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ARRANGEMENT OF ELASTIC FIBRES IN THE INTEGUMENT OF DOMESTICATED MAMMALS

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Abstract

The hairy skin of important domesticated mammals (12 species) was studied with scanning electron microscopy, transmission electron microscopy, laser scanning microscopy, and several light microscopical methods, to obtain more information about three-dimensional elastic fibre arrangement. It was obvious that there is a basic construction scheme of the elastic fibre meshwork as present in the upper and mid-dermis, with special regard to the size, number, and grouping of hair follicles. In the densely-haired species, in particular, a typical elastic mat with horizontal fibres is formed. In many of the sparsely-haired animals, the upper and mid-dermis show a sponge-like elastic system. In the rather massive, collagen-rich skin of large species, the lower two thirds of the dermis without hair follicles only possess a loosely-structured elastic network, but thick elastic sheets are found at the border zone with the hypodermis.

Specific features appear with regard to the type of mechanical strain exerted, different body regions, varying hair follicle density, or as connected with the anchoring of the hair follicle complex, blood vessels, and nerves.

Key Words: Elastic fibres, hairy skin, domesticated mammals, fibre arrangement, dermis, elastin.

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Introduction

In vertebrates, elastic fibres are present as highly branching structures in the connective tissues of various organ systems. In the integument (as best known from mammals, particularly humans) such fibroelastic elements are concentrated in the dermis, where they are closely interwoven with collagen fibres that form the bulk of the skin. In this way, the dermis is specifically constructed to resist mechanical strain, whereby the elastic fibres with their high capacity of expansion and relaxation ('rubber-like') are responsible for the physiological elasticity of this skin layer. This implies that the dermis permits large elastic deformations by requiring only low forces. Nevertheless, it can have a high tensile strength due to its collagen component (for literature cf. Ross, 1973; Gosline, 1978; Daly, 1982; Sage, 1982; Sandberg *et al.*, 1982; Ryhänen and Uitto, 1983; Meyer *et al.*, 1989).

The biomechanical properties of the dermis have been shown to be directly correlated with the architectural arrangement of the fibrous tissue involved (Daly, 1982). In this context, it has to be admitted, however, that relevant methods to visualize the specific patterns and three-dimensional distribution of the elastic fibre network are rather rare. Conventional histological techniques, for example, are unsuitable for this purpose, because light microscopy allows only limited estimation of spatial relationships due to the thinness of the sections. Successful attempts to appreciate the extensive three-dimensional organisation of integumental elastic fibres were done several years ago, based on thick frozen sections and specific histological staining (Shellow and Kligman, 1967), and were improved by a combination of autoclave methods with scanning microscopy (Tsuji *et al.*, 1979).

The techniques mentioned above gave good results in humans, but for animals, the information is still very limited, apart from observations in the pig which was chosen because of its suitability as a model for experimental research in human skin (Meyer *et al.*, 1981a; Meyer, 1986). Taking into consideration that the

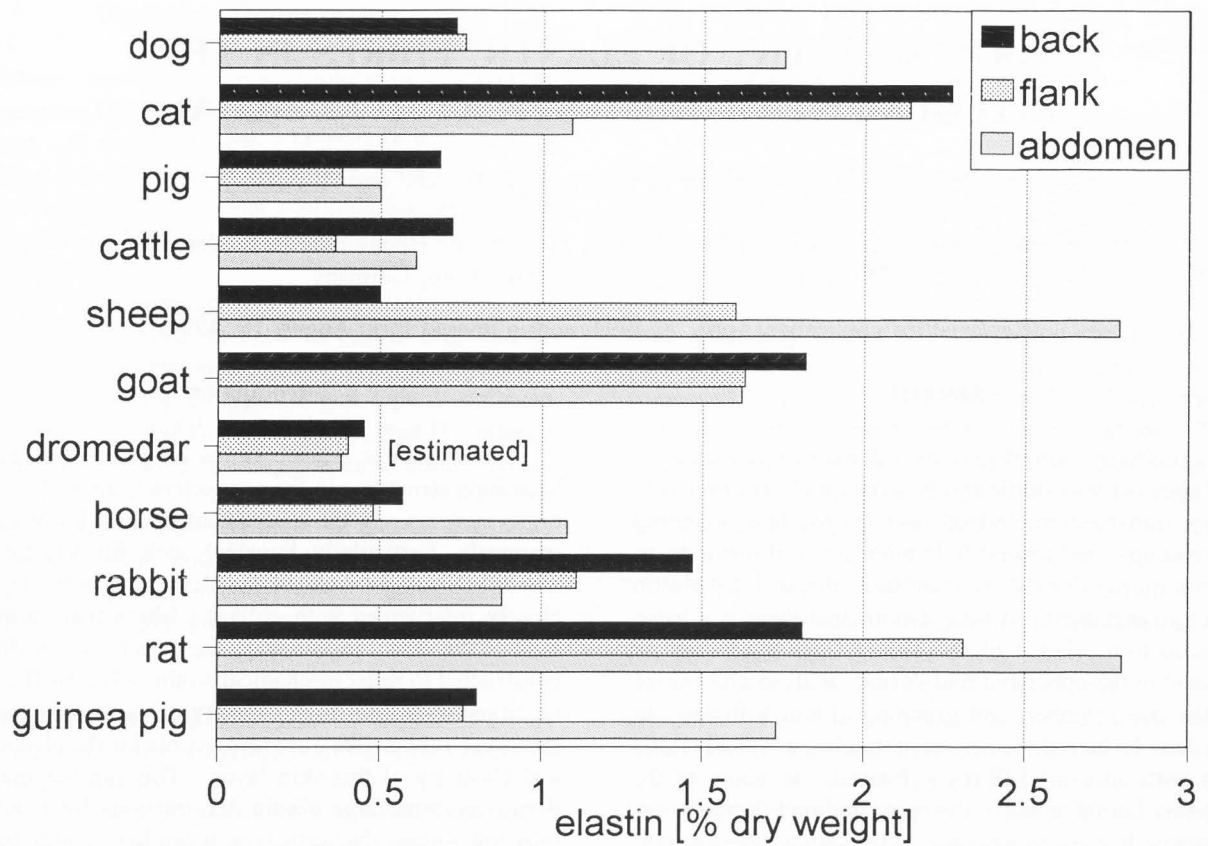


Figure 1. Comparative view of the elastin content of the integument of domesticated mammals (in percent of dry weight of tissue). The standard deviation was between 5-15% due to problems in depilation, or large blood vessels in dermis and hypodermis that could not be recognized during sampling.

amount and arrangement of elastic fibres may vary in the mammalian integument as related to the animal species and body region investigated (Dolnick, 1965; Montagna, 1971), the present study was designed to provide the first broad comparison of the spatial organisation of the elastic component in the skin of domesticated mammals. This approach is based on an equally broad methodological spectrum, and in particular includes the application of scanning microscopy. Since in the latter technique, concentrated solvents (ethanol, xylene, acetone, amyl acetate etc.) are used, shrinkage artefacts may change the true representation of the elastic fibre network. Although this problem has already been discussed earlier (Meyer and Neurand, 1987b), we have now also applied techniques that avoid dehydration and considerable shrinkage by mainly relying on thick frozen sections and fluorescence microscopy, and the specific capacity of modern confocal laser scanning microscopy.

Materials and Methods

For this study 12 important domesticated mammalian species were used (for the correct zoological nomen-

clature see Meyer *et al.*, 1993): dog (several mixed breeds, German Shepherd, Beagle; 6 females, 5 males), cat (several mixed breeds, German Shorthair; 7 females, 3 males), pig (several mixed breeds, German Landrace, German Yorkshire; 10 females, 3 males), cattle (German Black Pied, Friesian breed, 8 females); sheep (German Blackhead, Merino breed; 7 females, 1 male), goat (several mixed breeds, German Improved White, Angora breed; 9 females, 3 males), dromedary (Egyptian breed; 2 females, 2 males), horse (several mixed breeds, Hannover breed; 4 females, 2 males, 3 geldings), donkey (several mixed breeds; 3 females, 1 male), rabbit (several mixed breeds, German Giant; 6 females, 2 males), laboratory rat (Han/DA, Han/WIST; 8 females, 2 males), and guinea pig (several mixed breeds, 8 females, 2 males). The species were grouped as small (laboratory rat, rabbit, guinea pig, cat), medium-sized (dog, pig, sheep, goat), or large animals (cattle, dromedary, horse, donkey), and as sparsely-haired (less than 2000 hair follicles per cm²; pig, cattle, dromedary, horse, donkey) or densely-haired animals (3000-15000 hair follicles per cm²; all other species).

Skin specimens were generally taken from the hairy

skin (*integumentum commune*) of adult animals of medium age and three different body regions (back, flank, abdomen), and processed as described below. The skin material was obtained over a period of 10 years with the help of several institutes and clinics of the Veterinary School of Hannover.

