Since vertical tissue sections used for the study of the human cutaneous nervous system inherently allow visualization of only a small part of the mainly horizontally oriented cutaneous nerves, we searched for possibilities to extend this view. We now propose a method based on the immuno-staining of dermal sheet preparations for subsequent analysis by electron-, light- or laser scanning microscopy. Dermal sheet preparations for the first time allowed the imaging of the complex structure of the nerve end organ over several cm², and facilitated viewing of its topological relationship to other tissue components. We could visualize that the bulk of free ending nerve fibers ramified within 25 μm of the dermo-epidermal junction, whereas below that only larger nerve bundles were present. This method further allowed the detection and quantification of NCAM/CD56⁺ non-myelinating Schwann cells which envelope terminal axons within the dermis. Depending on the body region, we detected between 140 to over 300 individual terminal Schwann cells per mm² skin surface. Our method should allow the acquisition of new insights into the highly organized architecture of the skin nerve end organ. Its further application will give new impetus in the investigation of alterations of this skin compartment under pathological conditions.

Key words: skin nerves/Schwann cell/sensory end organ/nerve endings.

tool for studying cutaneous nerves (Wang et al, 1990; McCarthy et al, 1995; Kennedy et al, 1996; Johansson et al, 1999; Kennedy et al, 1999). Nevertheless, most of these studies have focused on the analysis of tissue sections, which is not practical for analyzing the innervation of large skin areas and thus overlooks the complexity of the nerve end organ.

In this study we introduce simple measures for the topographic examination of the cutaneous nerve system. We make use of dermal sheet preparations, in which nerve structures are readily accessible for immunostaining and subsequent analysis by light and fluorescence microscopy. We were able to reconstruct the intact nerve end organ in situ in three dimensions and to follow individual terminal nerve fibers for several millimeters. We find that the terminal nerve plexus is accompanied by single Schwann cells in a regular pattern and that it is organized in a highly complex spatial manner with other compartments in the skin. This novel approach will be useful in the characterization of the nerve end organ under physiologic conditions and may provide new insights into potential alterations during diseases of the skin.

Results

Removal of epidermis from skin biopsies makes the nerve end organ directly accessible to immunohistochemical analysis. Monoclonal antibodies against PgP9.5 (Daalsgard et al, 1989; McCarthy et al, 1995; Lauria, 1999) and NCAM/CD56 (Le Forestier et al, 1993) have been widely used for the analysis of nerve fibers in tissue sections, which, however, only provided a small window into the complex architecture of the nerve network in the skin. To circumvent this problem, these antibodies were reacted to dermal sheets, which were prepared as detailed in Fig 1. This resulted in the distinct staining of a regular network of fibers throughout the entire superficial dermis, which was further accessible for analysis by conventional light microscopy (Fig 2). The thickness of the terminal fibers stained for NCAM/CD56 (Fig 2A) ranged between an estimated 1 to 3 μm as calculated with a stage micrometer and continuously decreased toward the nerve endings. Virtually none of these endings appeared to be associated with a defined cellular structure, but ended freely. The nerve network could be analyzed throughout the entire surface of a dermal sheet, that is, areas up to several square centimeters. Individual fibers merged into nerve bundles which entered the deeper dermis and could be observed to a depth of more than 100 μm. Consistent with what we had observed in tissue sections, staining for PgP9.5 was not as strong as for NCAM/CD56. Skin appendages situated below the upper reticular dermis including hair follicles and sweat glands were not present in the dermal sheet preparations because they were below the range of the dermatome. Follicle shafts and sweat ducts were torn out of the dermal sheet during the separation procedure. We did not observe obvious differences in the immunostaining results between dermal sheets derived from autopsy skin samples up to 4 d postmortem and freshly collected skin samples. To investigate potential regional differences, dermal sheets were prepared from skin samples derived from thighs, lower leg, back of the foot, lower arm, breast, chest, and back. These tissues essentially showed a similar structural pattern of the dermal nerve plexus (data not shown). From these results we concluded that dermal sheet preparations represent an excellent tool to study the topography of the cutaneous nerve end organs.

