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ORIGINAL PAPER

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Immunolocalization of tight junction proteins in the adult and developing human brain

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Abstract The formation of endothelial tight junctions (TJs) is crucial in blood-brain barrier (BBB) differentiation, and the expression and targeting of TJ-associated proteins mark the beginning of BBB functions. Using confocal microscopy, this study analyzed endothelial TJs in adult human cerebral cortex and the fetal telencephalon and leptomeninges in order to compare the localization of two TJ-associated transmembrane proteins, occludin and claudin-5. In the arterioles and microvessels of adult brain, occludin and claudin-5 form continuous bands of endothelial immunoreactivity. During fetal development, occludin and claudin-5 immunoreactivity is first detected as a diffuse labeling of endothelial cytoplasm. Later, at 14 weeks, the immunosignal for both proteins shifts from the cytoplasm to the interface of adjacent endothelial cells, forming a linear, widely discontinuous pattern of immunoreactivity that achieves an adult-like appearance within a few weeks. These results demonstrate that occludin and claudin-5 expression is an early event in human brain development, followed shortly by assembly of both proteins at the junctional areas. This incremental process suggests more rapid establishment of the human BBB, consistent with its specific function of creating a suitable environment for neuron differentiation and neurite outgrowth during neocortical histogenesis.

Keywords Occludin · Claudin-5 · Tight junctions · Blood-brain barrier · Human brain development

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Introduction

Brain microvessels are provided with a complex functional unit, the blood-brain barrier (BBB), which is formed primarily by endothelial cells supported by other specialized cell types, such as pericytes and astrocytes, which create an efficient partnership ensuring finely tuned homeostasis and rigorous protection of the neuronal microenvironment (Cancilla et al. 1993; Balabanov and Dore-Duffy 1998; Rubin and Staddon 1999; Nag 2003). Among the components of the BBB that take on this task, are the tight junctions (TJs) which seal the interendothelial space between neighboring endothelial cells and are responsible for restricting both paraendothelial diffusion of solutes (barrier function) and movement of buoyant membrane lipids and proteins (fence function) (Tsukita et al. 2001). The molecular apparatus of TJs includes TJ-associated *peripheral* proteins, also called *adapter* proteins, such as the zonula occludens (ZO) 1, 2, and 3 and MAGI proteins, whose major role is the establishment of dynamic membrane–cytoplasm interlacing and TJ–cyto-

plasm feedback, as well as TJ-associated *transmembrane* proteins, such as the junctional adhesion molecules (JAMs), occludin, and claudins, which physically occlude the intercellular cleft domains (Fanning et al. 1998; Rubin and Staddon 1999; Wolburg and Lippoldt 2002; Gonzáles-Mariscal et al. 2003). Occludin and several members of the multigene claudin family copolymerize in order to form fibrillar heteropolymers, laterally linked to JAMs (Itoh et al. 2001) and appear on freeze-fracture replicas as paired strands of particles within plasma membranes of facing cells (Furuse et al. 1999; Tsukita and Furuse 1999; Sasaki et al. 2003). Recently, the general selective role accorded to TJ unity has been revised by the concept that each of its integral proteins could be appointed to exert a substrate-specific activity (Matter and Balda 2003; Nitta et al. 2003).

The pattern of distribution of occludin and claudins considerably differs between species and cell types. Occludin is ubiquitously distributed in different epithelial cells, and it is also expressed in large amounts in brain endothelial cells, however, it is undetectable in non-neuronal endothelia (Hirase et al. 1997). This protein was initially reported in chicken brain, subsequently described in porcine and rodent brain, and only recently demonstrated in human brain endothelial cells (Furuse et al. 1993; Hirase et al. 1997; Morcos et al. 2001; Papadopoulos et al. 2001; Plumb et al. 2002). In contrast, each of the claudin subtypes shows a characteristic, prevalent tissue distribution pattern (Furuse et al. 1993, 1998; Morita et al. 1999a). Claudin-1 prevails in mouse epithelial liver and kidney TJs, but it is also expressed in chicken, rat, and human endothelial cells with a slightly stronger expression in chicken brain (Furuse et al. 1998; Liebner et al. 2000; Lippoldt et al. 2000a). Claudin-3 is present in lung and liver and in brain endothelial TJs of mice and humans (Wolburg and Lippoldt 2002; Wolburg et al. 2003). Claudin-5 is expressed ubiquitously in endothelial cells and it has been demonstrated in chicken, rat, and human brain (Morita et al. 1999a, b; Liebner et al. 2000; Lippoldt et al. 2000a). In the brain, claudin-5 is expressed in the endothelial cells of all the vessel segments, whereas in other organs it appears restricted to some segments of the vasculature (for example, kidney arteries and arterioles; Morita et al. 1999b). Although the expression, biological activity, and regulation of these proteins has been extensively studied in epithelial cells (Goodenough 1999; Nusrat et al. 2000; González-Mariscal et al. 2003; Ivanov et al. 2004), limited information is available concerning the time course of their expression and junctional targeting in endothelial cells in developing brains (Morita et al. 1999b; Kniesel and Wolburg 2000; Wolburg and Lippoldt 2002). This study compares the localization of two TJ-associated molecules, occludin and claudin-5, in human adult cerebral cortex and fetal brain, by immunohistochemistry and confocal microscopy. The results provide data on BBB development in human brain and demonstrate early expression of occludin and claudin-5 and their adult-like distribution by midgestation, when the major waves of neuronal migration have ceased, and neuronal differentiation and cortex lamination begin.

