Ganglion Cell Differentiation and Intermediate Filaments in the Cervical Dorsal Root Ganglion of the Chick Embryo

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ABSTRACT. To examine the differentiation processes of cervical dorsal root ganglionic cells, vimentin, nestin and neurofilament-H protein were immunohistochemically detected in chick embryos from stages 15 through 43. During stages 15-17, migrating neural crest cells exclusively expressed vimentin. At stage 20, there was a marked decrease in vimentin, and appearance of nestin in most cells of the ganglionic primordia. However, a few Schwann precursor cells containing only vimentin were also observed. During stages 24-26, spindle-shaped precursors of pseudounipolar neurons containing neurofilament-H protein increased in number in the primordia of the ganglia. During stages 28-30, large round immature neurons containing neurofilament-H protein in both their perikaryon and neurites were recognized. At stage 36, the neurons contained neurofilament-H protein and transiently vimentin. In addition, the precursors of satellite cells, containing neither vimentin, nestin, nor neurofilament-H protein, could be observed around the neurons. At stage 43, three mature cell types could be distinguished; pseudounipolar neurons containing neurofilament-H protein, Schwann cells containing vimentin, and satellite cells containing neither vimentin, nestin, nor neurofilament-H protein. These results clearly demonstrate that differentiation of neural crest cells into neurons, Schwann cells and satellite cells, is reflected by the intermediate filament composition.

Key words : Dorsal root ganglion — Pseudounipolar neuron — Vimentin — Nestin — Neurofilament protein

Together with microtubules and microfilaments, intermediate filaments are the major structures in the cytoskeleton. Presently, nearly 50 different intermediate proteins are identified, and they are expressed either tissue specifically or developmental stage-specifically. In the development of the central nervous system, various intermediate filaments are expressed in association with neuronal-cell differentiation.[1-4] One of these, which sensitively reflect the differentiation processes of neural cells, is vimentin. At a very early stage of chick neural tube formation, differences in vimentin expression have already appeared in future motor areas and sensory areas.[4] In addition, marked changes in the expression of vimentin have been observed prior to morphological changes in various embryonic tissues.[5,6]

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With regard to the differentiation processes by which neuroepithelial cells evolve into neurons, both nestin and neurofilament protein have also been recognized as useful markers.[7-9]

Dorsal root ganglia are derived from the neural crest cells, and ganglionic cell components, such as the pseudounipolar neurons, satellite cells and Schwann cells, are derived from multipotential neural crest cells.[10-14] During ganglion development, programmed cell death has been detected,[15] but the origin of the cells undergoing cell death remains largely unknown. The present study using monoclonal antibodies for several intermediate filaments was designed to clarify not only the differentiation process of neural crest cells but also the origins of programmed death during dorsal root ganglion formation.

MATERIALS AND METHODS

Fertilized White Leghorn chicken eggs were obtained from Japan Lamb (Hiroshima, Japan), and allowed to progress to various developmental stages in an incubator at 38°C. All embryos were accurately staged by the criteria described by Hamburger and Hamilton (1951).[16] Forty-five embryos were employed for stages 15, 17, 20, 24, 26, 28, 30, 36 and 43. The cervical region of each embryo was fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 4 h at 4°C. After being washed in chilled phosphate-buffered saline (PBS) for 16 h, the tissues were dehydrated and embedded in paraffin. Deparaffinized serial sections, 3 μm thick, were subjected to the following histochemical procedures or stained with hematoxylin and eosin.

Immunohistochemistry for intermediate filaments

Vimentin, nestin and neurofilament-H protein were detected using the avidin-biotin peroxidase complex (ABC) system (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA, USA). Tissue sections were treated in 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After washing in PBS, they were incubated in 0.1% normal horse serum in PBS for 20 min to block nonspecific binding sites. Then they were exposed to three kinds of mouse monoclonal antibodies diluted with 0.1% bovine serum albumin in PBS for 1 h; anti-vimentin antibody (Vim3B4, Dako, Glostrup, Denmark; dilution 1 : 200), anti-nestin antibody (MAB353, Chemicon, Temecula, CA, USA; dilution 1 : 50), and anti-neurofilament-H antibody (N52, Sigma, St. Louis, MO, USA; dilution 1 : 200). Before exposing the tissue sections to anti-vimentin antidody, they were treated with 12 U/ml trypsin (EC 3.4.21.4, Type I, Sigma) for 20 min at 20°C. After incubation in primary antibodies, the sections were incubated in biotinylated horse anti-mouse IgG antibody for 45 min and rinsed in PBS. Then they were reacted with ABC reagent for 30 min, and a peroxidase substrate solution containing 0.005% hydrogen peroxide, 0.1% diaminobenzidine and 50 mM Tris-HCl buffer (pH 7.6) for 3 min. After immunostaining, they were counterstained with hematoxylin. PBS containing 0.1% bovine serum albumin or preimmune horse serum instead of primary antibodies was used for negative controls. No immunostaining was observed

in the negative control sections.

