Different distribution of S-100 protein and glial fibrillary acidic protein (GFAP) immunoreactive cells and their relations with nitrergic neurons in the human fetal small intestine

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Summary. The appearance, distribution and some histochemical features of non-neuronal cells (NN cells) associated with the myenteric plexus of human fetal small intestine have been studied by means of S-100 protein and GFAP immunocytochemistry between the 10th and 17th week of gestation. In addition, double labelling immunocytochemistry using an antibody raised against a constitutive isoform of nitric oxide synthase (nNOS) in combination with an S-100 protein antibody was applied to investigate the morphological relations between NN cells and nitrergic neurons in the developing gut wall. Cells with immunoreactivity for both glial-specific proteins are already present in the 10th week of gestation. While cells with S-100 protein immunoreactivity are located within the circular muscle layer as well as in the myenteric, and submucous plexuses, cells with GFAP immunopositivity are mainly restricted to the side of the myenteric plexus adjacent to the longitudinal muscle layer. In contrast to the dense network formed by S-100 protein immunopositive structures the GFAP immunopositive cells appear singly and do not connect into a network. Double-labelling immunocytochemistry reveals nitrergic fibers (NOS-IR) in close relation to the S-100 protein immunoreactive glial network. NOS-IR varicosities are in close association with the surface of those cells both in the circular muscle layer (CM) and in the tertiary plexus. It is concluded that two populations of NN cells with different locations and different immunohistochemical characters appear and develop together with the enteric ganglia in the human fetal intestine. The close morphological relation of NOS-IR fibers with S-100 protein immunopositive cellular network indicate a functional relationship between S-100 protein immunopositive cells and the nitrergic nerves during the early development of human enteric nervous system (ENS).

Key words: Non-neuronal cells, Development, Immunocytochemistry, Human intestine

Introduction

Myenteric ganglia are partly encapsulated by specialized populations of non-neuronal cells (NN cells) of different origin, even in early embryonic life (Daikoku et al., 1975; Boros and Fekete 1993). The cells, isolating nerve cells from other tissues of the gut wall, might have a crucial role in determining the ganglionic microenvironment necessary for the maturation of enteric ganglia. The cytodifferentiation of the NN cells associated with the myenteric plexus (MP) has been previously studied in rabbits (Gershon and Thompson, 1973), mice (Faussone-Pellegrini, 1985), guinea pigs (Gershon et al., 1973) and chicks (Le Douarin and Teillet, 1971; Gabella, 1989). Species differences in the chronological appearance of the different cell types as well as a basically different relationship of NN cells to axons or perikarya have been revealed. There are controversies as to their nature, significance and embryonic origin in the different species investigated, primarily due to the lack of distinctive markers that can be used at both the light and electron microscopic level. Although S-100 protein is considered a common marker for the enteroglia of the ENS (Ferri et al., 1982), a spectrum of enteroglial cells have been determined with considerable variations in their morphology, distribution and histochemical characteristics (Kobayashi et al., 1989). Several recent publications report antibodies raised against the proto-oncogen product, c-kit protein, which specifically labels interstitial cells (ICCs) in the gastrointestinal tract of the mouse and human (Ward et al., 1994; Huizinga et al.,...
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and nitrergic neurons in the developing intestine, this exclusively found in the supportive cells (Jessen and cells around the developing myenteric ganglia, in order to get information about the cellular and histochemical characteristics of the ganglionic microenvironment, necessary for the development of the enteric ganglia in the human fetal small intestine.

Because in the ENS GFAP and S-100-protein are exclusively found in the supportive cells (Jessen and Mirsky, 1983; Kobayashi et al., 1986), and S-100 protein is considered a general marker of enteroglial cells, we have utilized immunocytochemical techniques using antibodies against S-100-protein and GFAP to investigate the appearance and the distribution of the NN cells of glial origin in the developing human small intestine between the 10th and 17th week of gestation. Recent results indicate that nitric oxide (NO) also mediates neuronal-glial communication in the central nervous system (CNS). Glial cells have been viewed mainly as potential reservoirs of L-Arginine (Kerwin and Heller, 1994), the substrate for NOS, and at the same time as targets for neuronally derived NO (Murphy et al., 1995). There is now evidence for the expression of constitutive NOS in the astrocytes in the CNS (Agullo et al., 1995). The NO molecule as a highly reactive and lipophilic gas is capable of rapid diffusion into nearby cells where it mediates intra and/or intercellular communication, and its trophic effect have recently been demonstrated (Ogura et al., 1996). These data suggest that the distribution of NOS-IR within the fetal gut and the pattern of morphological relations between glial cells and nitrergic neurons are important factors for establishing the enteric microenvironment necessary for the final differentiation of the ENS. Since no data are available about the topographic relations of glial cells and nitrergic neurons in the developing intestine, this was defined as our second aim. We utilized a double-labelled immunocytochemical technique using a combination of S-100-protein and bNOS antibodies on whole-mounts and cryostat sections of human fetal small intestine to localize the NOS-expressing structures in relation to the S-100-protein-immunoreactive cellular network.