Materials and methods

Samples and tissue processing

Human autopsy specimens of normal adult cerebral cortex and fetal telencephalon were obtained from six adults (male, aged 55–65 years) and six fetuses (two for each of the examined ages: 12, 14, and 18 weeks of gestation), respectively. The sampling and handling of human specimens was done in accordance with the Ethics Protocols of the Department of Human Anatomy and Histology, University of Bari. Samples of parietal cerebral cortex and the lateral wall of the telencephalon including the pial meninges were collected and immersed in a fixative mixture of 2% paraformaldehyde plus 0.2% glutaraldehyde in 0.01 M phosphate-buffered saline (PBS), pH 7.2, for 2 h at 4°C. Cerebral cortex and telencephalon samples were sectioned at 20-μm intervals using a Vibratome (Leica, UK) and processed as free-floating sections, while the leptomeninges were removed from the underlying brain after fixation and processed as free-floating whole-mount preparations.

Immunohistochemistry

Dilutions of primary rabbit polyclonal antibodies, anti-occludin and anti-claudin-5 (Zymed Laboratories, CA, USA), in blocking buffer (BB; PBS, 1% bovine serum albumin, 2% fetal calf serum) were 1:50 and 1:100, respectively, and detected with a second-layer indirect immunofluorescence technique. The Vibratome sections were allowed to float freely with gentle agitation and immunostaining steps were as follows: (1) 30 min in BB, (2) 30 min in 0.5% Triton X-100 in PBS, (3) 1 h in one of the primary antibodies at room temperature (RT), and (4) 1 h in a fluorophore conjugate goat anti-rabbit IgG diluted 1:200 in BB (Alexa Fluor 488; Molecular Probes, OR, USA) at RT. The sections were washed three times for 10 min in PBS between each step and, when nuclei counterstain was required, TO-PRO™-3 iodide (Molecular Probes) diluted 1:10 K in PBS was added to the third wash after the conjugates. The sections were then transferred on subbed slides, carefully drained, coverslipped in Vectashield mounting medium (Vector Laboratories, CA, USA), and sealed with nail varnish. The negative control sections were prepared by substituting the primary antibodies with BB.

Single and double labeling with collagen type-IV were performed to enabled examination of the vessel course and visualize vascular wall details. Using the previously described immunohistochemical method, a monoclonal antibody anti-human collagen type-IV (diluted 1:50 in BB; clone CIV 22; Dako, Italy) was revealed with a fluorophore conjugate goat anti-mouse IgG Alexa Fluor 568. For double labeling, either anti-occludin or anti-claudin-5 revealed by a goat anti-rabbit IgG Alexa Fluor 488 (diluted 1:200 in BB; Molecular Probes) were added to collagen type-IV immunoreaction. The control sections were prepared: (1) by substituting the primary antibodies either with BB or with an inappropriate monoclonal antibody of identical subclass or a normal rabbit serum, or (2) mismatching the secondary antibodies.