Scanning electron microscopy (SEM)

Sections of the dermis, approximately 1 mm thick, were placed in distilled water and autoclaved at 110°C and 103 kPa for 6-15 hours in a high pressure autoclave system (Keller, Weinheim/Bergstrasse, Germany, type S-ECZ). The original method of Tsuji *et al.* (1979) had to be modified to fit the particular mammalian species used. The efficiency of the autoclaving process was controlled by standard light microscopy with orcein staining (see **Light microscopy** below). After autoclaving, the skin specimens were immediately immersed in Karnovsky's fixative (Karnovsky, 1965) for 4-6 hours at room temperature, and afterwards rinsed in 0.1 M cacodylate buffer (pH 7.4) for 1 hour at +4°C. After serial dehydration in graded ethanol (25-100%, +4°C), the tissue was placed in 100% xylene, and then dried very slowly for several weeks in an xylene saturated atmosphere in small, partly perforated glass jars (Meyer and Neurand, 1985, 1987b). After dehydration, several specimens were also critical-point-dried through CO₂ in a Polaron E 3100 Series I drying apparatus. Finally, all specimens were sputtered with gold in the Balzers SCD 040 sputter coater, and viewed in the Zeiss DSM 940 scanning electron microscope.

Transmission electron microscopy (TEM)

About 0.5-1 mm thick slices of the dermis were fixed in Karnovsky's fluid (Karnovsky, 1965) for 24 hours at +4°C, washed several times in 0.1 M cacodylate buffer (pH 7.4, +4°C), and postfixed in buffered 1% osmium tetroxide (Millonig, 1961). After dehydration in graded ethanol (25-100%, +4°C), the slices were embedded in Epon 812 (Serva) (Luft, 1961). Thin sections were cut with a diamond knife on a Reichert (Ultracut) ultramicrotome; stained with ethanolic uranyl acetate for 1 hour (Stempak and Ward, 1964), and lead citrate for 10 minutes (Reynolds, 1963); and examined in a Zeiss EM 10 transmission electron microscope operated at an accelerating voltage of 60 kV.

Laser scanning microscopy (LSM)

About 50 or 100 µm thick frozen sections of dermis material, that had been fixed for several days in 4% formalin with the addition of 2.5% calcium acetate (Lillie and Fulmer, 1976) containing 10% DMSO for better tissue permeation, were stained free floating in 0.1 or 1.0% aqueous eosine Y (Merck; excitation max. 515 nm, for specific staining characteristics see, Lillie, 1977)

for 10-30 minutes at room temperature. The sections were shortly washed in distilled water, mounted in glycerol and viewed in the Zeiss LSM 310 confocal laser scanning microscope. Sixteen optical section images were generated at a distance of 2.5 µm through the whole tissue at the following conditions: 512 x 512 pixels/8 bit, objectives Plan Neofluar 40/1.3 oil, helium neon laser 543 nm, emission filter OG 570. For the three-dimensional reconstruction split stereo images and red green stereo images were generated after digital contrast enhancement and a median filtering.

Light microscopy (LM)

For light microscopical purposes, several methodological strategies were followed:

(a) Small tissue blocks were fixed in Bouin's fluid for two weeks, then washed in 70% ethanol with addition of some drops of ammonia, dehydrated via graded ethanol (25-100%), and embedded via HistoSol (Shandon) in Paraffin (Histoplast, Serva). Ten µm sections were stained with 0.1 or 1.0% ethanolic acid orcein (Merck) at 70°C (after Taenzer-Unna from Lillie and Fulmer, 1976; for the reliability of this staining procedure, see Horobin and Flemming, 1979).

(b) Small skin blocks were fixed in 4% formalin-Ca (Lillie and Fulmer, 1976) containing 10% DMSO, and 50-100 µm thick frozen sections were stained, after air drying for several hours, for 1 hour in 1.0% ethanolic acid orcein at room temperature (Shellow and Kligman, 1967).

(c) Formalin-Ca fixed 20-50 µm frozen sections were stained for 2-5 minutes in saturated (0.39%) basic fuchsin (Merck) in phosphate-buffered saline (PBS) (pH 7.4), diluted 1:100, then rinsed in PBS and mounted in buffered glycerol (Pihlman and Linder, 1983). The elastic fibres were viewed under green excitation (525 nm) in a Zeiss fluorescence microscope with the epifluorescence condenser III RS.

(d) Formalin-Ca fixed 20-50 µm frozen sections and deparaffinized 25 µm sections of Bouin-fixed material were stained in a solution of equal parts of 0.5% aqueous magnesium II (Merck) and 1 N NaOH, and rapidly mounted and viewed in the staining solution (Müller and Firsching, 1991).

Estimation of elastin content

Fresh skin samples were carefully and extensively depilated with the help of a forceps, autoclaved (see **Scanning electron microscopy** above), dehydrated via graded ethanol, and the fat was removed with xylene. Afterwards the material was briefly dried at 60°C, homogenized in liquid nitrogen and extracted 4-5 times at 95-98°C in 9 volumes by weight of 0.1 N NaOH for 10-15 minutes. The insoluble residue (elastin) was

centrifuged, and the pellet was washed in water, then freeze-dried and evaluated gravimetrically. The whole procedure was a modification of the Lansing method as described by Giro and Davidson (1988).

Results

Our findings are a synopsis based on all methods applied, but they mainly rely on results obtained by SEM. From a comparative point of view, the typical arrangement and proportion of the elastic fibre network in the mammalian integument seems to be generally correlated with the relative size, number and grouping of hair follicles, or the mechanical strain exerted, and in this way regional differences are established. To a certain extent, this statement is confirmed by the varying amounts of elastin within the skin of the different animal species and body regions studied, i.e., rather large elastin amounts are only found in densely-haired species (Figs. 1; 2a-2c).

The general distribution of elastic fibers is illustrated in Figure 2. Findings in densely-haired species (Figs. 3 and 4) are compared to findings in sparsely-haired species (Figs. 5 and 6). The dermo-epidermal junction is illustrated in Figure 7. Figure 8 shows the interactions of elastic fibers with blood vessels and hair follicles.

In all animals, the elastic fibres are in very close contact with the enormous mass of collagenous fibre bundles. Findings based on the evaluation of frozen sections after fluorescence staining, which avoids dehydration, show a clearly uniform distribution of this elastic tissue, in particular in dermal regions without numerous hair follicles (Figs. 3c; 5f and 5g). Our TEM results corroborate the knowledge available about the typical ultrastructure of elastic fibres, with an accumulation of long tubular units, whereby, microfibrils form the wall of the tubule and its 'lumen' is filled with amorphous elastin (Fig. 2e). However, thick elastic fibres or branching elastic junctions or sheets of different sizes as visible in the skin of densely-haired species, especially in the large animals, are characterized by bundle formation with several 'normal' fibres included (Figs. 2d and 2e). In addition, TEM indicates that the outer microfibrillar coat of these elastic elements is densely interwoven with peripheral microfibrils of collagenous fibres.

Elastic fibres seem to be remarkably numerous and branching in the dermis of densely-haired species (cat, dog, sheep, goat) or the small fur-bearing animals (rabbit, guinea-pig, and rat). In these species, the elastic meshwork is normally extensively developed in the upper and mid-dermis (Figs. 2a and 3d). Within the latter part, in particular, comparatively thick elastic fibre 'bundles' form a close-meshed net that very distinctly

anchors and connects the hair follicles (Figs. 3a, 3d; 4a-4e). In the thin dermis of the small species, this anchoring network is more or less continuous with the elastic fibre system found in the upper dermis. In the medium-sized densely-haired mammals with long hair follicles, like sheep or goat, this upper network is relatively finer than the one in the mid-dermis. Horizontal sections show that in the latter skin part, all the hair follicles and hair follicle groups seem to be closely entwined by a regularly constructed elastic 'mat' (Figs. 4b, 4d and 4e). This mat is evidently limited to a region comprising the whole middle part, or second third part, respectively, of the hair follicle complex, ending at about the region where the arrector pili muscle joins the connective tissue sheath of the hair follicle. Moreover, hair follicle groups, or at least the primary hair follicles of these, are connected with each other by several (20-40) strong and parallel, horizontally arranged, peripheral elastic fibres (Figs. 3e and 5c). Such fibres probably enclose the hair follicle groups to form a subunit in the elastic mat system outlined above. In contrast to these findings, the deeper dermis in the densely-haired species studied is characterized by a clearly more wide-meshed and very loosely-structured elastic fibre network. It is integrated in the connective tissue of the hypodermis, i.e., there is no distinct border zone between the two skin layers.

