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Review

Growth factor action in neural crest cell diversification

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ABSTRACT

At the onset of their migration into the embryo, many neural crest cells are pluripotent in the sense that they have the capacity to generate progeny that consist of more than one cell type. More recently, we have found that there are pluripotent neural crest cell-derived cells even at sites of terminal differentiation. These findings support the notion that cues originating from the microenvironment, at least in part, direct neural crest cell type specification. Based on the rationale that growth factors that are known to support survival of neural crest cell derivatives may have additional functions in progenitor cell development, we have examined the action of pertinent growth factors. Trophic, mitogenic, antiproliferative and differentiation promoting activities were found. Stem cell factor (SCF) is trophic for pluripotent neural crest cells. Contrary to expectation, SCF plus a neurotrophin, rather than SCF alone, is trophic for committed melanogenic cells. Basic fibroblast growth factor (bFGF) is mitogenic both for pluripotent cells and committed melanogenic cells. However, the cells become dependent on another factor for survival. Whereas any neurotrophin tested can rescue bFGF-activated pluripotent neural crest cells, the factor that rescues melanogenic cells remains to be determined. Transforming growth factor β1 (TGF-β1) is a powerful antimitotic signal for all neural crest cells that overrides the bFGF/neurotrophin proliferative signal. Furthermore, SCF promotes differentiation of neural crest cells into cells of the sensory neuron lineage. Neurotrophin-3 (NT-3) specifically promotes high affinity uptake of norepinephrine by neural crest cells and is thus thought to play a critical role in the differentiation of sympathetic neuroblasts. In summary, our data indicate that neurotrophins and other pertinent growth factors affect survival, proliferation and differentiation of neural crest cells at multiple levels and in different lineages. Moreover, our findings emphasise the importance of the concerted action of combinations of growth factors, rather than of individual factors.

Key words: Neurotrophins; stem cell factor; fibroblast growth factor; transforming growth factor beta; norepinephrine transporter.

INTRODUCTION

Neural crest cells give rise to a wide spectrum of cell types and tissues in the adult vertebrate organism (for review, see Weston, 1970; Le Douarin, 1982). They include the autonomic and enteric nervous systems, most primary sensory neurons and some endocrine cells (e.g. adrenal medulla, C-cells of the thyroid). In addition the neural crest contributes to the septation of the cardiac outflow tract and gives rise to many cranial structures, such as facial bone, skin, the visceral skeleton, tooth papillae, the corneal epithelium, and meninges of the forebrain amongst others (Le Douarin, 1982). The neural crest is of clinical interest. Because of the variety of neural crest progeny, many congenital defects, familial diseases and malignancies are of neural crest origin. A few selected examples include some congenital heart defects, cleft lip/cleft palate, Hirschsprung's disease, neurofibromatosis, neuroblastoma and melanoma (Kissel et al. 1981; Riccardi & Mulvihill, 1981). Moreover, the neural crest is a valuable experimental system to study the mechanisms that control cell type specification. How does a particular neural crest cell

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decide to become a sensory neuron rather than a melanocyte, or to give rise to a smooth muscle cell rather than to an enteric neuron? At the molecular level, these mechanisms are largely unknown. It is clear however, that the migratory neural crest is a mixed population, consisting of cells that are pluripotent to different degrees and of cells that are committed to a particular cell lineage (Sieber-Blum & Cohen, 1980; Ito & Sieber-Blum, 1991; Ito et al. 1993; Sieber-Blum et al. 1993).

Several in vitro (Sieber-Blum & Cohen, 1980; Baroffio et al. 1988; Sieber-Blum, 1989*a*) and in vivo (Bronner-Fraser & Fraser, 1988) clonal analyses have shown that many neural crest cells are pluripotent in the sense that they can generate a diverse progeny. In addition to pluripotent cells there are at least 3 types of cells that are committed to a particular lineage. Some sensory neurons become committed very early, possibly before the onset of neural crest cell migration (Le Lièvre et al. 1980; Sieber-Blum, 1989*a*). Furthermore, in the trunk the majority of early migratory neural crest cells are committed to become pigment cells (Sieber-Blum & Cohen, 1980). In contrast, at cranial levels a significant portion of neural crest cells are committed to become smooth muscle cells (Ito & Sieber-Blum, 1991). The latter 2 populations of committed cells form inverse rostrocaudal gradients along the neuraxis, indicating that mechanisms during gastrulation are likely to affect neural crest cell differentiation (Wiskowski & Sieber-Blum, unpublished results). The neural crest is most diverse at posterior rhombencephalic levels. In addition to forming autonomic and sensory neurons and nerve supporting cells, neural crest cells from this region also migrate to the gut where they give rise to the enteric nervous system, they migrate through the branchial arches to the cardiac outflow tract, and they contribute to the formation of the lower jaw (Le Douarin, 1982). At this axial level pluripotent neural crest cells were observed that can give rise to at least 6 cell types: sensory neuroblasts, enteric neuroblasts, melanocytes, smooth muscle cells, chondrocytes, and connective tissue cells (Ito & Sieber-Blum, 1991; Sieber-Blum et al. 1993).