Confocal laser scanning microscopy

The sections were viewed under the Leica TCS SP2 confocal laser scanning microscope using a 40× and 63× oil-immersion objective lens with either 1× or 2× zoom factors. Alexa Fluor 488 was processed with an excitation at 488 nm and a detection range from 500 to 535 nm, and Alexa Fluor 568 with an excitation at 568 nm and a detection range from 580 to 620 nm. On the double-immunolabeled sections, a sequential scan procedure was applied during image acquisition of the two fluorophores. Confocal images were taken at

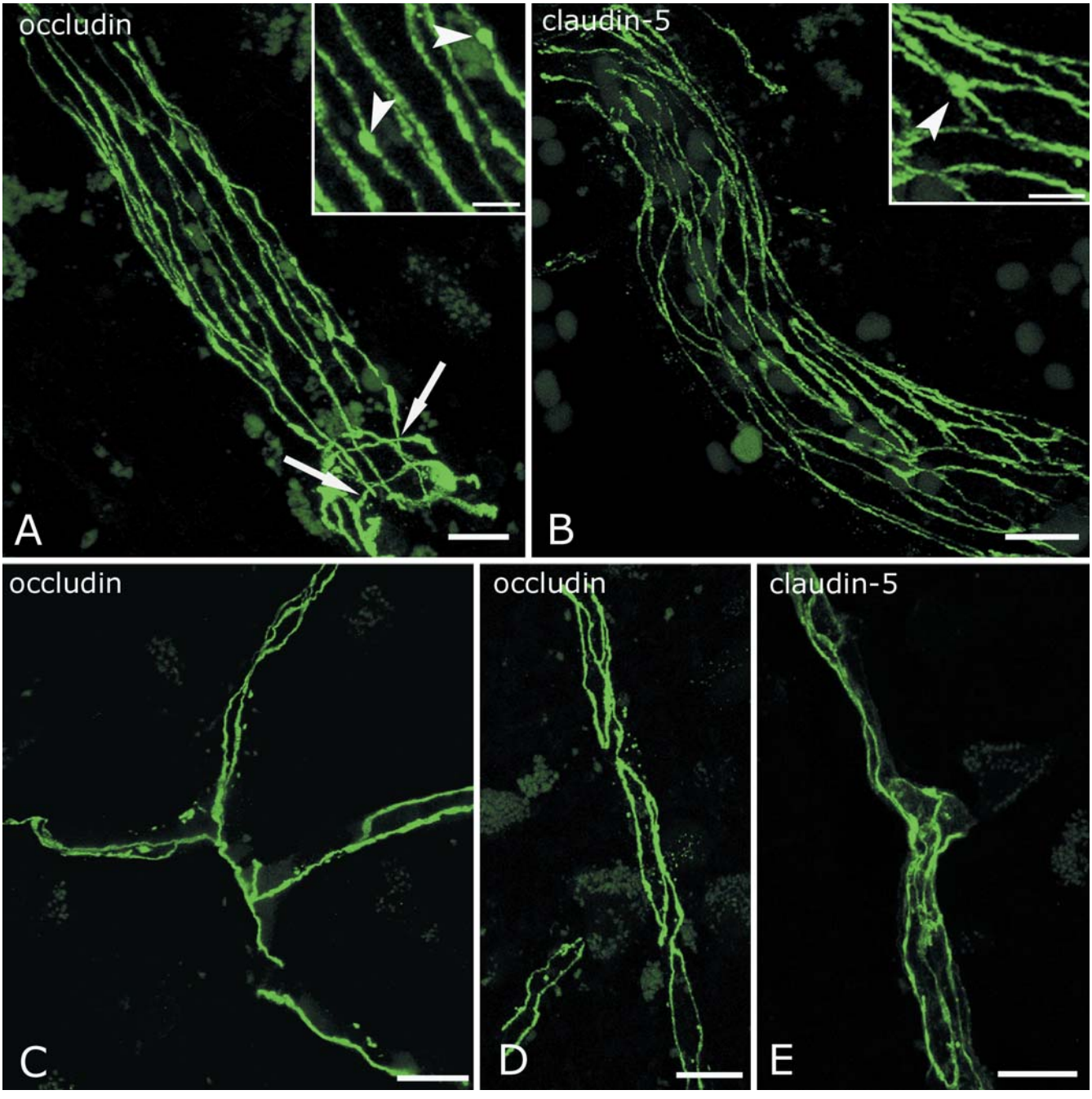


Fig. 1A–E Immunolabeling of adult cerebral cortex with occludin (**A**, **C**, **D**) and claudin-5 (**B**, **E**). Immunolabeling of large caliber vessels with both occludin (**A**) and claudin-5 (**B**) shows strong linear signals which clearly delineate the longitudinally and orthogonally (*arrows*) oriented endothelial profiles. *Insets* in (**A**) and (**B**) demonstrate details of immunoreactive tight junction plaques (*arrowheads*). Continuous, large bands of occludin (**C**, **D**) and claudin (**E**) immunoreactivity are present in the walls of the cortical microvessels. *Scale bars* 15 μm in **A–E**; 5 μm in *insets*

0.3-μm intervals through the *z*-axis of the section covering a total of 20 μm in depth. Single optical planes were separately analyzed in order to collect data at different levels along the vertical axis. Finally, multiple serial optical sections were compressed to obtain one image with extended focus (maximum projection). Single planes and maximum projections were recorded digitally, stored as TIFF files, and subsequently analyzed by Adobe Photoshop software (Adobe Systems, CA).