Cell death labeling

Cells undergoing DNA fragmentation have been identified using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL).[17] An apoptosis *in situ* detection kit (Wako, Osaka, Japan) was used. The tissue sections were treated with proteinase K (600 unit/ml) for 5 min at 37°C. Following washing in PBS, they were incubated in terminal deoxynucleotidyl transferase solution for label fragmented DNA with fluorescein-dUTP for 10 min at 37°C. After endogenous peroxidase activity was quenched in 3% hydrogen peroxide for 5 min at room temperature, the specimens were incubated in peroxidase-labeled anti-fluorescein antibody for 10 min at 37°C. Then they were visualized by diaminobenzidine solution for 5 min at room temperature. For positive controls, sections were incubated with DNase I for 15 min after protease incubation, and for negative controls, terminal deoxynucleotidyl transferase was omitted from the terminal deoxynucleotidyl transferase solution.

These experiments were approved by the Animal Research Committee of Kawasaki Medical School (No. 02-059, 2002) and conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School.

RESULTS

At stage 43, the dorsal root ganglia of the cervical spinal cord were located within intervertebral foramina, as shown in Fig 1. In the ganglia, three mature cell types were recognized, namely, large rounded pseudounipolar neuron, flattened satellite cells surrounding the soma of the neuron, and elongated Schwann cells along neurites (Fig 2a, b). Each cell type differed significantly not only in cell shape but also in the types and amounts of intermediate filaments. Large amount of neurofilament-H was located exclusively in the pseudounipolar neurons (Fig 2c). Only small amounts of vimentin could be recognized in Schwann cells lining nerve fibers (Fig 2d). No satellite cells contained vimentin or neurofilament-H protein.

Cervical dorsal root ganglionic cell differentiation and intermediate filament proteins

On the basis of the expression of intermediate filament proteins in developing ganglionic cells, five periods were distinguishable during stages 15 to 36.

Period I (stages 15-17)

At stage 15, small clusters of neural crest cells, as early primordia of the cervical ganglia, appeared on both sides of the neural tube (Fig 1a). As shown in Fig 3, the primordia consisted of a few neural crest cells. The neural crest cells were small and oval in shape and could be distinguished from surrounding mesenchymal cells. They were moderately vimentin-positive, whereas the mesenchymal cells around the primordia were weakly vimentin-positive. During this period, nestin and neurofilament-H protein

could not be detected in the primordia.

Period II (stage 20)

At stage 20, primordia of the cervical ganglia became enlarged (Fig 1b). They were located in the lateral vicinity of the neural tube and became

