Immunocytochemical Study on Neural Cell Adhesion Molecule in Rat Neural Crest Cells *in Vitro*

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ABSTRACT

The expression of neural cell adhesion molecule (NCAM) in rat trunk neural crest cells was studied *in vitro*. When the crest cells were cultured in a conventional medium without added factors, a small number of neuronal cells exhibited NCAM immunoreactivity. In a medium supplemented with 10 ng/ml basic fibroblast growth factor, numerous neuronal and epithelium-like cells were found to express NCAM immunoreactivity on their surfaces. On the other hand, NCAM expression in the crest cells was not influenced by nerve growth factor. These results suggest that the neural crest contains neuronal and non-neuronal precursors with different survival and growth factor requirements.

INTRODUCTION

A wide variety of cells share an embryonic origin in the neural crest, which appears in the neural primodium transiently in embryonic vertebrates[1]. After neural tube closure, they migrate along a well-defined pathway and differentiate into diverse functional cells, which include pigment cells, Schwann cells, some endocrine cells, and sensory and autonomic neurons[1]. In the course of the differentiation, the crest cells exhibit specific morphological and antigenic phenotypes, with narrowing of their developmental options.

The interest in the neural crest as a model system to study cell lineages during development in vertebrates resides in the fact that it is a pluripotential transitory structure from which a variety of cell types are derived. Since Cohen and Konigberg[2] established a primary culture system of the quail neural crest, *in vitro* differentiation of neural crest cells were extensively studied in avian and mammalian species. These *in vitro* studies have revealed that the differentiation of neural crest cells is highly dependent upon the composition of the culture medium[3,4].

Neural cell adhesion molecule (NCAM) is an integral membrane glycoprotein that participates in cell-to-cell interactions through its calcium-independent, homophilic binding[5,6]. Although NCAM was primarily characterized in neurons, the expression of this molecule is not confined to the nervous system, but is also observed in various cell types, including glial cells, endocrine cells, and skeletal and smooth muscles[7-11].

The molecular forms of NCAM are diversified in tissue- and developmental stage-specific manners by post-transcriptional and post-translational modifications. The diversity in the polypeptide portion of NCAM is generated both by alternative splicing and by differential polyadenylation site selection within

mRNA products coded by a single complex gene[12,13]. NCAM polypeptides are further diversified by polysialylation with an α2-8 linked polysialic acid[14,15]. NCAM varies in its content of polysialic acid from a maximum of about 30% to less than 10%[15,16]. The spatial and temporal expression of the differential forms of NCAM is thought to be involved in various developmental processes, such as cell segregation and axonal fasciculation, the pathfinding of axons, cell migration and neuromuscular interaction[17-21]. NCAM expression also contributes to the formation of sympathetic ganglia, which are composed of neural crest derivatives[22].

In this study, the expression of NCAM and highly polysialylated NCAM (high PSA NCAM) was investigated in the primary cultures of rat trunk neural crest cells in the presence or absence of neurotrophic factors.

MATERIALS AND METHODS

Neural crest cultures

Wistar rat (Charles River Japan) embryos were isolated at day 10 of gestation (day of vaginal plug = day 0 of gestation). Trunk neural tubes were isolated with Dispase (Godo Shusei) from the embryos at embryonic day 10 (E10) as described in our previous paper[23]. Isolated neural tubes were transferred onto a bovine fibronectin-coated glass coverslip (22 mm × 22 mm) in a 35 mm Petri dish (Nunc). Five neural tubes per dish were cultured in 1.5 ml of Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (1:1, Sigma) supplemented with 5% heat-inactivated fetal calf serum (Irvine), 0.5% rat embryo extract, 50 units/ml penicillin (GIBCO), and 50 μg/ml streptomycin (GIBCO). Preparation of rat embryo extract was detailed in our previous paper[23]. The explants were incubated at 37℃ in a humidified atmosphere of 5% CO_2 in air to allow neural crest cells to migrate away from the tubes. After 24 h of incubation, the explants were carefully scraped away with a tungsten needle, leaving crest cells on the substratum. One-half of the medium was changed every other day.