In comparison to the densely-haired species investigated, the sparsely-haired domesticated mammals, such as the pig, cattle, dromedary, horse, or donkey, show a relatively coarse but homogeneous, sponge-like elastic fibre meshwork, particularly in the upper dermis (Figs. 5a, 5b and 5d). In these animals, the hair follicles are also anchored by numerous elastic fibres or fibre bundles in the mid-dermis, although this elastic system appears quite light or loosely-structured (Figs. 4c, 4e-4g). The basic type of anchoring, however, is the same as described for animals with a dense hair coat. The sparsely-haired mammals studied generally have a rather thick dermis, where the hair follicles are restricted to the upper third, or even fourth part, with the exception of the pig, where the dermis is regularly penetrated by large primary hair follicles. It is obvious that in most of the animals, as in the densely-haired species, the lower part of the hair follicles, mainly the hair bulbs and the apocrine tubular glands present, are only connected to each other by a very wide-meshed elastic network. This feature is easily visible in the whole lower half of the dermis in most of the large domesticated mammals studied (e.g., horse, cattle, dromedary). Elastic fibres only line the ascending or descending blood vessels (Figs. 8e and 8f), the nerve trunks, or, if present, the alveolar tissue. In the transitional zone of dermis and hypodermis of the large species, however, this loose framework changes into one or several relatively thick, sheet-like

Elastic fibres in the integument

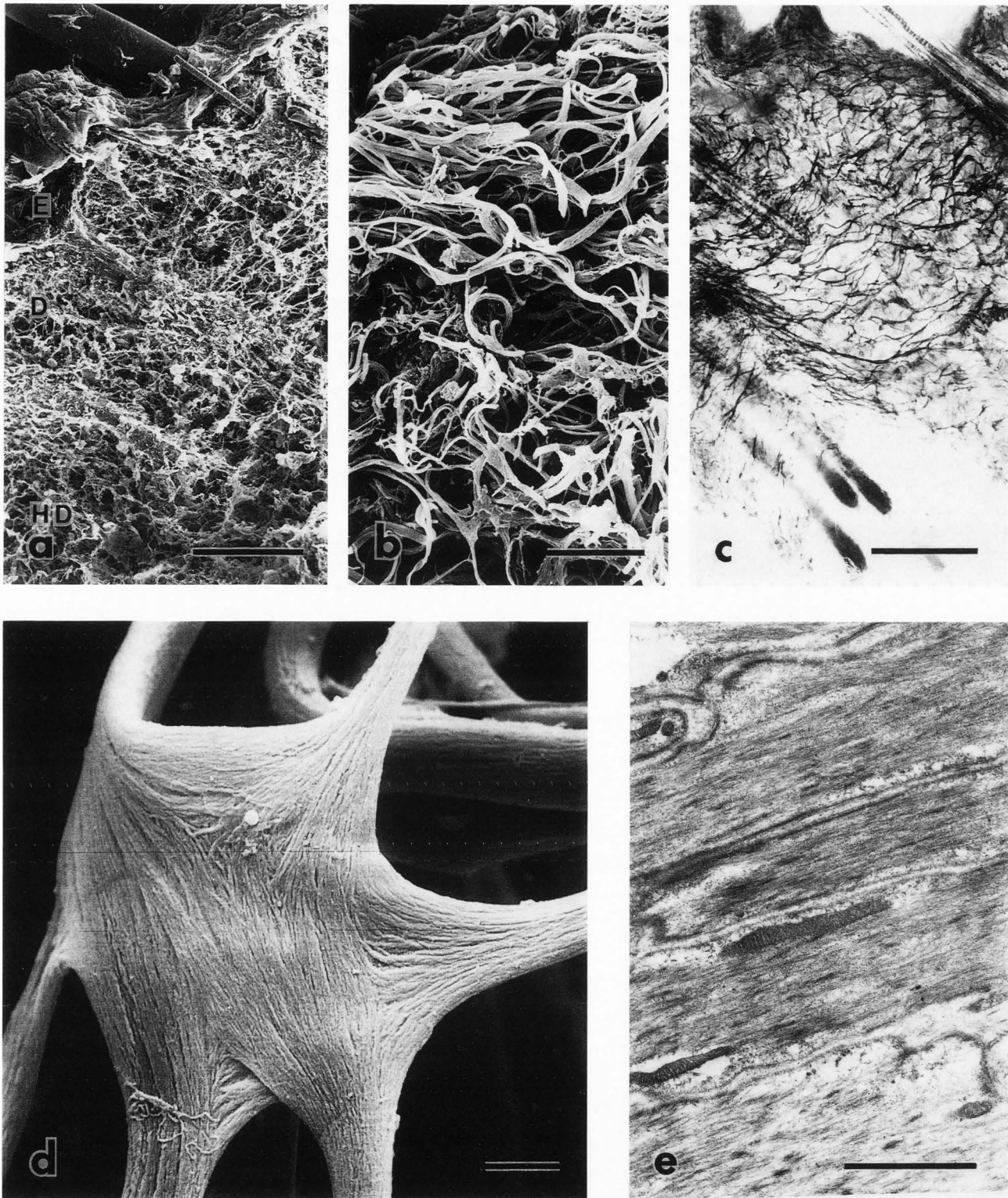


Figure 2. General aspects: high amounts of elastic fibres distributed uniformly throughout the skin (a: guinea pig, back, sagittal section, SEM), concentrated in the abdominal region (b: sheep, sagittal section, SEM), or confined to the upper half of the dermis (c: cat, back, sagittal frozen section, orcein); elastic fibre junctions were found in several species (d: dog, back, sagittal section, SEM), as well as the typical TEM structure of elastic fibre 'bundles' (e: goat, back). E = epidermis, D = dermis, HD = hypodermis. Bars = 100 μm (a); 10 μm (b); 200 μm (c), and 2 μm (d and e).

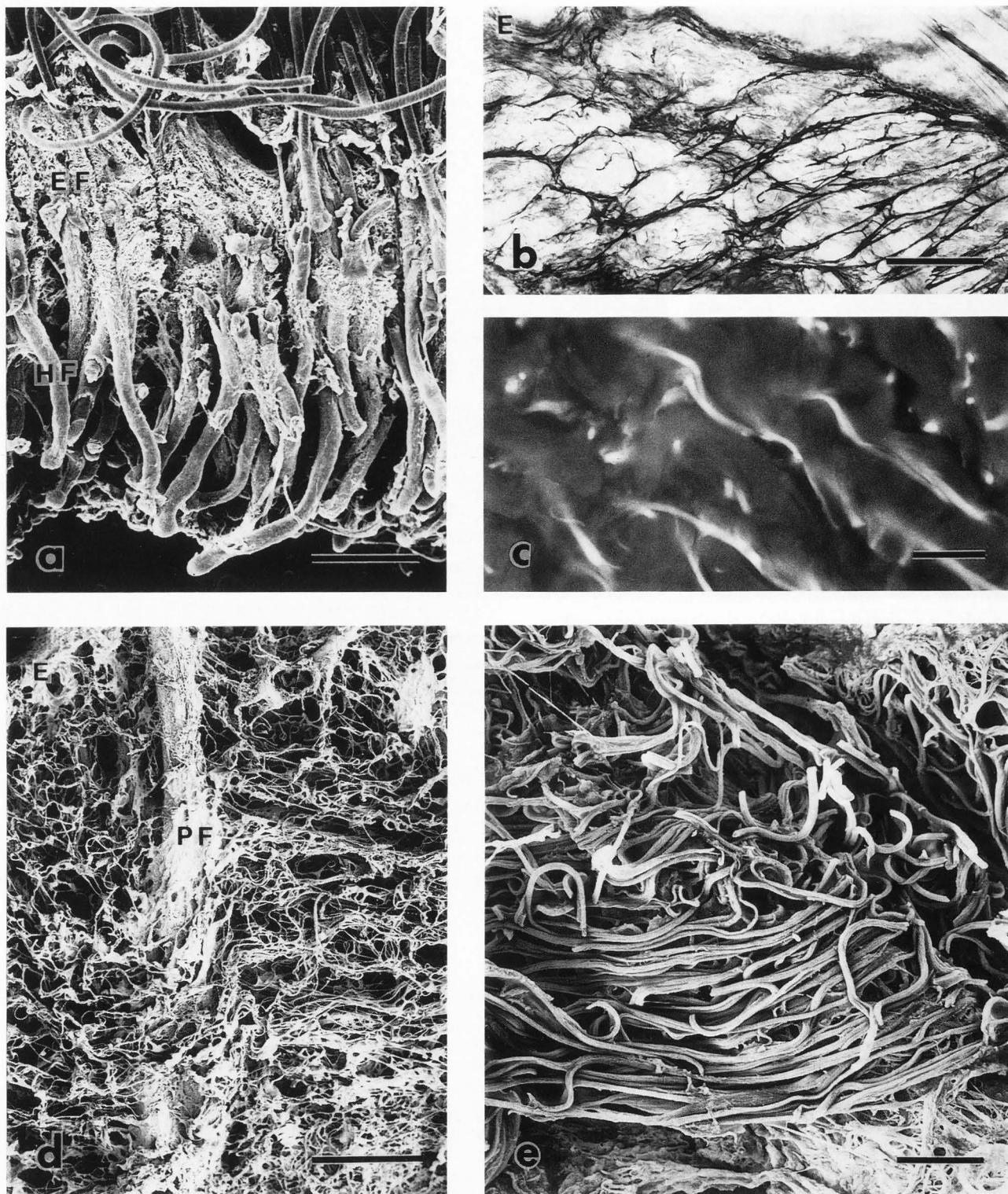


Figure 3. Densely-haired species: elastic mat system to anchor the hair follicles (a: sheep, back, sagittal section, SEM; b: dog, back, sagittal frozen section, orcein; d: dog, abdomen, sagittal section, SEM), with typical thick elastic fibres arranged parallel to the skin surface, i.e., horizontally (e: cat, back, sagittal section, SEM); the overall distribution of dermal elastic fibres is uniform (c: sheep, back, sagittal frozen section, basic fuchsin fluorescence). E = epidermis, EF = elastic fibre system between hair follicles (HF), PF = primary hair follicle. Bars = 200 μm (a, b, and d); 20 μm (c); and 10 μm (e).