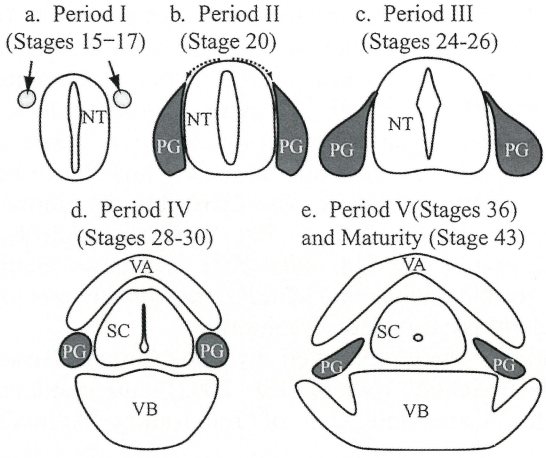

Fig 1. Schematic illustration of development of the cervical ganglia and spinal cord. On the basis of the expression of intermediate filament proteins in developing ganglionic cells, five periods (Period I-V) were distinguishable during stages 15 to 36. **a**) Period I (stages 15-17) : Neural crest cells migrate from dorsal neuroepithelium, and are grouped (*arrows*) on the dorsal side of the neural tube (*NT*). **b**) Period II (stage 20) : The primordia of the ganglia (*PG*) become located in the lateral vicinity of the neural tube (*NT*), and neural crest cell migration still continues (*dotted arrows*). **c**) Period III (stages 24-26) : The primordia of the ganglia (*PG*), in particular the ventral portion, markedly increase in width. **d**) Period IV (stages 28-30) : The primordia of the ganglia (*PG*) are located within the intervertebral foramina between the vertebral arch (*VA*) and vertebral body (*VB*). *SC* : spinal cord. **e**) Period V (stage 36) and maturity (stage 43) : Primordia of ganglia (*PG*) in the intervertebral foramina became laterally extended. *SC* : spinal cord, *VA* : vertebral arch, *VB* : vertebral body.

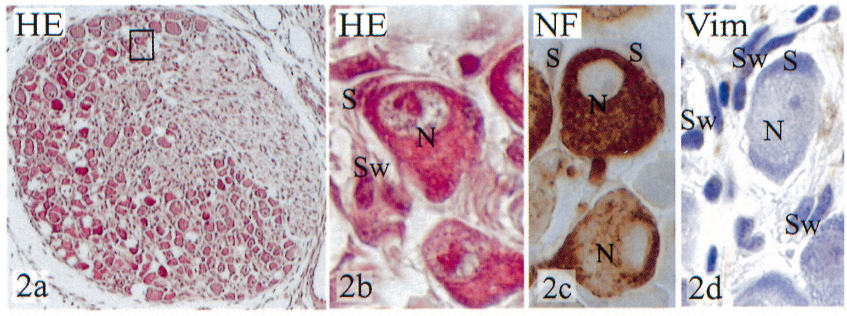

Fig 2. A cervical ganglion at stage 43. **a**) A low-power micrograph of a ganglion. HE staining. ×260. **b**) A micrograph of the rectangle in (**a**) : At stage 43, a ganglion is composed of large round pseudounipolar neurons (*N*), flattened satellite cells (*S*) and elongated Schwann cells (*Sw*). ×1400. **c**) Neurofilament-H protein staining : Pseudounipolar neurons (*N*) contain large amounts of neurofilament-H protein, while satellite cells (*S*) are neurofilament-H protein-negative. ×1400. **d**) Vimentin staining : Schwann cells (*Sw*) contain small amounts of vimentin, but, either pseudounipolar neuron (*N*) or satellite cell (*S*) is vimentin-negative. ×1400.

teardrop in shape (Fig 4a). The primordia were approximately 40 μm in width and 160 μm in length, and were characterized by the presence of two cell types; numerous spindle-shaped cells and a few elongated cells. The

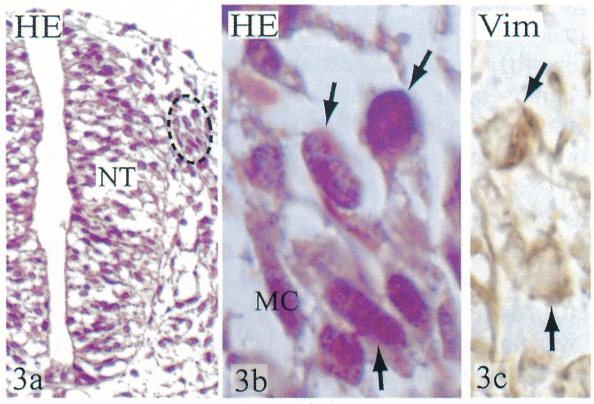

Fig 3. The primordium of a cervical ganglion during period I (stage 17). HE staining. **a**) A low-power micrograph: A primordium is indicated by the broken line on the dorsal side of a neural tube (*NT*). ×330. **b**) The primordium consists of round or ovoid neural crest cells (*allows*). *MC*: mesenchymal cells. ×1600. **c**) Vimentin staining: Migrating neural crest cells (*allows*) contain a moderate amount of vimentin. ×1600.

