particular there was a decrease in papillary network. The fluorescence staining was only seen around capillaries, at the dermal-epidermal junction there was a thin border.

By scanning electron microscopy the superficial dermis appeared to be disordered. Collagen bundles were irregular, thin, and often separated by conspicuous interfibrillar spaces (Fig. 3b).

Transmission electron microscopy showed short and thin fascicles of collagen fibres, separated by empty spaces corresponding to ground substance. At higher magnification most of the fibres showed a normal structure with a characteristic periodicity. However, occasional numbers of fibres did not show the typical periodicity; moreover, their extensions appeared

to separate and form microfibrils, giving a "brushed" aspect (Fig. 4b). Elastic fibres were not seen in the papillary dermis. Fibroblasts were less elongated and flattened (Fig. 5). The cytoplasm was composed of moderate amounts of poorly developed rough endoplasmic reticulum, numerous dense bodies of the lysosomal type and frequent membrane-bound lipid vacuoles. The cytoskeleton was characterized by voluminous fascicles or bundles of microfilaments, which occupied large cytoplasmic areas, but showed no contact with the plasma membrane (Fig. 6b). The close association between collagen fibres and fibroblasts was not readily seen and consequently the cell population was isolated within the interstitial matrix. The morphological modifications and their semi-quantitative valuations are summarized in Table 1.

The fragmentation of collagen fibres, the absence of elastic material and the occurrence of quiescent fibroblasts without any direct contact with collagen fascicles represent the morphological changes of ageing dermis.

Discussion

The loss of fibroblast interstitial matrix adhesiveness and the architectural changes of the cytoskeleton of this cell constitute the most striking morphological observations of dermal ageing. They could explain in part the problems of protein metabolism commonly seen with ageing. The close interrelationship between fibroblasts and collagen (Amblard 1980; Robert 1980) and the important role of fibronectin in several functions mediating cell adhesion, cell-to-cell interactions and cell-matrix interactions are well known. The function of fibronectin as an "adhesion glycoprotein" (Fleischmajer et al. 1980b) or a molecular "glue" (Mc Donagh 1981; Fyrand 1979; Pena and Hughes 1978) was shown in recent studies that established the presence of a binding site between the alpha chain of collagen and one of the two polypeptide chains of fibronectin (Engvall and Ruoslahti 1977; Mosher and Furcht 1981; Ruoslahti et al. 1981). An "actin binding site" with strong affinity for actin was also found on the fibronectin chain (Keski et al. 1980; Ruoslahti et al. 1982).

In the present study, the immunofluorescent procedures with antifibronectin show a decrease in dermal fibronectin among subjects aged 60–75. To our knowledge, these changes in the cellular fibronectin distribution with ageing have not been reported with "in vivo" studies. However, in accordance with the age of the culture "in vitro", studies by Vogel et al. (1981) with cultures of fibroblasts showed a decrease in fibronectin at the cell surface. The loss of cell-matrix adhesion in elderly individuals associated with a decrease in fibronectin suggests that this glycoprotein is an important factor in maintaining the integrity of cell matrix.

Our ultrastructural findings revealed important morphological changes in the cytoskeletal architecture. Such variations have been described in cultured fibroblasts with the gradual ageing of the culture (Pena 1980). It is well known that the cytoskeleton integrity relies on ATP and the inhibitors of basic metabolism reduce the amount of cellular ATP (Bershadsky et al.

1979 and 1980) and favour the destruction of cellular cytoskeleton. Moreover, a decrease in oxygen consumption and ATP reduction are also observed during senescence. Consequently, knowing the essential role of cytoskeleton in the migration and the excretion of secretion, it can be postulated that the changes observed in aged fibroblasts could represent a deficiency in pro-collagen transport within the cell, an altered secretory process and finally a loss of close relationship with fibronectin. We have not focused our study on the alterations of plasma membrane. Since the interstitial fibronectin-cytoskeleton relations are induced by the membrane, it could be hypothesized that any change within the plasma membrane would affect these relations. Studies by electron microscopy performed on tissue cultures using the freeze-fracture technique have shown morphological variations of plasma membrane between elderly and young individuals (Kelley and Skipper 1977). It can then be postulated that these changes are also present "in vivo" during ageing.

As expected, our morphological findings in the fibroblastic cell population confirm previous investigations: rarefaction of cytoplasmic organelles, disappearance of elastic fibres, organization of collagen in small fascicles and increase in ground substance are phenomena characteristic of dermal ageing (Bouissou et al. 1973). They are in agreement with biochemical studies (Robert et al. 1972), (Maurel et al. 1980) which showed a decrease in the rate of collagenous protein metabolism, and in that of elastin and an increase in glycoaminoglycan metabolism. These observations confirm well-known data: with ageing there is a decrease in the turnover of collagen, whereas its amount remains invariable.

The observation of lipid vacuoles in the aged fibroblast may represent the morphological expression of a dysfunction in lipid metabolism. It has been already demonstrated that the fibroblast plays a role in the metabolism of lipoproteins (Brown et al. 1981). Therefore, with ageing this metabolism is modified and lipoproteins (particularly LDL) are stocked within dermis. Biochemical determination of cutaneous cholesterol and apoprotein B (Bouissou et al. 1981) and immunotyping procedures with anti-apoprotein B provided evidence of this lipoprotein accumulation within the dermis (Pieraggi et al. 1982).

The process of dermal ageing is probably related to a dysfunction of the cell-interstitial matrix unit, in which fibronectin, cytoskeleton and the plasma membrane are deeply involved. The resulting progressive morphological changes correlate with the alterations in fibroblastic metabolism, particularly the protein moiety. Even if we have a better understanding of mechanisms involved in the ageing of the cell and its dysfunction, we have to recognize that the indigenous agents to the inductive process are poorly known.

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