Results

Adult cerebral cortex

The arterioles which penetrate the cerebral cortex show a distinct linear pattern of immunoreactivity with both occludin and claudin-5 antibodies (Fig. 1A, B). The immunoreactivity corresponds to the cell–cell contact areas

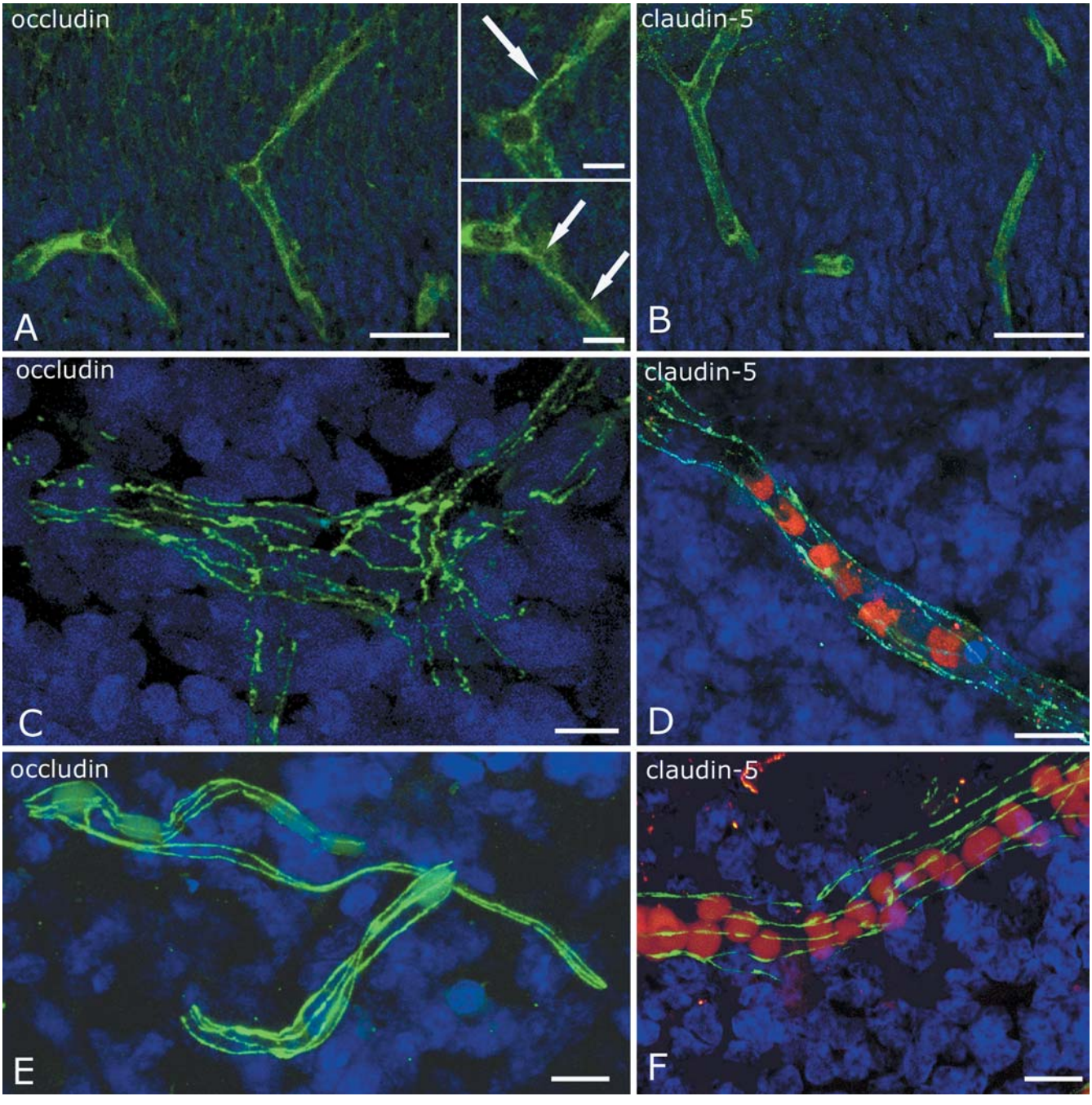
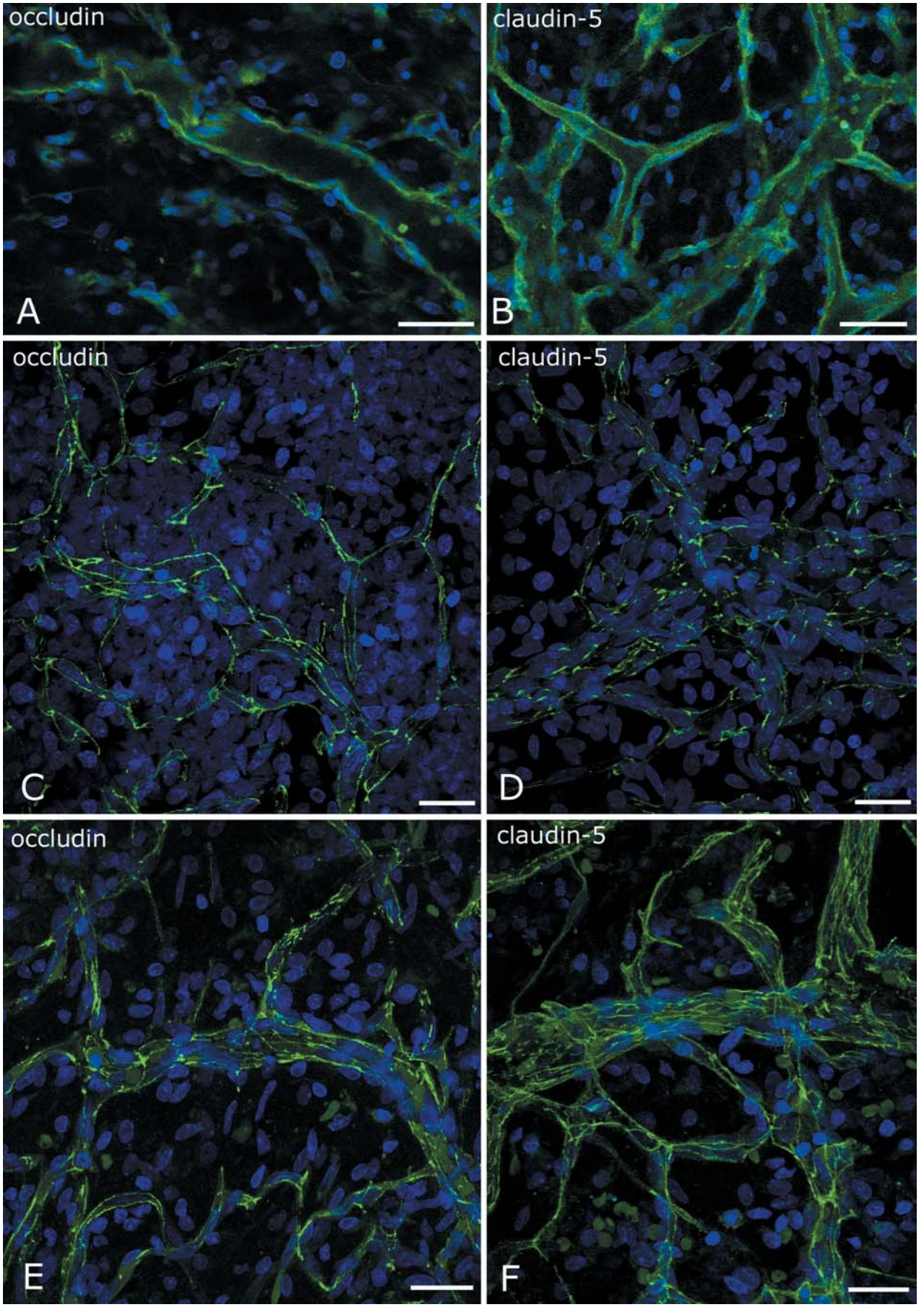