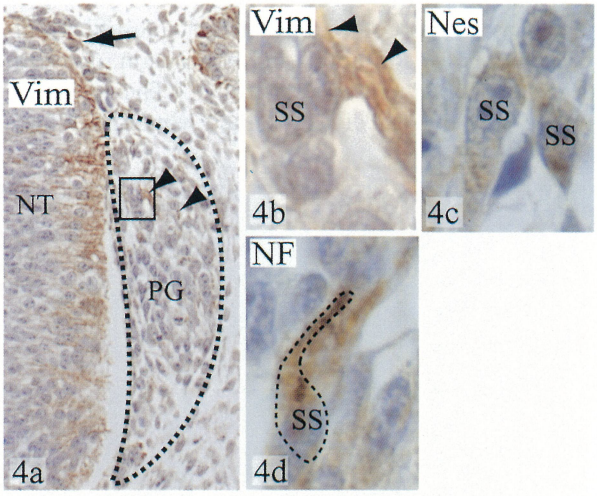

Fig 4. The primordium of a cervical ganglion during period II (stage 20). **a**) A low-power micrograph of vimentin staining: Teardrop shaped primordium of the cervical ganglion (*PG*), located in the lateral vicinity of the neural tube (*NT*), consists of numerous spindle-shaped cells. A migrating neural crest cell (*arrow*) in the mesenchyme between the dorsal ectoderm and the ganglionic primordium contains a large amount of vimentin. A few elongated cells in the primordium are also vimentin-positive (*arrowheads*). ×270. **b**) Vimentin staining within the rectangle in (**a**): Spindle-shaped cells (*SS*) have a negligible amount of vimentin, but the elongated profiles are vimentin-positive (*arrowheads*). ×1300. **c**) Nestin staining: Spindle-shaped cells (*SS*) have a moderate amount of nestin. ×1300. **d**) Neuofilament-H protein staining: Both perikaryons and extensions of the spindle-shaped cells (*SS*) are neurofilament-H protein-positive. ×1300.

spindle-shaped cells had an oval nucleus measuring 4×6 μm, and the primordia contained numerous mitotic cells and a few cells with neurite-like extensions. The mesenchymal tissues between the ganglionic primordium and the dorsal ectoderm contained a few migrating neural crest cells, which contained a large amount of vimentin (Fig 4a). The spindle-shaped cells in the ganglionic primordia, however, contained a markedly decreased amount of vimentin (Fig 4b), and expressed nestin (Fig 4c). In some of the spindle-shaped cells, both somas and neurite-like extensions could be identified and they were neurofilament-H protein-positive (Fig 4d). In contrast, the elongated cells were still strongly vimentin-positive (Fig 4b).

Period III (stages 24-26)

The alar and basal plates could easily be distinguished in the cervical spinal cord, and the primordia of the cervical ganglia had increased to approximately 210 μm in length. The width had decreased on the alar plate side, but there was an increase on the basal plate side to 110 μm (Fig 1c). The primordia of the ganglia consisted of numerous large cells and a few small, elongated cells. The majority of the large cells were spindle-shaped, and a few cells changed their profile from spindle to large and round (Fig 5a). Therefore, three types of cells were distinguishable ; large spindle-shaped cells, large round cells and small elongated cells. A distinct neurite became evident in the large round cells.

The large spindle-shaped cells contained small amounts of nestin and vimentin (Fig 5b) similar to the spindle-shaped cells during period II, but in a few cells, there was a moderate amount of neurofilament-H protein (Fig 5c). The large round cells contained neither nestin nor vimentin, but they were positive for neurofilament-H protein. The small, elongated cells increased in number and exclusively contained vimentin (Fig 5b).

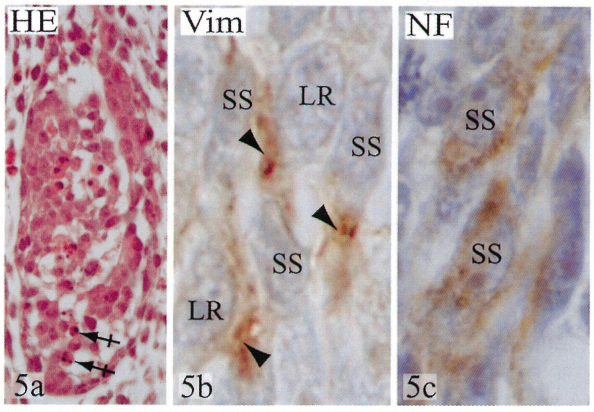

Fig 5. The primordium of a cervical ganglion during period III (stage 26). **a**) HE staining : The major cell type is spindle-shaped, and the ganglion contains numerous pyknotic cells (*barred arrows*). ×190. **b**) Vimentin staining : A moderate amount of vimentin can be observed only in the small elongated cells (*arrowheads*), but large spindle-shaped cells (*SS*) contain small amount of vimentin and the large round cell (*LR*) is negative. ×1400. **c**) Neurofilament-H protein staining : A moderate amount of neurofilament-H protein can be seen in the large spindle-shaped cells (*SS*), particularly in their perikaryon and neurite areas. ×1400.