In some experiments, 50 ng/ml 2.5S nerve growth factor (NGF, Sigma) or 10 ng/ml basic fibroblast growth factor (bFGF, from bovine pituitary, Sigma) was added to the culture medium from the beginning of the explant culture.

Indirect immunofluorescence analysis

At day 2 and 4 *in vitro*, the neural crest cells were fixed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h. The fixed cells were washed several times with phosphate-buffered saline (PBS, pH 7.4), and treated with 0.25% Triton X-100 in PBS for 10 min. The permeabilized cells were blocked with 1% bovine serum albumin in PBS for 30 min. The cells were then incubated with appropriately diluted primary antibodies for 1 h. The primary antibodies used were monoclonal antibody (MAb) 12E3 (1:5,000, mouse IgM) which reacts with the PSA portion in high PSA NCAM[24,25], rabbit polyclonal antiserum to rat NCAM (1:250, NA1206, AFFINITI Res. Products) which binds to the polypeptide moiety common to the three isoforms of rat NCAM, and rabbit polyclonal antiserum to chicken tau (1:200, Sigma). After being washed with PBS, the specimens were incubated for 30 min with FITC-conjugated goat anti-mouse IgM antibody (1:200, Cappel) or TRITC-conjugated goat anti rabbit IgG antibody (1:100, Cappel). After removal of unbound antibodies with PBS, the specimens were embedded in glycerol-PBS (9:1) containing 0.1% p-phenylenediamine[26] (Wako), and observed under Olympus VANOX microscope equipped with fluorescein optics. As negative controls, BALB/c control

ascites fluid (Cedarlane Lab.) and preimmune rabbit serum (GIBCO) were substituted for the respective primary antibodies. No specific immunoreaction was detected in these negative controls. All the immunocytochemical procedures were performed at room temperature.

In some experiments, the fixed cells were incubated with *Vibrio cholerae* neuraminidase (0.05 IU/ml, Sigma) in 50 mM acetate buffer (pH 5.0) containing 4 mM $CaCl_2$ and 0.2 mM eth-ylenediaminetetraacetic acid at 37°C for 2 h. As a non-enzyme control, the cells were incubated with the hydrolysis solution alone. These cells were examined by double-immunofluorescence staining using MAb 12E3 and polyclonal antiserum NA 1206 as described above.

RESULTS

Neural crest cultures

The neural tube explants adhered to the fibronectin substratum within 2 h. Neural crest cells began migrating away from the explants after 5 h of incubation, and rapidly increased in number. Fig. 1 shows a phase-contrast image of cell outgrowth at 24 h *in vitro*. Some crest cells were polygonal in shape and others showed stellate morphology. The effects of the fibronectin substratum were detailed in our previous paper.
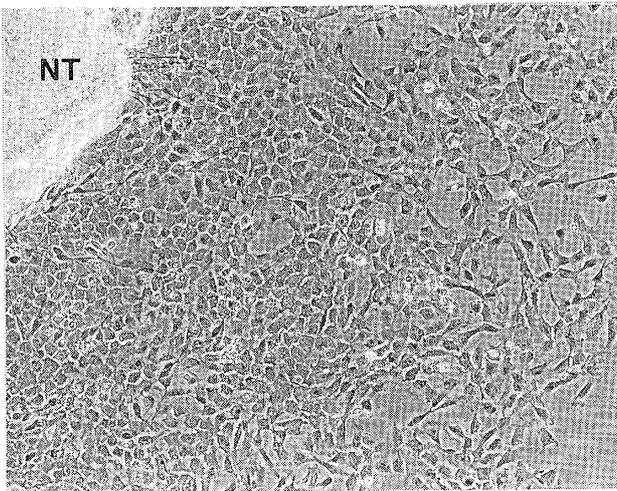

Fig. 1. Phase-contrast photomicrograph of neural tube explant (NT) and neural crest cell outgrowth. Isolated neural tubes were cultured for 24 h in a medium supplemented with 5% fetal calf serum and 0.5% rat embryo extract. × 30

At day 4 *in vitro*, the morphology of the neural crest cells was diversified. The crest cells showed fibroblast-like, epithelium-like or neuron-like morphology. Basic FGF, but not NGF, in the medium markedly increased the number of the crest cells during the 4-day-incubation when compared to controls without added factor (not shown).