Fig. 2A–F Fetal telencephalon at 12 (**A**, **B**), 14 (**C**, **D**), and 18 (**E**, **F**) weeks of development. At 12 weeks, intense endothelial cytoplasm immunoreactivity is present in microvessels with occludin (**A**) and claudin-5 (**B**), and signs of an initial linear cell membrane assembly with occludin are present (**A**, *insets*; *arrows*). At 14 weeks, there is linear and beaded staining at the endothelial borders of microvessels with occludin (**C**) and claudin-5 (**D**). Nearly continuous bands of occludin (**E**) and claudin-5 (**F**) immunoreactivity characterize the microvessel walls at 18 weeks. *Scale bars* 15 μm in **A**, **B**, **D**; 5 μm in *insets*; 10 μm in **C**, **E**, **F**

of the endothelial cells and, outlining the endothelial profiles, reveals sequences of parallel and regular endothelial stripes and rhomboid scales as well (Fig. 1A, B). The immunolabeling appears continuous and oriented along the long axis of the vessel, except where collaterals originate and orthogonally oriented endothelial cells are visualized (electronic supplementary Fig. I available at http://dx.doi.org/10.1007/s00418-004-0665-1). In these

Fig. 3A–F Fetal leptomeninges at 12 (**A**, **B**), 14 (**C**, **D**), and 18 (**E**, **F**) weeks of development. At 12 weeks, there is diffuse endothelial staining with occludin (**A**) and claudin-5 (**B**). At 14 weeks, interrupted lines of occludin immunostaining (**C**) and beaded claudin-5 immunoreactivity (**D**) are present in the meningeal vessels. At 18 weeks, there is a more continuous linear immunoreactivity for both proteins (**D**, **E**). *Scale bars* 30 μm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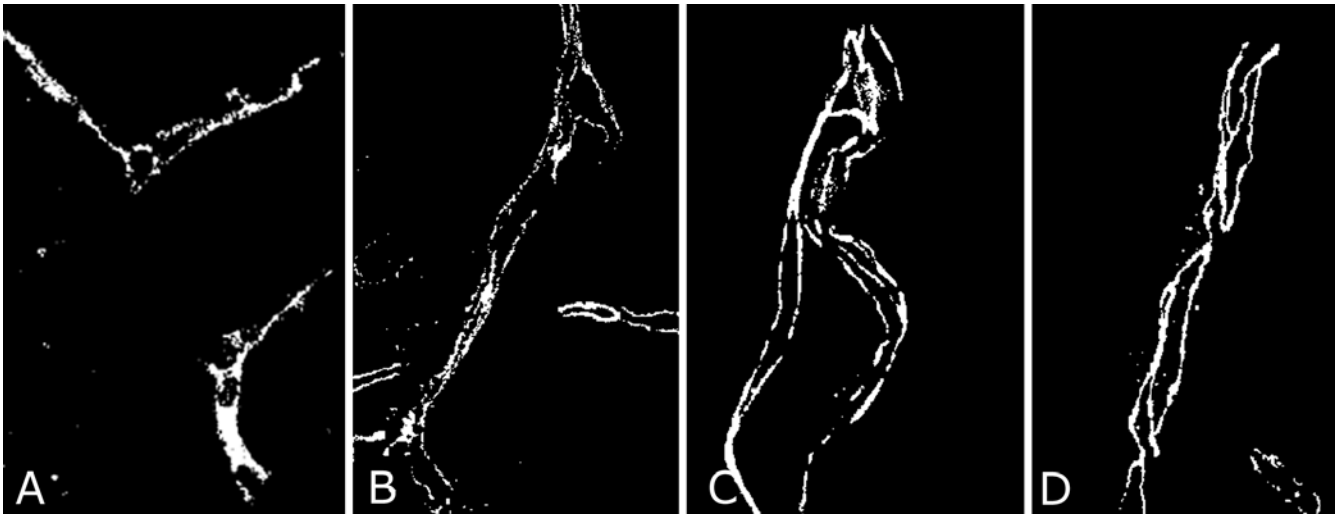

Fig. 4A–D Occludin immunolabeling of telencephalon microvessels at 12 (**A**), 14 (**B**), and 18 (**C**) weeks of development and in the adult cerebral cortex (**D**). The binary image conversion emphasizes the chronological changes in occludin expression, as well as the similarities between late fetal (**C**) and adult (**D**) immunoreactivity

large vessels, the regular continuity of the staining appears accentuated by small plaques of immunoreactivity located either along the linear labeling or at the edges where endothelial cells meet (*insets* in Fig. 1A, B). The same linear and continuous pattern of staining is also detected on the cortex microvessels with both occludin and claudin-5 antibodies (Fig. 1C–E). These vessels are marked by one to three large bands of immunoreactivity which run parallel to the long axis of the vessel for its entire length and bifurcate on the larger vessel tracts, delimiting single endothelial profiles (Fig. 1C–E).