Period IV (stages 28-30)

The neural tube was located within the cartilaginous vertebral canal of the primordium of the vertebrae. The primordia of the cervical ganglia were approximately 280 μm in length and 80 μm in width, and were located within the intervertebral foramina in the lateral vicinity of the basal plate of the neural tube (Fig 1d). They had long fibrous connections to the spinal cord as dorsal root, and consisted of four cell types; large spindle-shaped cells, large round cells, small polygonal cells and elongated cells (Fig 6a). Some polygonal cells were observed in close contact with the large round cells (Fig 6a, b).

The large cells, both spindle-shaped and round, exclusively contained neurofilament-H protein both in their perikaryon and in neurites (Fig 6b), but nestin and vimentin could not be detected. Vimentin, nestin and neurofilament-H protein were not detected in the small polygonal cells. The elongated cells, which were along neurites, exclusively contained vimentin.

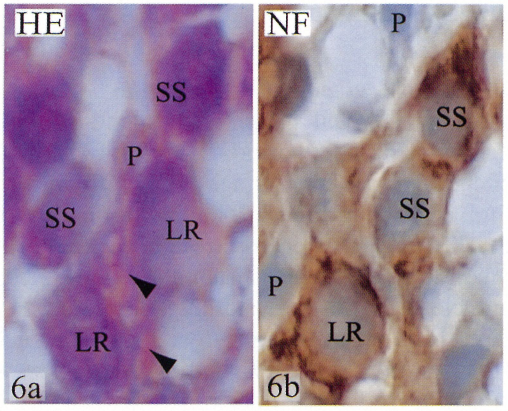

Fig 6. The primordium of a cervical ganglion during period IV (stage 28). **a**) HE staining: The primordium contains four types of cells; large spindle-shaped cells (*SS*), large round cells (*LR*), small polygonal cells (*P*) and elongated cells (arrowheads). $\times$ 1500. **b**) Neurofilament-H protein staining: Both large spindle-shaped cells (*SS*) and large round cell (*LR*) contain moderate to large amounts of neurofilament-H protein in their perikaryonic and neurite areas. Small polygonal cells (*P*) are negative. $\times$1500.

Period V (stage 36)

The primordia of the ganglia extended laterally within the intervertebral foramina, and exhibited teardrop profiles (Fig 1e). In the primordia, the large spindle-shaped cells disappeared, and three cell types could be distinguished as seen in stage 43; namely large round cells, satellite cells surrounding the large round cells, and elongated cells along neurites (Fig 7a). Large round cells in the ventrolateral area of the primordia were larger in cell size than those in the dorsomedial area. The large round cells in the ventrolateral area were strongly neurofilament-H protein-positive (Fig 7b), and many of them also contained vimentin (Fig 7c). In the satellite cells surrounding the large round cells, the three kinds of intermediate filament proteins could not be detected. The elongated cells exclusively contained vimentin.

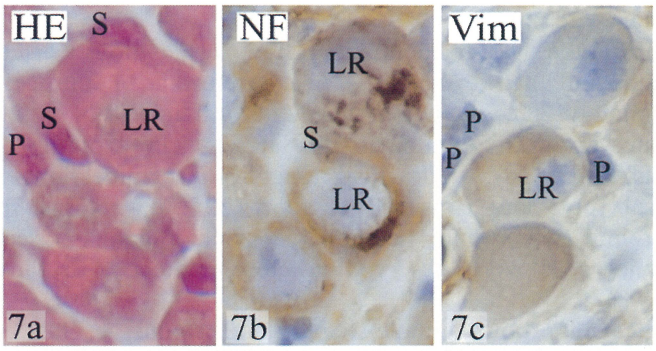

Fig 7. The primordium of a cervical ganglion during period V (stage 36). **a**) HE staining: Satellite cells (*S*) can be observed around large round cell (*LR*). Polygonal cell (*P*) is also seen close to the large round cell. × 1400. **b**) Neurofilament-H protein staining: The large round cells (*LR*) contain a copious amount of neurofilament-H protein, but the satellite cell (S) is negative. ×1400. **c**) Vimentin staining: The large round cell (*LR*) is vimentin-positive, but the polygonal cells (*P*) are negative. ×1400.