Terminal nerve fibers are regularly enveloped by single Schwann cells. Along the course of nerve fibers, preferentially at junctions, we observed NCAM/CD56+ protrusions with diameters ranging from 5 to 15 μm (Fig 2A, arrows). Hematoxylin staining of dermal sheets demonstrated that...
each of these protrusions contained a nucleus and, thus, represented cellular elements. These cells were tightly attached to the nerve fibers (Fig 2B, arrowheads) and appeared to ensheathe terminal nerve endings. To get an impression as to the number of Schwann cells per square millimeter of skin, we counted individual nuclei of NCAM/CD56$^+$ stained cells associated with nerve fibers in some samples. In two samples derived from chest skin $323 \pm 8.3$ and $287 \pm 38.1$ (mean $\pm$ SD) Schwann cells per square millimeter of skin were found. In one sample of each breast skin, thigh skin, and abdominal skin, $246 \pm 12.6$, $147 \pm 22$, and $186 \pm 4.8$ cells were found, respectively. To further characterize the NCAM/CD56$^+$ cells, immunoelectron microscopy of dermal sheets was performed. NCAM/CD56 immunoreactivity was detected on both the outer membrane and the invaginations of cells with ultrastructural features of Schwann cells. Embedded within the cytoplasm of Schwann cells, one or several axons of unmyelinated nerve fibers were visible (Fig 3). These results demonstrate the arrangement and distribution as well as the high frequency of terminal Schwann cells within the superficial dermis and suggest an important role for these cells in the biology of terminal nerve endings.

Three-dimensional reconstruction allows visualization of the nerve end organ in its topographic relationship with other tissue components. Having established the topography of the dermal nerve network by light microscopy, we subjected immunostained dermal sheets to confocal laser scanning microscopy to reconstruct the network in its three dimensions. A total of 84 planes per sheet with a thickness of 600 nm each was acquired, and the three-dimensional reconstruction of such a data set is shown as a shadow projection in Fig 4A–C and as maximum intensity projection in Fig 4D. To visualize the dermal capillary plexus, sheets were double stained with anti-vWF (Fig 4B–F, red; Bukh et al, 1986). Larger trunks of the nerve end organ were found to intertwine with the superficial capillary plexus, whereas no obvious topographic relation existed between the terminal nerve endings and blood vessels. No direct contact between nerve endings and endothelial cells were observed in this study. Nevertheless, this might be due to the fact that anti-vWF staining decorates cytoplasmic Weibel Palade bodies and not the endothelial cell membrane. Further studies analyzing the topologic relationship of nerve endings with the subepidermal capillary plexus in the skin are currently ongoing. Nearly all penicillate nerve endings spread out within 25 $\mu$m below the dermoepidermal junction, as shown by restricting the analysis to the 40 superficial planes obtained by laser scanning microscopy (Fig 4F). Below this level, preferentially larger nerve trunks were present (Fig 4F). Thus, our illustration of the nerve end organ in three dimensions ascertains its highly complex arrangement and its association with other dermal components.

Figure 2
Depiction of the superficial cutaneous nerve network in dermal sheet preparations by immunostaining. Dermal sheets were immunostained for NCAM/CD56 (A,B) or PgP9.5 (C) as specified in experimental procedures and analyzed by light or fluorescence microscopy. Arrows in A mark some of the protrusions that were found regularly in the course of the terminal nerve endings. For B, cell nuclei (arrowheads) in sheets immunostained for NCAM/CD56 were counterstained with hematoxylin. These dermal sheet preparations were derived from thigh (A,B) and breast skin (C).
In this study, we introduce a novel approach for the analysis of the nerve end organ of human skin in situ. Our method is based on the separation of the dermis from the overlying epidermis at the basement membrane (Juhlin and Shelley, 1977; Kitano and Okada, 1983), which makes superficial dermal structures directly accessible for immunostaining procedures and subsequent microscopic examination. Using confocal laser microscopy and computer-assisted three-dimensional reconstruction, our study extends previous attempts to illustrate the spatial architecture of dermal nerves (Winkelmann et al., 1967; Kennedy et al., 1994; Guinard et al., 1998; Griffin et al., 2001) and visualizes the spatial relationship of the nerve end organ to other dermal structures such as the blood capillary system.