Fetal telencephalon

At 12th week of gestation intense staining for both occludin and claudin-5 is recognizable in the telencephalon microvessels, on primary vessel trunks, which radially penetrate the future cerebral cortex, and on their peripheral branches located in the deep ventricular zone (Fig. 2A, B). Immunolabeling for both proteins, occludin and claudin-5, is punctate and present diffusely in the endothelial cytoplasm (Fig. 2A, B). On a few microvessels, occludin staining also appears as linear immunoreactivity which stands out above the labeled cytoplasm and is likely to correspond to primary assemblies of occludin molecules at the interface of adjacent endothelial cells (*insets* in Fig. 2A). With claudin-5, despite the strong cytoplasmic immunoreactivity, signs of a linear signal are rarely observed and are invariably weaker. Two weeks later, at the 14th week of gestation, growing vascular side branches anastomose, forming a regular network (electronic supplementary Fig. II available at http://dx.doi.org/10.1007/s00418-004-0665-1). The diffuse cytoplasmic immunoreactivity to occludin and claudin-5 changes completely into a discrete labeling restricted to the endothelial cell–cell contacts (Fig. 2C, D). Occludin immunoreactivity is intense and frequently consists of linear tracts interrupted by focal discontinuities, or of rows of fine puncta (Fig. 2C). The immunoreactivity to claudin-5 is mainly formed by rows of either distinct or fused puncta, linearly arranged along the endothelial margins (Fig. 2D). Some of the small microvessels show a more continuous junctional immunoreactivity to both proteins with thin, diaphragm-like tracts along the intense, linear signal (electronic supplementary Fig. III available at http://dx.doi.org/10.1007/s00418-004-0665-1). Later on, at midgestation (18 weeks) the pattern of immunostaining changes again from a preferentially punctate staining to an almost uninterrupted linear signal (Fig. 2E, F). Occludin immunoreactivity is strong and consists of large bands oriented along the long axis of the vessel (Fig. 2E). At this developmental age, a continuous staining pattern is also observed with claudin-5, consisting of thin linear bands along the long axis of the vessel with few areas of discontinuous immunostaining (Fig. 2F).

Fetal leptomeninges

In order to complement the study of fetal brain microvessels, a parallel study was carried out on microvessels of the telencephalic leptomeninges. In the meningeal microvessels, the timing of occludin and claudin-5 expression and the distribution of the staining pattern were similar to those of the telencephalon microvessels (Fig. 3A–F). At the 12th week of gestation, an intense cytoplasmic immunoreactivity was observed to both proteins on large caliber and small vessels (Fig. 3A, B). At the 14th week, as in the brain endothelial cells, the observed punctate staining diffusely in the cytoplasm changed into a linear labeling along junctions with both occludin and claudin-5. The prevalence of short immunoreactive tracts interrupted by negative ones,

gave a typical beaded appearance to the vessel walls (Fig. 3C, D). At midgestation this intermittent pattern of immunoreactivity changed into more uniform linear staining (Fig. 3E, F).

Discussion

Analysis of sections of the cerebral cortex immunolabeled for occludin and claudin-5 by laser confocal microscopy allows visualization of the TJ-associated molecules in the vessel walls and provides details of TJ organization. In penetrating large vessels and in cortex microvessels, a common feature of *mature* TJs is their continuous profile and orientation along the long axis of the vessel. However, while the TJs of large vessels show thin, linear staining, the bands of immunoreactivity in small vessels tended to be thicker suggesting more extensive junctional regions, consistent with their greater involvement in BBB functions (Nagy et al. 1984). Another important feature of the large caliber cortical vessels is the presence of small plaques of immunoreactivity that could correspond to a different local organization of the TJ proteins and, as has been suggested by freeze-fracture studies of stroke-prone spontaneously hypertensive rats, may be considered indicators of a dynamic state of TJs (Lippoldt et al. 2000b).