Expression of NCAM peptide in neuronal and non-neuronal cells derived from neural crest

In control cultures, a small number of neuron-like cells exhibited immunoreactivity for NCAM peptide (Fig. 2A). Entire cell surfaces, including long neurites, were intensely stained with anti-NCAM antibody. Double immunofluorescence staining revealed that NCAM-positive cells also exhibited immunoreactivity for tau proteins, suggesting that they were neuronal cells (Fig. 2 A and B). Fibroblast-like or epithelium-like cells in the cultures did not show NCAM or tau immunoreactivity.
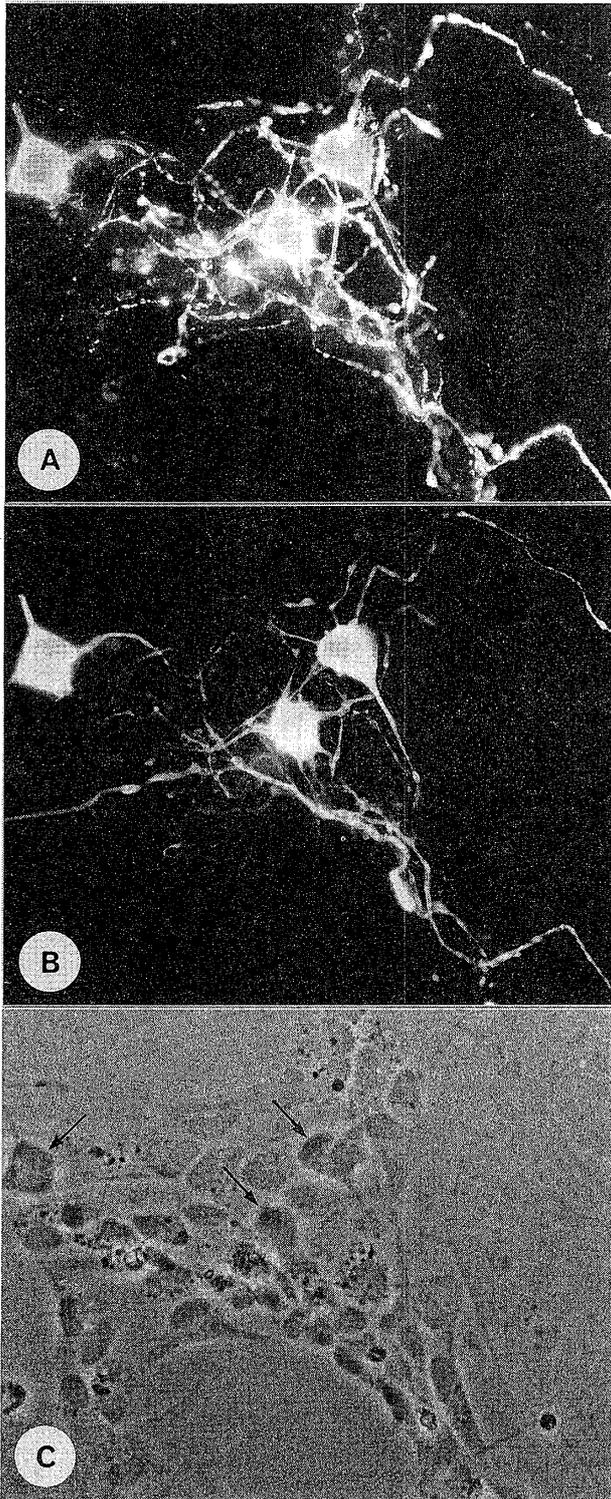
Fig. 2. Expression of NCAM peptide (A) and tau proteins (B) in neural crest cells at day 4 *in vitro* in a medium supplemented with 5% fetal calf serum and 0.5% rat embryo extract. C shows the corresponding phase-contrast. Arrows indicate the cell bodies of NCAM- and tau-immunoreactive cells. × 300