Materials and methods

Tissues

Intestinal segments of 10-, 12-, 14- and 17-week-old human fetuses (n=3 for each gestational age) were obtained immediately after legally approved or spontaneous abortions. The human studies have been reviewed by the University Medical Committee for Ethical Affairs and have therefore been performed in accordance with the ethical standards laid down in an appropriate version of the 1964 declaration of Helsinki.

Immunocytochemistry

Segments of small intestine were ligated and distended using a modified Zamboni fixative (Scheuermann et al., 1987) and fixed overnight at 4°C. After washing with phosphate buffered saline (PBS) at pH 7.4, small pieces were embedded in Tissue Tek (Miles) and cryostat sections (15um) were cut. Larger pieces were used for whole-mount preparations. Sections and whole-mounts were incubated overnight at room temperature with anti S-100 protein (Dakopatts Z311, final dilution 1:400) and anti GFAP (Amersham RPN 1106, final dilution 1:5). After incubation with the primary antibodies the tissues were rinsed in PBS and incubated with biotinylated goat anti rabbit secondary antibody. Sections and whole-mounts were incubated overnight at room temperature with anti S-100 protein (Dakopatts Z311, final dilution 1:400) and anti GFAP (Amersham RPN 1106, final dilution 1:5). After incubation with the primary antibodies the tissues were rinsed in PBS and incubated with biotinylated goat anti rabbit secondary antibody. Sections and whole-mounts were incubated overnight at room temperature with anti S-100 protein (Dakopatts Z311, final dilution 1:400). After incubation in the primary antisera the tissues (whole-mounts and cryostat sections) were washed and incubated for 6hrs in a mixture of species-specific secondary antibodies conjugated to FITC (Jackson, final dilution 1:100) or biotin (Amersham, final dilution 1:50). Following secondary antisera incubation, tissues were washed and incubated overnight in streptavidin-biotinylated horseradish peroxidase complex (RPN 1051 Amersham, diluted 1:100). The immunoreaction was visualized using 3,3'-diaminobenzidine (DAB) in the presence of H2O2. Double-labelling immunofluorescence histochemistry was performed by overnight incubation at room temperature in a monoclonal mouse anti-neuronal NOS (anti-bNOS) antiserum (Affinity, final dilution 1:200) in combination with a rabbit S-100-protein antiserum (Dakopatts Z311, final dilution 1:400). After incubation in the primary antisera the tissues (whole-mounts and cryostat sections) were washed and incubated for 6hrs in a mixture of species-specific secondary antibodies conjugated to FITC (Jackson, final dilution 1:100) or biotin (Amersham, final dilution 1:50). Following secondary antisera incubation, tissues were washed and incubated overnight in streptavidin-Texas red (Amersham, final dilution 1:100). Specimens were then mounted in PBS-buffered glycerol and viewed in a Zeiss Axioshot microscope equipped with epifluorescence and in a Zeiss LSM 410 confocal microscope equipped with an argon (excitation wavelength of 488 nm) and a Helium-Neon ion laser (excitation wavelength of 543 nm) as light sources. The preparation were viewed with Zeiss Oil immersion (magnification: 40X (NA:1.3) and 63X (NA:1.4)) objectives and scanned at 0.9 μm steps to obtain a Z-series of up to 20 optical sections. Then, maximal value projection reconstructions were made using the Imaris software running on a Silicon Graphics indigo 2 workstation.

Results

Using specific antibodies against S-100 protein and GFAP on whole-mount preparations and on cryostat
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Fig. 1. Immunostaining for S-100 protein in cryostat sections of human fetal small intestine in the 17th week of gestation. Cells with S-100 protein immunoreactivity were distributed along the myenteric plexus (large arrowheads), within the circular muscle layer (arrows) and in the inner (asterisks) and outer (arrowheads) submucosal plexuses. Bar: 72 μm.

Fig. 2. Immunostaining for GFAP in a cryostat section of human fetal small intestine in the 17th week of gestation. Immunoreactivity (arrowheads) was restricted to the side of the myenteric ganglia adjacent to the longitudinal muscle layer (asterisks). Bar: 44 μm.

Fig. 3. Immunostaining for S-100 protein of a whole-mount preparation of human fetal small intestine in the 17th week of gestation. Cells with S-100 protein immunoreactivity (arrowheads) interconnect (arrows) and form a network in the plane of the myenteric ganglia. Bar: 35 μm.