Elastic fibres in the integument

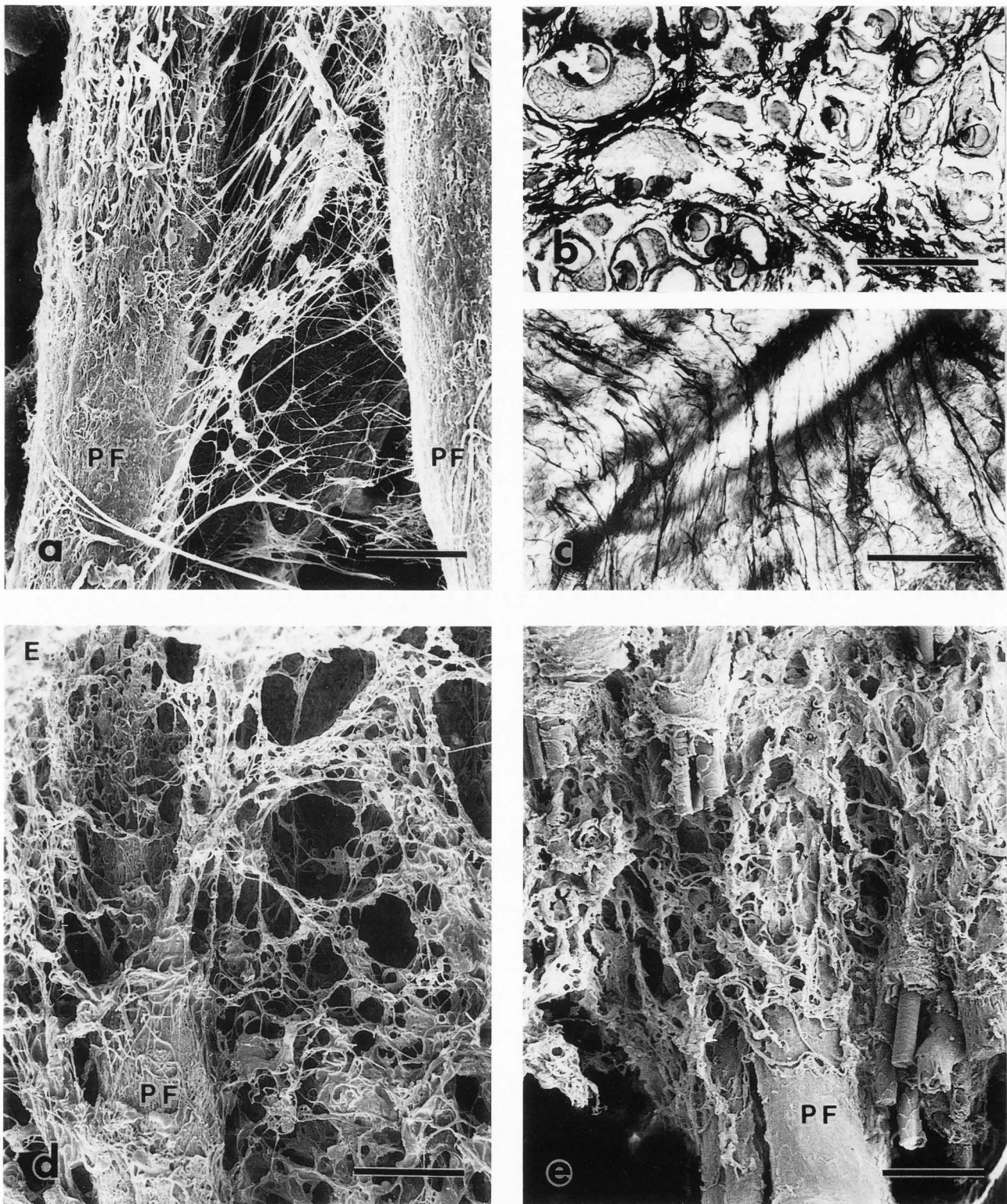


Figure 4. Densely-haired species: anchoring and interweaving of hair follicles with fine elastic fibres (a: goat, back, deep dermis, SEM), or within a rather coarse elastic network (b: sheep, back, upper dermis, horizontal section after autoclaving and subsequent paraffin embedding, orcein; c: dog, back, deep dermis, club hair ending, sagittal frozen section, orcein; d: dog, abdomen, upper dermis, SEM; e: cat, abdomen, deep dermis, SEM). E = epidermis, PF = primary hair follicle. Bars = 20 μm (a); 200 μm (b and c); 100 μm (d); and 50 μm (e).

elastic elements that distinctly separate both skin layers. These elastic sheets are normally also connected with elastic fibres accompanying blood vessels or nerve trunks and give rise to some medium-sized or thin fibres that descend into the hypodermis (Figs. 6a-6e).

With respect to specific features of the elastic fibre system formed in the skin of all domesticated mammals studied, several aspects were evaluated in detail as follows. In the upper dermis of most of the larger species, many thin or delicate elastic fibres are observed that are arranged perpendicularly to the surface, terminating at the underside of the epidermis (Figs. 7a and 7b). TEM reveals microfibrils of tubular structure to be inserted into the basal membrane. These microtubular-like fibrils can be distinguished from the neighbouring collagenous microfibrils by their smaller diameter (about 6-15 nm), by the peculiarity that they branch before joining the basal membrane, and by the fact that typical cross-banding is missing (Fig. 7f). In the densely-haired species fine elastic fibres often form a tight subepidermal network or plexus closely contacting the basal membrane (Figs. 7d and 7e), and clearly connected to the elastic mat system of the upper dermis through medium-sized fibres (Figs. 7c).

Moreover, fine as well as thick elastic fibre elements are a permanent feature of the hair follicle, i.e., it is covered by a more or less regular network that is continuous with the adjacent sponge-like elastic system of the dermis. Elastic fibres are heavily concentrated around thick and deeply inserted primary hair follicles, and this is particularly obvious in the pig and the horse (Figs. 5d and 5e). In the pig, such fibres are connected to the sheet-like elastic system of the border zone of dermis and hypodermis (Figs. 6d; 8c and 8d). In general, a direct elastic connection between all hair follicles of a hair follicle group can be recognized, as typical for fine fibres between secondary hair follicles. If present, the basal end of telogen hair follicles (club hairs) is densely studded with elastic fibres that are arranged in a 'star-like' manner, whereby the fibres anchor in the basal membrane of the club end and branch off radiating (Fig. 4c). In all animals studied, the *musculus arrector pili* passes the upper and mid-dermis without clear contact with the elastic system. A close connection of this muscle with elastic fibres is restricted to the tendon-like attachment to the hair follicle and the undersurface of the epidermis.

The sebaceous glands are normally only rarely in contact with the dermal elastic connective tissue. This is in contrast to the integumental blood vessel system. Even after intense autoclaving, it can be seen that the internal elastic laminae or sheaths of thick arterioles are interwoven with and thus, anchored in fine and branching elastic fibres in the general elastic meshwork (Fig.

Figure 5 (on the facing page). Sparsely-haired species: homogeneous elastic network (a: pig, back, horizontal section, SEM; b: horse, abdomen, sagittal section, SEM), with a typical strong anchoring of hair follicles (c: cattle, abdomen with horizontal elastic fibres between hair follicles, sagittal frozen section, orcein; d: horse, back, horizontal section, SEM; e: horse, abdomen, deep dermis, lateral view of a primary hair follicle with surrounding elastic network); LSM-3D-computer reconstruction generally confirms the SEM results (f: dromedary, back, uniform horizontal arrangement of elastic fibres around a primary hair follicle; g: cattle, back, fine elastic fibres reaching the connective tissue sheath of a hair follicle; f and g: 100 μm frozen sections, eosin fluorescence; these structures are best visible with the help of red-green stereo images). E = epidermis, HEF = horizontal elastic fibres, PF = primary hair follicle. Bars = 50 μm (a and b); 200 μm (c); 20 μm (d); and 10 μm (e-g).

8a). In this way, capillaries surrounding the hair follicle can also be followed (Fig. 8b). While such findings cannot be made often, they are detectable in both densely-haired and sparsely-haired species.