In fetal telencephalon microvessels, an adult-like pattern of the endothelial TJs is achieved by midgestation, when strong, near continuous labeling is observed especially with occludin. Vascularization of the human telencephalon does not start until around the 8th week of development within the primitive meningeal layers by in situ differentiation of endothelial cell precursors (vasculogenesis). From these new-formed leptomeningeal vessels, and according to the angiogenic mechanisms of vascular budding and sprouting, penetrating vessels invade the nervous wall, lengthen, and anastomose, giving rise within a few weeks to an intraneural vascular network (Bär 1980; Norman and O'Kusky 1986; Marín-Padilla 1987; Risau 1993, 1997; Plate 1999). Occludin and claudin-5 are already expressed in the primary vessels of the 12th week telencephalon and by midgestation (18 weeks) they show dramatic changes (Fig. 4A–C for a schematic summary). The most critical change occurs between 12 and 14 weeks of fetal development, when occludin and claudin-5 immunoreactivity shifts from the endothelial cytoplasm to the endothelial borders and concentrates in linear, discontinuous tracts that may correspond to simple, incomplete networks of junctional strands. Soon after, a near-continuous staining pattern is detectable as assembly of proteins at the junctional areas proceeds, and more extended and complex strands form. Formation of telencephalon endothelial TJs reflects the progressive process observed in rodent and avian brain (Roncali et al. 1986; Vorbrodt and Dobrogowska 1994, 2003; Kniesel et al. 1996; Vorbrodt et al. 2001; Bertossi et al. 2002), but our results indicate that in humans the whole program begins earlier and proceeds faster. In fact, as primate neocortex histogenesis differs greatly from that of rodents, both quantitatively and qualitatively as well as in terms of the relative sequences of complex changes (Meyer et al. 2000; Smart et al. 2002), it may need controlled environmental conditions supported by competent BBB activities. Our previous studies, in agreement with the few reports carried out in human and other species (Saunders 1977; Dziegielewska et al. 1979; Møllgård and Saunders 1986; Bauer et al. 1993, 1995), corroborate the concept of a precocious 'working attitude' of the human fetal BBB. During human telencephalon development, the endothelial cells express high levels of GLUT-1 (glucose transporter isoform 1) with an adult-like asymmetrical distribution on the opposite endothelial membranes (Virgintino et al. 1998a, 2000), a subcellular localization that seems to be regulated by a fence function of the TJs (Gerhart et al. 1989; Dobrogowska and Vorbrodt 1999; Lippoldt et al. 2000b) and that temporally corresponds to the presence of an endothelial barrier to endogenous serum albumin (Virgintino et al. 2000).

The slight timing difference observed in membrane targeting between occludin and claudin-5 supports the different roles attributed to these molecules during TJ formation. In other non-mammalian and mammalian species, occludin appears first in the cell membrane, followed by cingulin, ZO-1, and claudin-5 (González-Mariscal et al. 2003). Although studies carried out on occludin-deficient mice have demonstrated that occludin is not required for formation of TJ strands, recent data have shown that it is responsible for sealing of TJs and regulation of their barrier properties (Hirase et al. 1997; Lacaz-Vieira et al. 1999; Saitou et al. 2000; Wolburg and Lippoldt 2002).

Regarding leptomeningeal TJ development, the overlapping sequence of events observed in the telencephalic and meningeal vessels suggests that occludin and claudin-5 expression and distribution are independent of the modes (vasculogenesis versus angiogenesis) and timing of vessel formation and, conversely, are related to the telencephalon histogenetic program by means of neural cell signals derived from the microenvironment (Rubin and Staddon 1999; Bauer and Bauer 2000). The possible candidates for this signaling role are envelopes of perivascular astrocytes and astrocyte endfeet forming the glial limiting membrane underlying the leptomeningeal sheet. Several studies have shown that astrocytes participate in the induction of barrier properties in endothelial cells by direct contact and/or releasing of soluble factors (Janzer and Raff 1987; Laterra et al. 1990; Cassella et al. 1996; Kacem et al. 1998; Pekny et al. 1998; Sobue et al. 1999; Abbott 2002; Willis et al. 2004). In human fetal telencephalon, radial glia cells make contacts with the walls of telencephalon microvessels and terminate by forming the glial limiting membrane at the leptomeningeal surface (Marín-Padilla 1995; Virgintino et al. 1998b; Bertossi et al. 1999). These early relationships suggest the possible involvement of radial glia cells in the endothelial expression of TJ-associated proteins of both telencephalon and leptomeningeal microvessels.

Our morphological approach does not provide direct information on the TJ functional state, but it is noteworthy

that increased electrical resistance, predictive of a restricted paracellular flow, has been correlated with the presence of occludin in the endothelial cell junctions (Hirase et al. 1997), as well as with a collagen type-IV enriched vascular substratum (Tilling et al. 1998). Collagen type-IV has been demonstrated to induce TJ formation in endothelial cells in vitro, thus directly influencing the expression of occludin (Tagami et al. 1992; Savettieri et al. 2000). Therefore, the demonstration in the 14-week fetus of occludin and claudin-5 concentrated at the endothelial junctional domains and the presence of a collagen type-IV positive vascular basal lamina strongly suggest an early onset of barrier functions in human telencephalon microvessels.

In conclusion, our studies of fetal development, from the 12th to the 18th week of gestation, which corresponds to the peak time period of telencephalon corticogenesis and angiogenesis, show that the telencephalon microvessels undergo a combination of growth and differentiation which matches the metabolic demands of the progressively expanding neocortex and guarantee a constant chemical environment and a protected habitat for the differentiating neurons.

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