Fig. 4. Immunostaining for S-100 protein of whole-mount preparations of human fetal small intestine on the 17th week of gestation. Cells on the surface of the submucosal plexus interconnect (arrows) and form a network. Bar: 30 μm.

Fig. 5. A higher magnification photograph of GFAP immunostaining in a cryostat section. CM: circular muscle layer; LM: longitudinal muscle layer; arrows: myenteric ganglia; arrowheads: GFAP reaction. Bar: 45 μm.

Fig. 6. Immunostaining for GFAP in whole-mount preparations of human fetal small intestine in the 17th week of gestation. Immunopositive cells (arrowheads) appear to be separated from each other and from the immunopositive plexuses (arrows). Bar: 40 μm.
sections of human fetal small intestine, the early appearance and the different distribution of NN cells expressing one of these two glial specific proteins were revealed (Figs. 1-6.). Two types of NN cells with glial phenotype were demonstrated in the fetal gut. They appeared together, intermingled with the primordial ganglion cells in the 10th week of gestation. Their distribution changed gradually and by fetal week 14 the two populations of cells were distributed differently. Either S-100 protein or GFAP immunoreactivity was expressed in cells adjacent to the primordial myenteric ganglion cells in the 10th week of gestation. Around the 17th week of gestation, when all the tissue layers in the intestinal wall were well differentiated, cells with strong S-100-protein immunopositivity appeared along the longitudinal and circular muscle side of the myenteric ganglia, within the circular muscle layer and in the myenteric and submucous plexuses (Fig. 3.). In the whole-mount preparations S-100 protein immunopositive cells were interconnected, and formed multi-layered networks, at the level of the MP (Fig. 3.), between the circular muscle layer and the MP and within the submucous plexus (Fig. 4). In the 17-week-old fetuses cells with GFAP immunoreactivity were mainly clustered to the side of the MP that was adjacent to the longitudinal muscle layer (Figs. 2, 5). In the whole-mount preparations most of the GFAP immunoreactive cells appeared to be single, not connected to each other or to immunopositive fibers running within the plexus (Fig. 6). After double labelling with the combination of NOS/S-100 protein antibody the majority of the NOS-IR fibers either smooth or varicose were running within the plexuses or among the smooth muscle cells. However, part of these NOS-IR fibers formed baskets around the S-100 protein-IR cells (Fig. 7).

Discussion

Evidence has been provided that the microenvironment from which neurons originate is critical in determining the ultimate pathway of differentiation (Le Douarin and Teillet, 1971). The sequential appearance of the various types of enteric neurons (Gintzler and Hyde, 1983) suggests a changing microenvironment within the intestinal wall at the different stages of embryonic development. The current study has revealed the cellular elements that constitute the earliest and most immediate environment of the developing human myenteric
ganglia. Utilizing light microscopic immunocytochemical techniques and antibodies against glial-specific proteins like S-100 protein and GFAP, two distinct types of NN cells of a glial phenotype and/or of glial origin were demonstrated in association with the MP. Although they appeared together around the 10th week of gestation, their distribution already differed greatly around the 14th week of gestation. Single cells with GFAP immunoreactivity were clustered to one side of the MP in the vicinity of the longitudinal muscle layer, while the cells with S-100-protein immunoreactivity were widely distributed in the intestinal wall and frequently formed multilayered cellular networks both in the MP and in the submucous plexuses. Based on these results and on data from the literature (Ferri et al., 1982; Jessen and Mirsky, 1983; Kobayashi et al., 1989) it can be concluded, that two main populations of glial cells, one expressing S-100 protein, the other GFAP immunoreactivity, appear within and/or around the MP at the very beginning of ganglionic morphogenesis. Thus these cells provide the first morphological elements of the ganglionic microenvironment, necessary for the ganglionic morphogenesis. Being glial cells they might be involved in providing the mechanical support, isolation and nutrition for the developing ganglia. Later on in the development or in mature ganglia enteroglial cells are mostly found within the ganglia. In other locations they might have been replaced by cells differentiating later in the intestinal wall. Recent publications point out the importance of the interrelationship between nitricergic neurons and astrocytes (Agullo et al., 1995; Murphy et al., 1995). There is evidence suggesting that glial cells serve as a potential reservoir of L-Arginine (Kerwin and Heller, 1994) and also as a main target of NO (Murphy et al., 1995). Utilizing a double-labelling immunocytochemical method a close morphological relation was revealed between NOS-IR fibers and S-100 protein-IR cells in the developing human fetal intestine. NOS-IR varicosities on the glial cell surface might function as communication sites between glia and nitricergic nerves. Although the nature of this communication is not clear, the glial cells closely related to nitricergic nerves might directly benefit from the trophic effect of NO, which has recently been reported (Ogura et al., 1996).

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