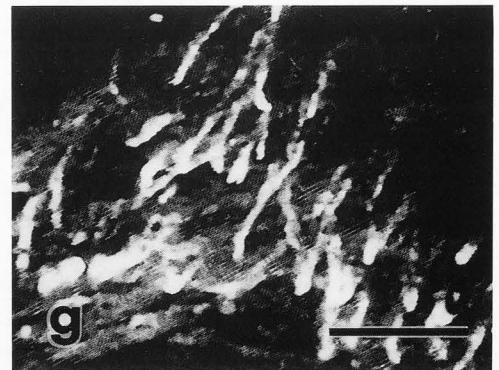
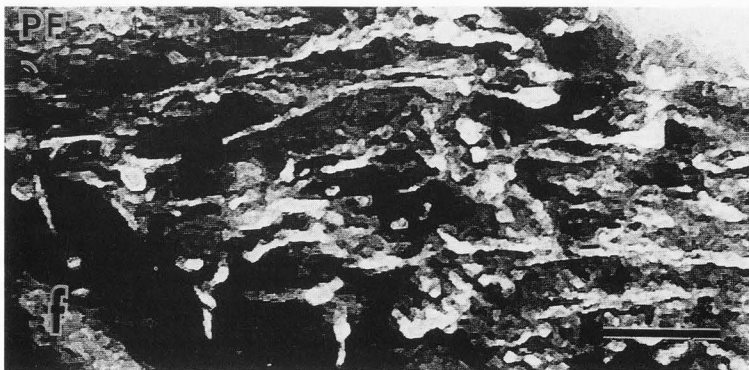
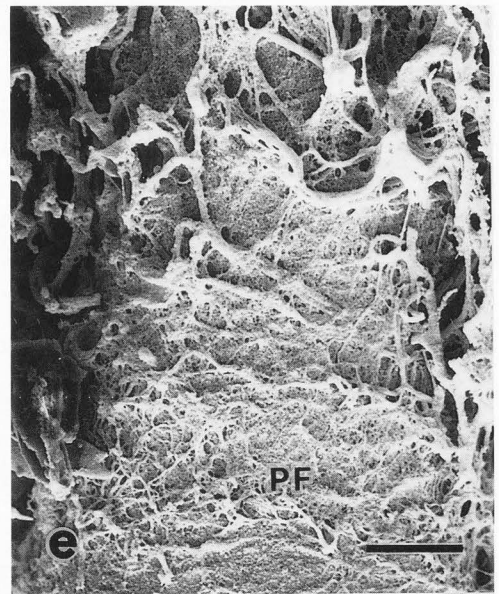
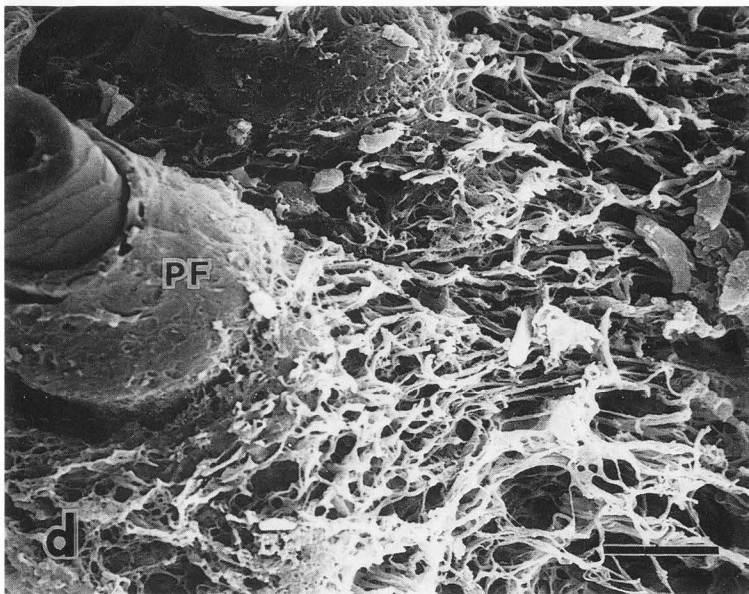
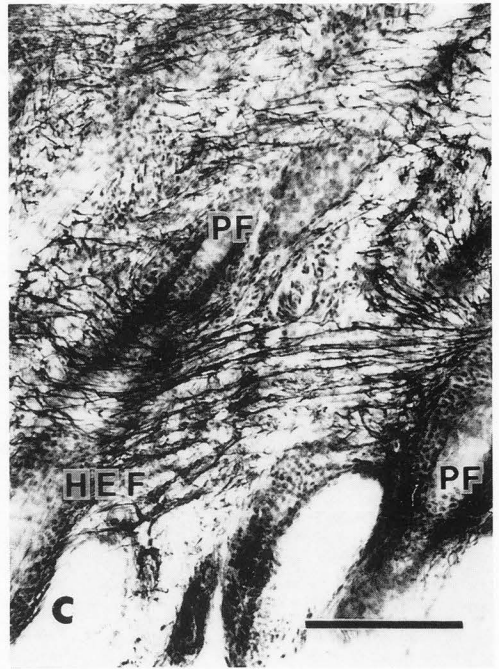
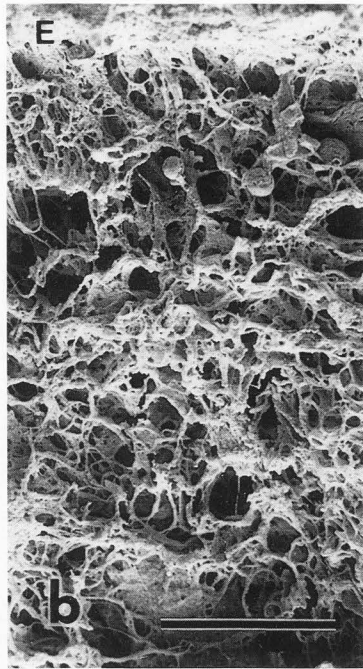
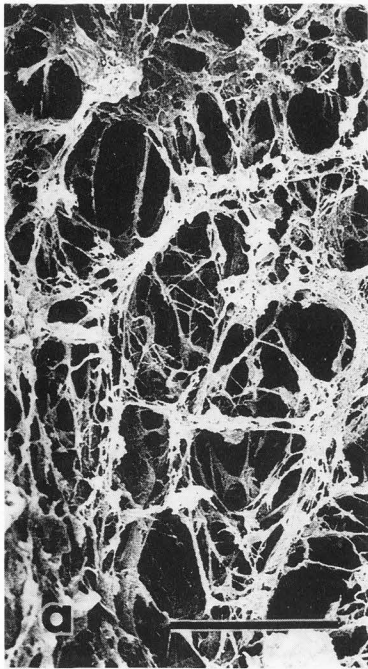
As far as regional differences in the arrangement and content of elastic fibres are concerned, one feature is common to all animals studied: the comparatively largest amounts of elastic tissue show regional variation and are related to high numbers of hair follicles and their abundantly developed, surrounding and anchoring elastic network (see e.g., rat, cat, goat, or sheep, Fig. 2b). Its fibres are closely connected with the elastic sheath of the cutaneous muscle. In sparsely-haired animals, the abdominal region also appears to contain a mass of elastic tissue, as demonstrable in the pig, but, to some extent, also in the large species (horse, donkey, cattle), where the horizontally arranged collagen fibre bundles of the lower dermis in the lateral and abdominal body regions seem to be entwined with thick elastic fibres (Figs. 6b and 6c), or a striking net of several densely interwoven sheet-like elastic elements is formed that may contain large blood vessels (Figs. 8e and 8f).

Clear differences with regard to the sex of the animal cannot be ascertained, neither with regard to the structure nor the amount of the elastic fibre system present in the skin of the mammals investigated.

Discussion

Using SEM, supplemented by several other microscopy methods, we were able to demonstrate a specific multidirectional elastic fibre system in the integumentum commune (hairy skin) of domesticated mammals. This elastic component is best visible in the dermis, where it