The importance of the cutaneous nerve system is best exemplified under conditions where functional changes lead to alterations of skin homeostasis. For example, in peripheral neuropathy, a reduced number of nerve endings is among the earliest events (McCarthy et al., 1995; Kennedy et al., 1996; Kennedy et al., 1999); in leprosy, nerves are a target of Mycobacterium leprae infection (Haimanot and Melaku, 2000); and in certain allergic diseases, such as atopic dermatitis, nerves may play a pathogenic role in the exacerbation and/or maintenance of disease (Winkelmann, 1960). Several of these conditions are very common and lead to enormous morbidity of affected patients, thus urging for the inclusion of the nerve plexus in pathogenic and therapeutic considerations. Although a key role of the nerve end organ in disease has been well realized, the study of its changes has been hampered by the fact that imaging of nerves is inherently difficult. Early efforts by manual illustrations of the nerve plexus from histologic sections gave some insight into its anatomy, but were not feasible for the performance of large-scale systematic disease studies. More recently, the availability of immunoreagents and laser scanning microscopy has made the selective visualization of nerves within the complex dermal microenvironment possible.
possible (McCarthy et al, 1995; Kennedy et al, 1996; Kennedy et al, 1999). Nevertheless, to date, most studies have focused on tissue sections, which fail to grasp the complexity of the nerve plexus.

Confocal laser microscopy facilitates the visualization of structures within tissues to a depth of at least 100 μm, which, at a vertical diameter of 50 to 100 μm, includes the entire superficial dermis. Importantly, the architecture of all compartments within the dermis is preserved in dermal sheets. Consequently, the relationship of the nerve end organ with adjacent structures can be studied in detail over large tissue areas. We have used NCAM/CD56 in combination with vWF, which is expressed by blood vessel endothelial cells, to examine the anatomical topography of these compartments. Interestingly, large nerve bundles were found in a close spatial association with the vascular plexus, with small nerve fibers left to end freely in the dermis. With the present methodology more in depth questions such as the relationship of the nerve and vascular plexus during healing of skin wounds or during inflammatory skin diseases can be addressed.

Three-dimensional reconstruction of images obtained by this method have demonstrated that the termination of the cutaneous nerves into penicillate nerve endings is confined to an area of 25 μm immediately below the dermoepidermal junction. In deeper layers mainly larger nerve bundles were detectable. Investigations of epidermal sheets, which corresponded to the respective dermal sheet preparations, revealed only a sparse number of nerve endings, irrespective of the separation technique used (data not shown). This finding was surprising, however; because the presence of epidermal nerve endings has been well documented (Arthur and Shelley, 1959; Hilliges et al, 1995; Kennedy et al, 1996; Johansson et al, 1999; Kennedy et al, 1999; Lauria, 1999), we assume that in the course of the separation techniques used, epidermal nerve fibers might get lost or damaged and are no longer detectable. We are currently trying to adapt our separation technique to extend the analyses to epidermal sheets.

In addition to a three-dimensional view of the cutaneous nerve plexus, we could demonstrate that terminal nerve endings are regularly enveloped by single NCAM/CD56+ Schwann cells. Schwann cells in the skin have been described in earlier electron microscopic studies (Cauna, 1973; Orfano and Mahrle, 1973; Breathnach, 1977). Nevertheless, their abundance and spatial distribution had previously, to the best of our knowledge, not been analyzed. The finding that terminal Schwann cells are numerous and regularly located at the dermoepidermal junction raises several intriguing questions. Could it be that they play a role in the pathogenesis of skin diseases? Are terminal Schwann cells, for example, the first targets of mycobacteria and herpes viruses? What is their role in peripheral neuropathies—is the number and/or distribution of these cells disturbed in the course of these diseases? These questions cannot be answered by the use of conventional techniques that are based on sectioning of tissues. Thus, we propose that our approach will provide some of this much-needed information and might also contribute to novel diagnostic and therapeutic strategies.

Materials and Methods

Skin samples Split thickness skin was obtained from different body locations at autopsy (back of foot, n = 5; thigh, n = 5; back, n = 5; chest, n = 2), from skin samples derived from mammary (n = 2) and abdominal reduction surgery (n = 2), and from areas of clinically normal skin adjacent to skin tumors (n = 3). All experimental procedures were performed according to the World Medical Association Declaration of Helsinki and approved by the Regional Committee for Medical Research Ethics.