Adding bFGF to the culture medium markedly increased the number of NCAM-positive cells. Some of these NCAM-positive cells displayed epithelium-like morphology (Fig. 3 A-C), and others showed neuron-like morphology (Fig. 3 D-F). The NCAM-positive cells with neuron-like morphology showed intense immunoreactivity for tau (Fig. 3 E), whereas those with epithelium-like morphology did not (Fig. 3B).
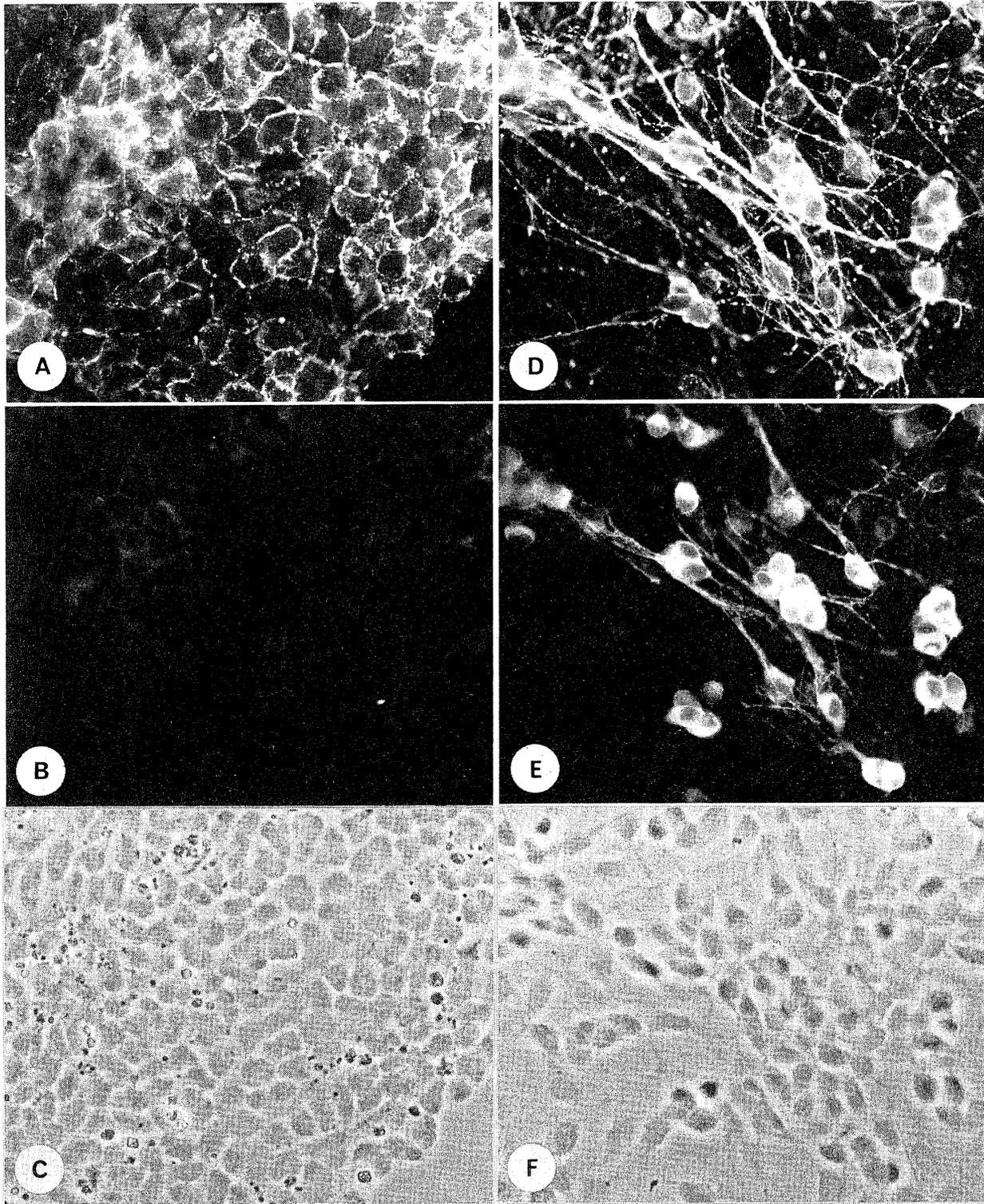
Fig. 3. Expression of NCAM peptide (A, D) and tau (B, E) in neural crest cells at day 4 *in vitro* in a medium supplemented with 5% fetal calf serum, 0.5% rat embryo extract and 10 ng/ml bFGF. C and F show the corresponding phase-contrast images. × 300