Elastic fibres in the integument



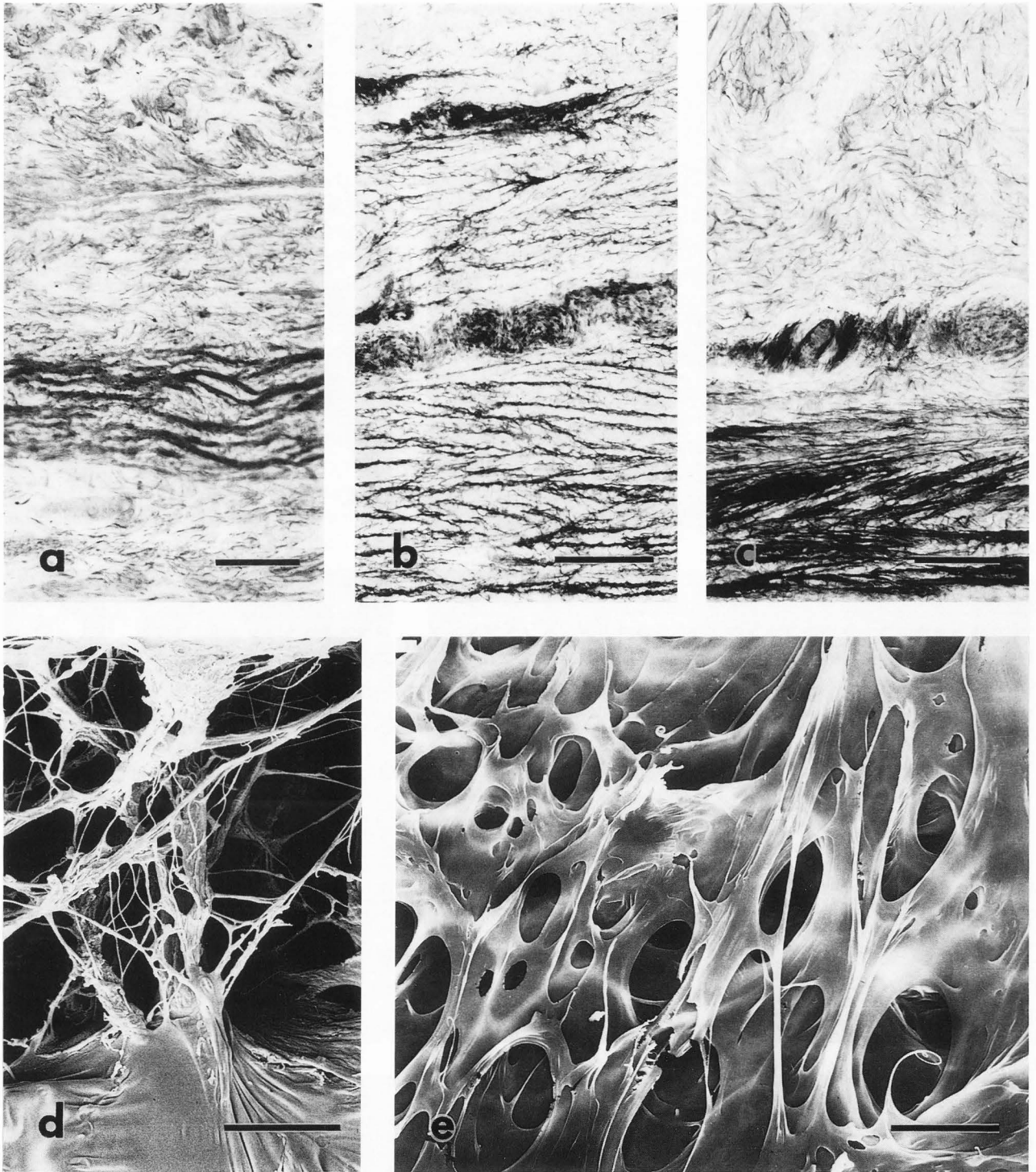


Figure 6. Sparsely-haired species: transitional zone of dermis-hypodermis with elastic sheet systems (a: horse, back, deep dermis with thick sheets, sagittal frozen section, magneson II; b: horse, abdomen, deep dermis with a regularly alternating horizontal arrangement of thin horizontal elastic sheets and thick collagenous fibre bundles, sagittal frozen section, orcein; c: cattle, flank, deep dermis with densely interwoven elastic fibres to form a thick sheet, sagittal frozen section, orcein; d: pig, abdomen, lateral view of an elastic sheet that is connected via elastic fibres to the upper dermis, SEM; e: pig, abdomen, elastic sheet from a surface view, horizontal section, SEM). Bars = 100 μm (a); 200 μm (b); 50 μm (c); and 20 μm (d and e).

Elastic fibres in the integument

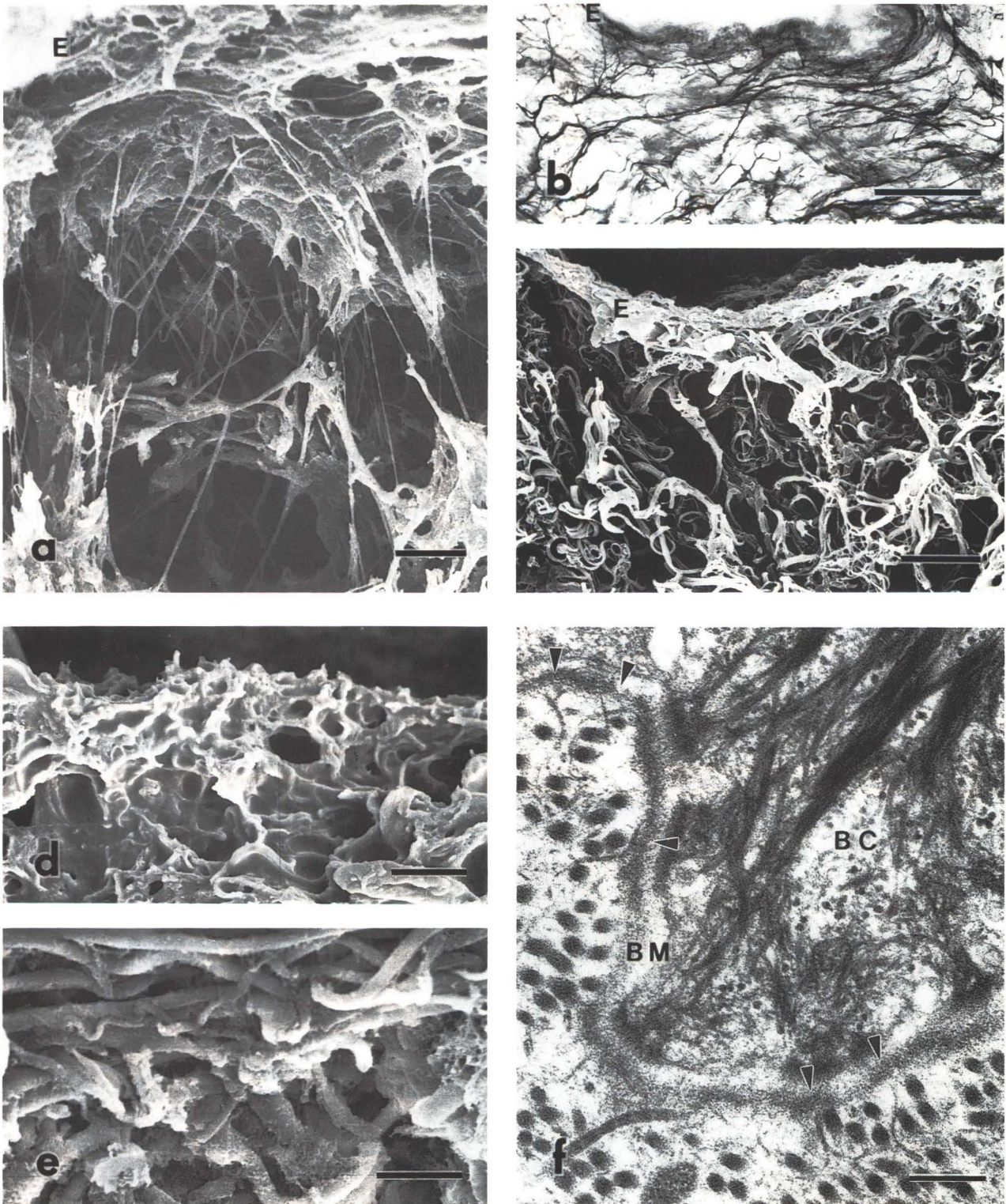


Figure 7. Dermo-epidermal junctional zone: specific contacts as visible from thin elastic fibres terminating at the undersurface of the epidermis (**a**: pig, back, SEM; **f**: pig, back, part of the basal membrane of a basal cell, arrowheads mark the anchoring elastic microfibrils, TEM); densely-haired species often develop a subepidermal elastic network or plexus (**b**: dog, back, upper dermis, sagittal frozen section, orcein; **c**: sheep, back, upper dermis, SEM) that is also seen at high magnification (**d**: dog, back, surface view of a plexus, SEM; **e**: guinea pig, back, lateral view of fine and interwoven elastic network fibres, SEM). E = epidermis, BC = basal cell, BM = basal membrane. Bars = 10 μm (**a**, **c** and **d**); 200 μm (**b**); 5 μm (**e**); and 200 nm (**f**).