Cell death in developing cervical dorsal root ganglia

The primordia of the cervical ganglia contained small cells with pyknotic and fragmented nuclei during the periods III and IV. The cell number with pyknotic nuclei reached a peak at stage 26, with three to five cells per ganglionic primordium section profile. The pyknotic cells were 3 to 4 μm in diameter, and were diffusely scattered throughout the primordia. The cells had a few, small condensed nuclear fragments measuring approximately 1 μm in diameter, and clearcut positive staining appeared in the TUNEL assay (Fig 8a). The dying cells were phagocytosed by mesenchymal cells in the primordia. During period IV, TUNEL-positive cells were decreased in number, and they disappeared after period V.

During period III, the majority of pyknotic cells contained nestin (Fig 8b) and, a few were neurofilament-H protein-positive (Fig 8c). The relationships between the cell differentiation stages in cervical ganglia and intermediate filaments are summarized in Fig 9.

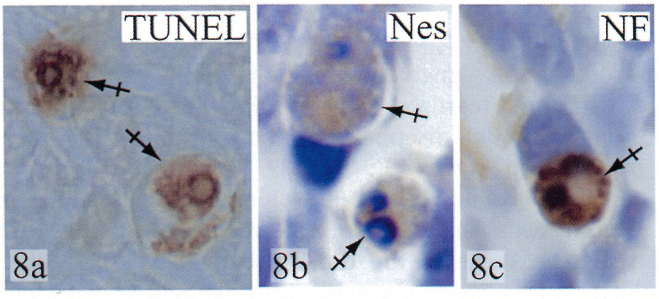

Fig 8. Dying cells in period III (stage 25). **a**) TUNEL staining: TUNEL positive cells (*barred arrows*) are diffusely scattered throughout the primordia of the ganglia. ×1700. **b**) Nestin staining: Pyknotic cells contain a small amount of nestin (*barred arrows*). ×1700. **c**) Neurofilament-H protein staining: A barred arrow indicates a neurofilament-H protein-positive cell with a pyknotic nucleus. ×1700.

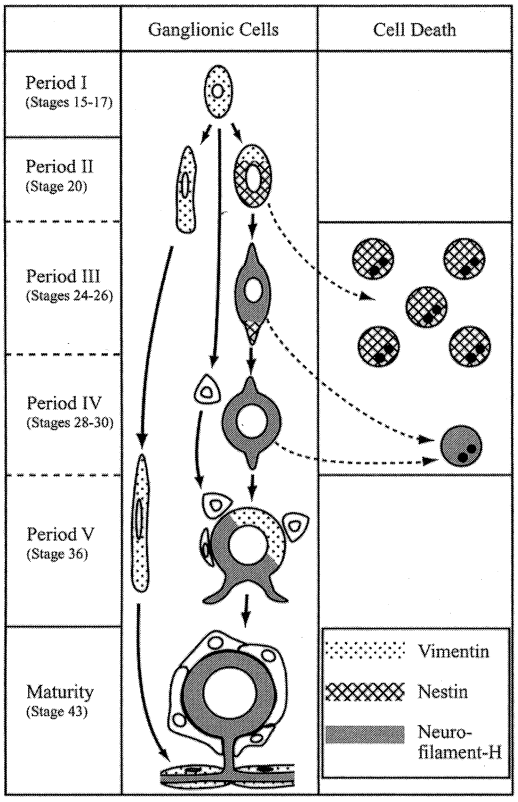

Fig 9. A diagram illustrating ganglionic cell differentiation and the expression of intermediate filament proteins. Developmental pseudounipolar neurons change their expression of intermediate filament proteins with morphological change as follows; vimentin → nestin → neurofilament protein → vimentin+neurofilament protein → neurofilament protein. Schwann precursor cells exclusively contain vimentin throughout their development. Ganglionic cells undergoing apoptosis during period III and IV can be assumed to originate from nestin-positive and neurofilament-H protein-positive spindle-shaped cells, mainly from the precursors of pseudounipolar neurons prior to satellite cell differentiation.