Preparation of dermal and epidermal sheets The separation protocol for dermal and epidermal sheets is based on previously described procedures (Juhlin and Shelley, 1977; Kitano and Okada, 1983). Briefly, split thickness skin was floated on either 2.4 U per mL Dispase (Roche, Vienna, Austria) in PBS, pH 7.4, or on a 3.8% ammonium thiocyanate solution in PBS, pH 7.4 (Fig 1). After 30 min at 37°C, the epidermis was removed from the underlying dermis with watch-maker’s forceps and washed in PBS. The size of dermal sheets ranged between 20 and 300 mm². For immunostaining purposes, sheets were fixed in acetone at 4°C for 20 min or in 4% paraformaldehyde at 4°C for 2 h. For immunoelectron microscopy, dermal sheets were incubated with the first antibody, that is, anti-neural cell adhesion molecule (NCAM)/CD56 before fixation with paraformaldehyde (see below).

Immunostaining procedures After fixation, dermal and epidermal sheets were soaked for several minutes in Tris-buffered saline (pH 7.5). Sheets fixed with paraformaldehyde were additionally treated with 0.5% Triton X-100 in Tris-buffered saline for 45 min. Subsequently, sheets were incubated in blocking buffer consisting of 2% bovine serum albumin and 10% normal serum (species of the second step immunoreagent) in Tris-buffered saline at room temperature for 30 min. Sheets were then incubated overnight at 4°C with the primary antibodies, that is, anti-PgP9.5 (Mouse IgG2b; Serotec, Oxford, UK), anti-NCAM/CD56 (mouse IgG1; Becton Dickinson, San Jose, CA) or anti-von Willebrand factor (vWF, rabbit serum; Dako, Glostrup, Denmark) diluted in blocking buffer in the respective dilutions. Sheets were soaked in the appropriate biotinylated second step reagents, that is, biotinylated sheep anti-mouse Ig (Amersham Pharmacia Biotech, Buckinghamshire, UK) or biotinylated goat anti-mouse Ig; (Amersham Pharmacia Biotech) at room temperature for 1 h (37°C) followed by incubation with the SteptABC complex (Dako) and immunohistochemistry was performed as previously described (Weninger et al, 1999). For immunofluorescence, the second step reagents were Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 546 goat anti-rabbit IgG (both from Molecular Probes, Eugene, OR). After additional washings immunostained sheets were mounted in either Aqua-mount (BDH Laboratory Supplies, Poole, UK) or Neo-Mount (Merck, Darmstadt, Germany). Immunofluorescent sheets were analyzed on either a laser confocal microscope (LSM 510, Karl Zeiss, Jena, Germany) or a conventional immunofluorescence microscope (Olympus). Images were processed using an Octave Workstation (Silicon Graphics, Inc.) with the Huygens (Scientific Volume Imaging) Imaris and colocalization (Bitplane AG, Zurich, Switzerland) software package. This software package also contains features that enable the measurement of distances within samples analyzed.

Determination of the Schwann cell number To determine the number of Schwann cells per square millimeter of skin, we performed hematoxylin counterstaining of sheet preparations previously stained immunohistochemically for NCAM/CD56. We then counted individual nuclei of NCAM/CD56+ stained cells associated with nerve fibers. Three independent visual fields have been analyzed in each dermal sheet preparation.

Immune electron microscopy For immune electron microscopy unfixed dermal sheet preparations were incubated with mouse anti-NCAM antibody for 2 h at 20°C in tissue culture medium. After
extensive washing in PBS, the sheets were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer. Further processing proceeded as previously described (Vass et al., 1986). Briefly, bound antibody was detected by a biotin/avidin technique, using species-specific biotinylated anti-mouse immunoglobulin (Amer-sham, UK) and peroxidase-labeled avidin (Sigma, St. Louis, MO). Peroxidase reaction was developed with 3,3’-diaminobenzidine tetrahydrochloride (Sigma). The material was then routinely fixed in osmic acid and embedded in epoxy resin. For control material was processed in the absence of the primary antibody or by using an irrelevant monoclonal antibody of the same immunoglobulin class. Thin sections were analyzed on a Jeol 100 × electron microscope.

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