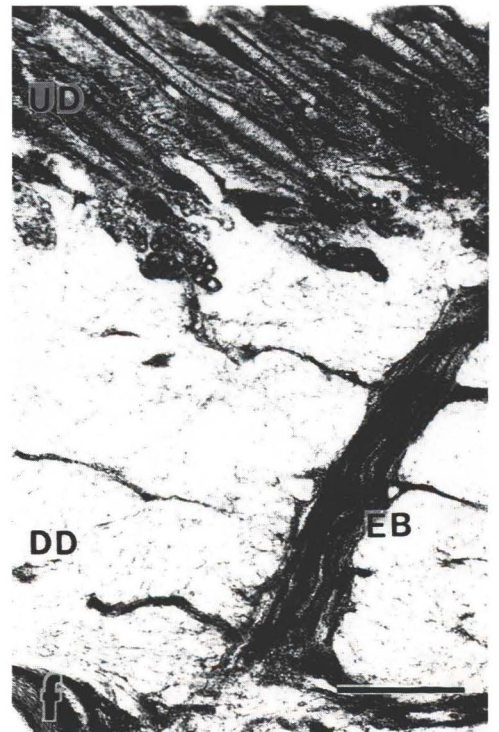
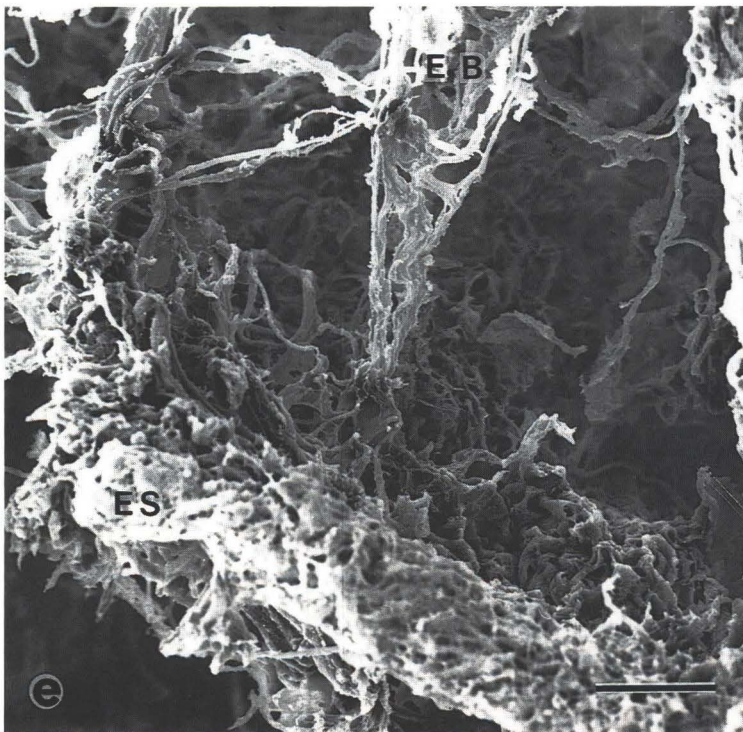
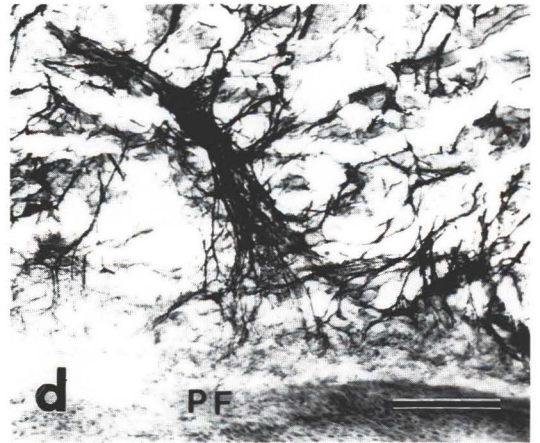
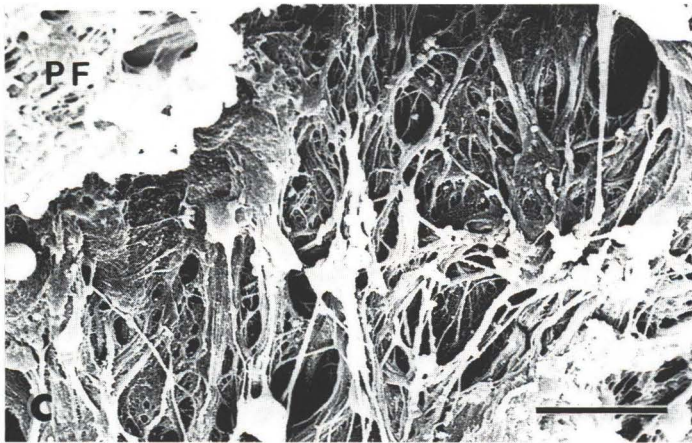
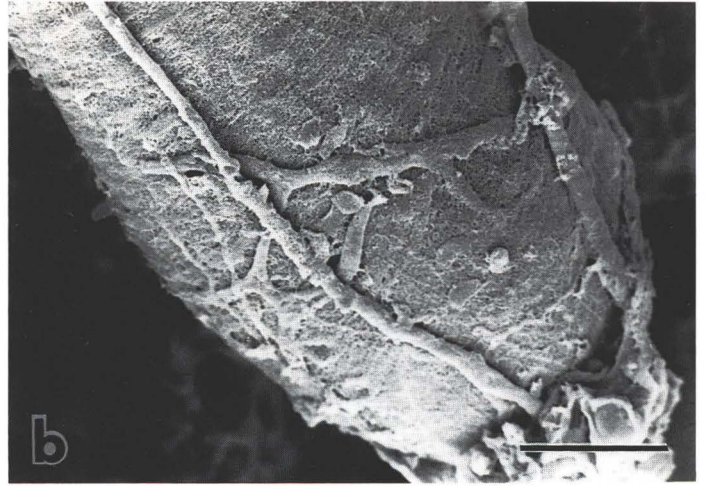
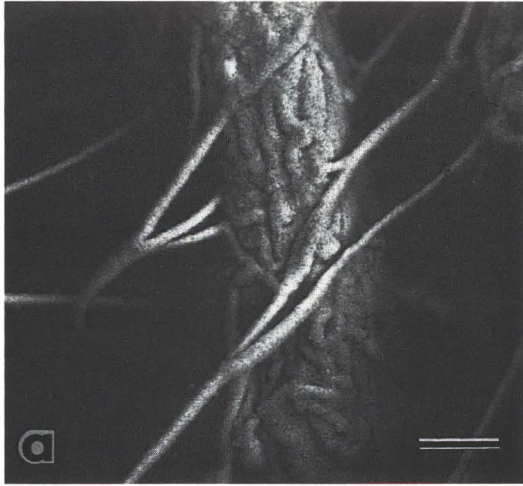


Figure 8. Specific aspects: demonstration of the elastic intima of arterioles to be anchored in the elastic dermal network (a: pig, back, SEM), or as connected to the elastic sheath of the lower part of a hair follicle (b: cat, abdomen, SEM); in sparsely-haired species, the large primary hair follicles are strongly anchored by broad elastic bundles in the deep dermis (c: pig, back, surface view, SEM; d: pig, back, sagittal frozen section, orcein); large species show a strong interweaving of elastic elements at the border zone of dermis-hypodermis with thick elastic fibres accompanying blood vessels (e: cattle, back, deep dermis, lateral surface view, SEM; f: horse, back, dermis with elastic fibres concentrated in the upper third, sagittal frozen section, orcein). ES = elastic sheet system, EB = elastic fibre system surrounding blood vessels, PF = primary hair follicle, DD = deep dermis, UD = upper dermis. Bars = 2 μm (a); 20 μm (b); 5 μm (c); 100 μm (d); 10 μm (e); and 0.5 mm (f).

forms a sponge-like network that is modified by the size, number, and grouping of the hair follicles, and by the type of mechanical strain exerted, as, for example, in the thick integument of large animals. In this way, regional differences are established, as found, in particular, for abdominal skin.

Even though variations of elastic fiber content with the mammalian species and the body region studied were found, the great abundance of elastic fibres found after removal of collagen and ground substance, is striking. Considering that elastin only comprises between 0.5 and 2.5% of dermis by weight (in humans 0.6% cf., Uitto, 1979; Ryhänen and Uitto, 1983), these fibres must be extremely light. This is obvious in the rather thin skin of small or medium-sized, densely-haired mammals with a typical elastic anchoring of all hair follicles, so that these structural elements can be moved in various ways as normally necessary for maintaining optimal insulation effects of the hair coat. Such mobility is probably assisted by very low amounts of elastic fibres as found at the basal part of primary hair follicles. These relatively long skin elements pass between hypodermal fat cells, and thus can be shifted more easily. The fact that the arrector pili muscle is not directly tied to the surrounding elastic meshwork, but only by its tendon-like elastic connection to the hair follicle and the epidermis, respectively, generally confirms the view, that the elastic system of the dermis aids hair movement.

To a certain extent, our findings corroborate the light microscopical results of Dolnick (1965) and Meyer *et al.* (1981b), who reported that heavy masses of elastic fibres exist throughout the dermis in small fur-bearing mammals (nutria, otter, mink, cat), although a typical arrangement of these fibres was not described. Similarly, several authors have found elastic fibres to surround

the hair follicles or to serve as attachment of the arrector pili muscle (Mikhailova, 1958; Strickland and Calhoun, 1963; Dolnick, 1965; Sar and Calhoun, 1966; Kozłowski and Calhoun, 1969; Meyer *et al.*, 1981b), also in sparsely-haired species (cattle, horse, pig) (Goldsberry and Calhoun, 1959; Marcarian and Calhoun, 1966; Talukdar *et al.*, 1972; Meyer *et al.*, 1981a, Meyer, 1986; see also Arao and Perkins, 1969). It was in the pig that, for the first time, SEM was used to demonstrate a considerable content of elastic tissue with the basic structure of elastic fibre arrangement as a homogeneous flat 'sponge' between epidermis and hypodermis (Meyer *et al.*, 1981a; Meyer, 1986), showing clear similarities to human skin (Tsuji *et al.*, 1979; Tsuji, 1982). Therefore, an 'elastic solidity' may be developed by quite thick skin that normally lacks large numbers of hair follicles (Meyer and Neurand, 1987a,b).

Specific differences with regard to the age, we used only adult animals of medium age, and sex of the domesticated mammalian species studied could not be verified, which is in some contrast to observations in humans (Vitellaro-Zuccarello *et al.*, 1994). Regional differences of specific interest may be discussed with regard to the abdominal skin, where relatively large amounts of elastic tissue have been observed, especially in sparsely-haired animals like the pig. Here, such variation seems to be combined with a characteristic arrangement of collagen fibre bundles (Meyer, 1986). The latter aspect can also be evaluated in large species like the horse, donkey, cattle, or dromedary, because these mammals often show a close interweaving of elastic fibres with collagen fibre bundles in the lateral and abdominal body regions, which could be related to the development of elastic sheets in the border zone of dermis and hypodermis. Such sheets have first been reported in porcine skin (Meyer, 1986), and these structures have also been demonstrated consistently in the deep dermis of normal human skin (Tsuji, 1988). In view of specific functional properties of elastic sheets, the opinion of Tsuji (1988), who concluded that they may be the base of an elastic fibre system that could prevent hypertension of the skin on stretching, can be followed. This is especially true of the lateral and abdominal integument in the horse or cattle, that needs high extensibility because of the varying intestinal volumina during feeding. In this way, the close intertwining of elastic elements with horizontally arranged collagen fibre bundles, as observed in the present study, may be equally important.