DISCUSSION

Our present study showed that, on the basis of the expressions of intermediate filament proteins, the development of the cervical ganglia could be characterized by the following three issues; (1) the developmental changes of the pseudounipolar neurons, (2) the fate determination of Schwann- and statellite-precursor cells and, (3) the origin of apoptotic cells which appeared in the primordia of the cervical ganglia.

As the earliest step in the formation of the dorsal root ganglia, the neural crest cells are known to move from the neural tube ventrolaterally to the sides of the neural tube.[18,19] As reported previously, the first intermediate filament expressed by neuroepithelial cells is nestin,[7,20,21] and the intermediate filament protein of developing neuroblasts in the neural tube changes from nestin to vimentin.[9] Thereafter, the vimentin filaments finally change to neurofilament in neurons.[2,9,22,23] As shown in our results, the

neural crest cells in the primordia of the cervical ganglia contained vimentin exclusively. A large amount of vimentin is expressed in morphologically transforming cells in histogenesis[4,5] and disease.[24-27] Vimentin can easily be assembled and disassembled either in dephosphorylation or in phosphorylation.[28-30] Therefore, it seems reasonable to assume that the vimentin filaments in the neural crest cells are involved mainly in cells migrating to the ganglionic primordia. In the primordia of the ganglia during period II, two cell types were distinguished ; spindle-shaped cells and elongated cells. Although the elongated cells continuously expressed only vimentin, the spindle-shaped cells co-expressed nestin and vimentin. As is well known, nestin filaments are one of the most common intermediate filaments contained in undifferentiated neurogenic cells.[6,7,21,31] Therefore, the nestin-positive spindle-shaped cells may be considered to be the precursors of pseudounipolar neurons.

During period III, the large spindle-shaped cells, precursors of pseudounipolar neurons, lost both nestin and vimentin, and showed an increase in the amounts of neurofilament-H protein. During period IV, the precursors of the neurons changed their profiles from spindle to round, and both perikaryon and neurites of the large round cells exclusively contained neurofilament-H protein. During period V, vimentin reappeared temporarily in the precursors of the neurons. At this stage, the precursors of the neurons changed from bipolar to pseudounipolar profiles.[19] Transient vimentin reappearance in the precursors of the neurons during period V can be considered to reflect morphological change from bipolar to pseudounipolar neurons for the following three reasons. First, vimentin participates in cell shape formation through interconnection to other cytoskeletal components.[32-36] Second, among intermediate filament proteins, vimentin is the most sensitive to phosphorylation and dephosphorylation.[37] Third, phosphorylation of vimentin causes a specific disassembly of the cytoskeletal architecture of the cells, and the cytoskeletal architecture can easily be reconstructed by dephosphorylation.[28,38] Therefore, vimentin is considered to be one of the intermediate filaments most responsible for changes in cell shape. Indeed, vimentin is observed with marked cell profile changes in other tissues.[5,6,9,38,39]

Neural crest cells can differentiate into not only pseudounipolar neurons but also satellite cells and Schwann cells in the dorsal root ganglia.[13,14] As shown in our results, the primordia of the cervical ganglia during period II contained a few vimentin-positive cells with elongated profiles. On the basis of their morphological features and distribution, these elongated cells are considered to be precursors of Schwann cells, and they exclusively express vimentin in a similar manner to the mature Schwann cells observed at stage 43. In contrast, neither the small polygonal cells nor satellite cells contained vimentin or neurofilament-H protein, and the small polygonal cells closely surrounded large round neural somas during period IV. Therefore, these small polygonal cells may be the precursors of the satellite cells. However, further investigation of the differentiation process of satellite cells in association with the expression of glial fibrillary acidic protein (GFAP) must be carried out.

During neuronal cell differentiation in the dorsal root ganglia, large-

scale cell proliferation and cell death exist side by side.[15,40] As shown in our results, large numbers of pyknotic cells appeared during periods III and IV, and these pyknotic cells could correspond to TUNEL-positive cells. Most of the dead cells contained nestin, and no vimentin could be identified. Therefore, after cell migration into the primordia of the cervical ganglia, some of neural crest cells which began to differentiate into neurons were considered to have undergone programmed cell death. Neurofilament-H protein could also be identified in a few dying cells during periods III and IV. Programmed cell death possibly occurred either in the spindle-shaped or large round cells in association with morphological changes from bipolar to pseudounipolar neurons.

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