When rat embryo extract was not added to the culture medium, neither neuron-like cells nor NCAM-positive cells appeared in the crest cell cultures irrespective of bFGF supplement.

NCAM peptide and PSA double staining results

At day 4 *in vitro*, the crest cell cultures were subjected to double staining for NCAM peptide and high PSA NCAM. Staining for these two antigens was virtually superimposal (Fig. 4), irrespective of the added bFGF, though a few epithelium-like cells in the bFGF-treated cultures showed only NCAM peptide immunoreactivity (Fig. 4 C and D, asterisks).
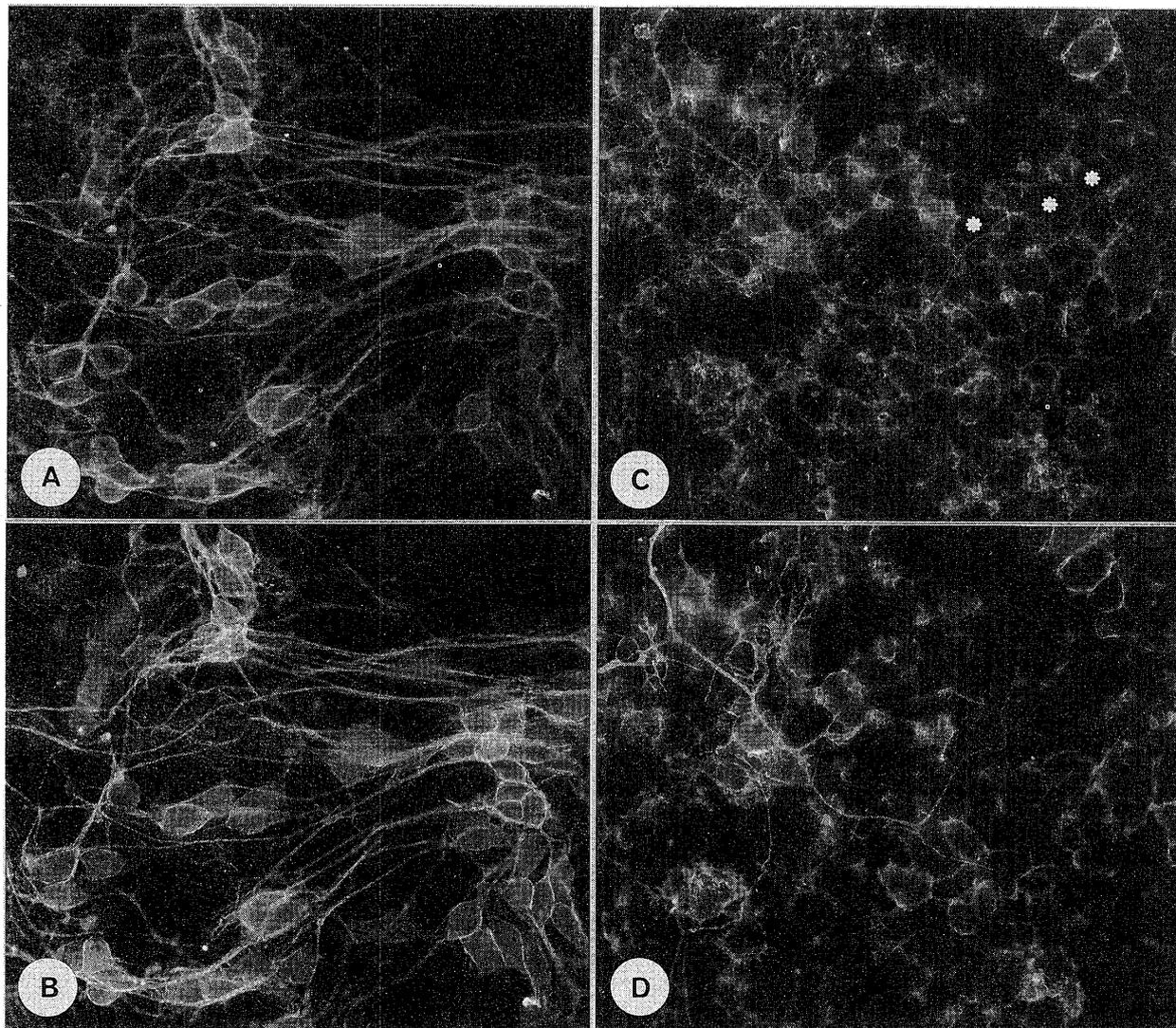