The dermo-epidermal junctional area is another feature of interest with regard to the specific functional involvement of elastic fibres in the skin. Fine elastic fibres that terminate at the undersurface of the epidermis have often been found in domesticated mammals (Dick, 1947; Montagna and Yun, 1964; Sar and Calhoun,

1966; Kozłowski and Calhoun, 1969; Meyer *et al.*, 1981a,b; Meyer, 1986). The occurrence of elastic microfibrils inserting in the basal membrane, as recognized best in thick epidermis such as that of the pig, was also reported in humans. These microfibrils have been shown to be continuous with the microfibrils associated with elastin in dermal elastic fibres (Kobayasi, 1977; Odland, 1983). The presence of a subepidermal elastic plexus has until now only been demonstrated in thick human skin that is normally under constant mechanical strain, like in the palmar or plantar regions (Schmidt *et al.*, 1974; Cotta-Pereira *et al.*, 1978). In contrast to these findings, such a plexus in animals was found in this study to be extensively developed in densely-haired species, and in direct contact with the elastic network of the upper dermis. It seems reasonable to assume that a dense hair coat with large numbers of hair follicles which has to be moved elastically, exerts considerable mechanical stress on the usually thin epidermis, which thus needs an effective protection during frequent mechanical deformation.

In addition, in arterioles of the skin, the elastic lamina is present as an interrupted layer between endothelial and smooth muscle cells (Braverman, 1989). The integration by interweaving of this elastic lamina into the overall elastic system of the mid- and deep dermis that was visible in our SEM preparations, may not only be a general anchoring of blood vessels, but also be important during blood movement. In the same way, the regularly occurring elastic tissue framework lining the blood vessels of the deep dermis and hypodermis in the large species studied, should be understood as a self-regulating transport aid during varying fluid movement in the skin.

The different methods applied during our investigation have proven to be very effective in the demonstration of the typical elastic network in the skin of domesticated mammals. The autoclaving technique, as coupled to SEM, obviously preserves the *in vivo* spatial arrangement of elastic tissue, because methods avoiding dehydration, such as the use of thick frozen sections and fluorescence staining, with special regard to laser scanning microscopy and three-dimensional reconstruction, generally confirm the SEM findings. This is supported by the fact that the exposition of elastin by autoclaving of collagen is as effective as, for example, enzymatic digestion of the latter, although this procedure, likewise, produces good results (e.g., Ushiki and Murakumo, 1991; Bartels *et al.*, 1993). Moreover, it has been shown that air-drying may cause less shrinkage of the skin material than standard critical-point-drying (Albrecht *et al.*, 1976; Boyde, 1978; Liepins and De Harven, 1978; Tsuji *et al.*, 1979), and we could improve this method by achieving slower drying in a xylene-saturated atmosphere (Meyer and Neurand, 1985, 1987b). We concede, how-

ever, that irrespective of the advantages of autoclaving and SEM, control of normal elastic tissue structure as based on methods that avoid considerable tissue shrinkage should always be done simultaneously, so that misinterpretations can be avoided. Nevertheless, all results confirm the view that collagen masks the minor elastic component of the dermis, particularly with respect to the application of conventional histological technique using paraffin embedding (Tsuji *et al.*, 1979; Meyer *et al.*, 1981a; Sandberg *et al.*, 1981; Tsuji, 1982; Godeau *et al.*, 1986). This means that interpretations of the spatial organisation of elastic fibres exclusively based on this methodological approach should be abandoned.

Our comparative investigation has clearly demonstrated that remarkable amounts of elastic fibres exist in the integument of different mammalian species, and that these fibres are typically arranged according to the varying hair follicle size, grouping, and density, or skin structure as related to the body size. By its close interweaving with the collagen fibre bundles, this extremely plastic component probably enables the skin to sustain a higher mechanical stress than would be predicted from their normal structure (Elden, 1980; Meyer and Neurand, 1987a,b). Moreover, the development of a specific elastic anchoring system for the hair follicles, creating excellent conditions to move these structural elements as typical for mammals, is of similar importance. This is especially true of the maintenance and improvement of insulation effects of the hair coat by erecting primary hair follicles. From the phylogenetic point of view, the evolution of elastin, that appears to be an exclusive acquisition of vertebrates, may have proceeded very rapidly but it may not have been related to the development of efficient, highly pressurized circulatory systems as assumed previously (Sage and Gray, 1977; Sage, 1982). Such an evolution may simultaneously have improved other organ systems, such as the lungs or the integument, to resist increasing mechanical demands, particularly in the amniote groups. The latter animals seem to possess the most effective elastin, which functions 'rubber-like' and energy-saving (Gosline, 1978; Sage, 1982). Both aspects, rapid evolution as well as the adaptive structural and functional modification of elastin (Sage and Gray, 1977), may be based on the broad evolutionary versatility of the fibroblasts/fibrocytes involved in elastin formation. In view of the essential inductive influences of these ectomesenchymal (neural crest) cells on the origin of feather follicles and hair follicles (e.g., Sengel, 1976; Meyer and Röhrs, 1986), a close co-evolutionary process in the adaptive variation of elastin and these complex skin appendages could be presumed. Both feathers and hairs, can be moved very precisely and effectively by the specific arrangement of elastic tissue (for birds see, Petry, 1951), and, thus, insulation

of the animals by plumage or hair coat is improved, so that homiothermy is supported.

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Discussion with Reviewers

B. Forslind: In electron microscopy, physiological buffers are generally used not only as vehicles for the fixatives but also for rinsing purposes after fixation. Physiological buffers, e.g., phosphate buffers are used for extraction of proteins by biochemists. Assuming that the fixative has indeed stabilized all components of the tissue structures at cellular and subcellular levels, what is the rationale of using physiological buffers for rinsing? Please comment on your experience of using water instead of physiological buffers in the rinsing step(s) of the preparation sequence.

Authors: We generally do not rinse skin specimens in water instead of physiological buffers for TEM or SEM purposes. Our experience over a period of more than 20 years includes only the use of phosphate buffer or cacodylate buffer, but until now we did not find any significant differences in cell or tissue ultrastructural quality when these two buffers are compared.

B. Forslind: In view of your statement that there is an abundance of elastin fibres in the dermis, I suppose that this could be an effect of shrinkage of the original sample volume and swelling of the elastin. Have you made any approximate evaluation of the actual linear and volume shrinkage of your specimens and possible elastin swelling as an effect of autoclaving and other preparation methods?

Authors: This problem has already been discussed previously (see e.g., Meyer and Neurand, 1987). For more than 10 years, we have regularly done controls of all the domesticated species investigated to define the effects of shrinkage by the use of unfixed and fixed frozen skin sections (including confocal SEM in this study), and the embedding of skin material in specific methacrylate resins that avoid shrinkage artefacts [see e.g., Hanstede and Gerrits, *J Microsc* 131: 79-86 (1983)]. This experience has shown that autoclaved skin specimens show a species-related linear or volume shrinkage of 10-35%. This shrinkage is generally taken into account in the description and interpretation of skin structure as far as the use of autoclaving methods and SEM are concerned. Summarizing our findings in the demonstration of integumental elastic fibres, we do not think that this methodical approach causes severe changes in the basic arrangement pattern of these skin elements.

B. Forslind: In human skin, the appearance of the elastic tissue undergoes changes with increasing age as well as duration of sun exposure [Tsuji and Hamada, *Brit J Derm* 105: 57-63 (1981); Tsuji, *J Cut Pathol* 11: 300-308 (1984)]. You have not indicated corresponding effects in your animals. Please comment.

Authors: We normally use only adult animals of known age. This implies that we do not use domesticated mammals that show typical symptoms of senescence. Such material is rather rare, even in the Veterinary School of Hannover. However, such an approach would be very interesting and promising for the future.

C. Doillon: In human skin, and using similar techniques as developed in the present paper, are the elastic fibers closely distributed nearby hair follicles as found in animals?

Authors: As far as we know, elastic fibres around hair follicles have not been demonstrated in humans by autoclaving and SEM. Any comparable findings exclusively depend on standard histological techniques.

C. Doillon: Using autoclave technology that basically removes collagen by denaturation, do you think that gelatin residue can mask or coat elastic fibers?

Authors: We do think that gelatin residues can coat elastic fibres to a certain extent. This implies that for proper SEM results, all skin specimens have to be controlled histologically after the autoclaving procedure. This is best done by paraffin histology and orcein staining. In this way, any material masking elastic fibres can be identified to avoid misinterpretation of the SEM findings (see, e.g., Tsuji *et al.*, 1979; Meyer *et al.*, 1981a).

C.W. Kischer: There are differences in elastin content among all the animals tested as shown in Figure 1. But, what about possible differences among breeds, for example in the case of dogs, sheep, etc.? Would this be significant?

Authors: From our first results on elastin content in mammalian skin as given in this study, we think that differences between breeds may exist, in particular with varying hair density, hair quality, and body size, especially in dogs. However, we have not investigated enough material until now. For the present study, we tried to avoid large variations in animal size, and we mostly used mixed breeds.

C.W. Kischer: The views of elastic fibers by the several methods were obtained under no-load conditions. Do you think that a different architecture might be observed if the skins were placed under load condition?

Authors: We do not think that load conditions would change the basic architecture of the integumental elastic fibre system present. Nevertheless, specific changes could occur but cannot be verified by SEM technique. However, a modified histological fluorescence technique using rapidly frozen sections could be effective for such an investigation.