Fig. 4. Localization of NCAM peptide (A, C) and high PSA NCAM (B, D) in neural crest cells at day 4 *in vitro* in a medium supplemented with 5% fetal calf serum, 0.5% rat embryo extract and 10 ng/ml bFGF. In most cells, the staining for NCAM peptide is superimposable with that for high PSA NCAM. A few epithelium-like cells express NCAM with a reduced amount of PSA (asterisks in C). × 300

Pretreatment of the crest cells with neuraminidase completely abolished immunoreactivity to MAb 12E3, suggesting that the immunodeterminant of the MAb 12E3 antigen is indeed the sialic acid moiety of NCAM. As expected, immunoreactivity for NCAM polypeptide was retained even after neuraminidase treatment (data not shown).

DISCUSSION

It is well-known that the differentiation of neural crest cells *in vitro* is greatly influenced by the composition of the culture medium. Our previous study showed that catecholaminergic differentiation of rat trunk neural crest cells was supported by rat embryo extract supplemented in the medium[23]. The present results further confirmed the effects of rat embryo extract to support the expression of neuronal antigenic phenotypes, such as NCAM and tau proteins. However it remains to be established whether the inductive information provided by the extract is of an "instructive" or "permissive" character.

In the crest cell cultures, bFGF, but not NGF, markedly increased the number of both neuronal and non-neuronal cells, suggesting that bFGF promotes the survival and/or mitogenesis of neuron precursors and other uncommitted neural crest-derived cells. These results are consistent with the general belief that bFGF supports the survival or proliferation of crest-derived cells, whereas NGF does not[27].

According to a previous report by Biosseau *et al.*[22], NCAM expression was linked with neuronal phenotypes in rat neural crest derivatives. The present study also showed that NCAM expression was restricted in tau-positive neuronal cells in the control experiments without added factor. However, NCAM expression was observed not only in neuronal cells but also in epithelium-like cells (Fig. 3 A-C), when the crest cells were cultured in bFGF-supplemented medium. Similarly, Kalcheim previously reported that bFGF stimulated the survival of HNK-1-immunoreactive non-neuronal cells in quail crest cell cultures without influencing their mitogenetic activity in a serum-free chemically defined medium[28]. These results support the view that the neural crest contains neuronal and non-neuronal precursors with different survival and growth factor requirements in the early stages of its ontogeny.

Basic FGF is a pituitary-derived growth factor, which biologically promotes DNA synthesis in not only fibroblasts but also various types of cells. Previously Kalcheim and Neufeld found bFGF immunoreactivity in the extracellular matrix associated with mesenchyme just dorsal to the spinal cord anlagen of E4 quail embryo[29]. This location is part of the migratory route through which neural crest cells enter the dermis. They also found bFGF immunoreactivity in dorsal root ganglia, the representative neural derivatives of the neural crest[29]. These results suggest that bFGF can be of relevance to the understanding of neural crest development *in vivo*. Although the mechanisms that mediate the effects of bFGF on neural crest cells are still unknown, the culture system in the present experiments provides us with a useful model system to investigate neuronal differentiation of mammalian neural crest cells. Since NCAM is thought to be implicated in adhesive interactions between the crest-derived cells[30], this model system would also be useful for studying gangliogenesis.

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