Knowledge of the time in development and the location in the embryo, in which the pool of pluripotent neural crest cells disappears is likely to aid in elucidating the mechanisms that direct neural crest cell diversification and cell type specification. For this reason, we performed clonal analyses of neural crest derived tissues. By visual inspection and immunocytochemistry, cultures were assayed for clones that had developed from individual cells and contained more than one cell type. Clones founded by pluripotent cells were observed in all tissues tested. In the trunk ectoderm, neural crest cells immigrated at embryonic day 3.5. As expected, the majority (79%) of these ectodermal neural crest-derived cells were committed to the melanogenic lineages. However, contrary to expectation, 20% were derived from pluripotent cells, forming mixed clones that contained pigment cells, autonomic neuroblasts, sensory neuroblasts and possibly other cell types (Richardson & Sieber-Blum, 1993). Substantial portions of the colony-forming cells in dorsal root and sympathetic ganglia were pluripotent to various degrees (Duff et al. 1991). Moreover, pluripotent neural crest cells en route to the cardiac outflow tract and the intestine were present within the posterior branchial arches (Ito & Sieber-Blum, 1993). In summary, pluripotent neural crest cells are found not only in the migratory neural crest, but also at sites of terminal differentiation. These findings further support the notion that embryonic microenvironmental signals encountered by migrating neural crest cells are involved in neural crest cell type specification.

Mixed clones derived from sympathetic and dorsal root ganglia were not equivalent. The portion of site-appropriate neuroblasts predominated over site-inappropriate neuroblasts (Sieber-Blum et al. 1993). Thus clones derived from sympathetic ganglia contained 10-fold more sympathetic neuroblasts than clones from dorsal root ganglia. Vice versa, there were about 10 times more sensory neuroblasts in DRG-derived clones than in sympathetic ganglion-derived clones. These quantitative origin-dependent differences in mixed clones indicate that the probability of forming a site-appropriate neuron increases in the appropriate site and decreases at inappropriate sites. This mechanism is similar to the one observed in the haematopoietic system, where pertinent growth factors have been shown to change the probability of cell type specification (Metcalf, 1983; Ponting & Dexter, 1988). We have therefore examined the actions of pertinent factors on neural crest cell survival, proliferation and differentiation.

TROPHIC EFFECTS

Stem cell factor (SCF) has been found to be important in erythropoiesis (Anderson et al. 1990). It is the ligand of the proto-oncogene, *c-kit* (Huang et al. 1990). SCF is defective in the mouse mutant, *Steel* (*Sl*; Copeland et al. 1990), whereas *c-kit* is mutated in the *Dominant White Spotting* mouse (*W*; Chabot et al. 1988). The coat colour is affected in both mouse

mutants, indicating that the SCF/*c-kit* system is involved in melanogenesis. SCF is known as a trophic factor for melanogenic cells and melanocytes (Morrison-Graham et al. 1990; Nishikawa et al. 1991; Murphy et al. 1992; Steel et al. 1992; Morrison Graham & Weston, 1993; Lahav et al. 1994). In our serum free culture system (Sieber-Blum & Chokshi, 1985; Zhang et al. 1997*a*), SCF alone had no detectable effect on melanogenesis (Langtimm-Sedlak et al. 1996). In contrast, when SCF was combined with a neurotrophin, the size of pigmented colonies increased, indicating either a mitogenic or a trophic action of the factor combination. To distinguish between the 2 possibilities, bromodeoxyuridine incorporation studies were performed. They indicated that the SCF/neurotrophin combination is trophic, not mitogenic. Equivalent results were obtained when nerve growth factor (NGF), brain derived growth factor (BDNF), or neurotrophin-3 (NT-3) were combined with SCF (Langtimm-Sedlak et al. 1996). Since the trophic effect was not specific for a particular neurotrophin, this may indicate that the mechanism involves activation of the nonselective neurotrophin receptor, p75NTR.

While melanogenesis was not noticeably affected, there was a marked increase in the size of colonies that were formed by pluripotent cells in the presence of SCF (Langtimm-Sedlak et al. 1996). BrdU incorporation studies showed that this is also a trophic effect. Interestingly, the trophic effect of SCF on pluripotent neural crest cells (and/or their immediate progeny) was neutralised by all of the 3 neurotrophins tested (NGF, BDNF, NT-3). Since p75NTR can mediate apoptosis (Chao, 1995), this may indicate that neurotrophins can actively eliminate pluripotent neural crest cells. This may be a mechanism functioning during gangliogenesis, when the pool of pluripotent neural crest-derived cells decreases.

In summary, our data indicate that SCF is a survival factor for pluripotent neural crest cells. Moreover, the combination of SCF and a neurotrophin, but not SCF alone, is trophic for committed melanogenic cells. These data indicate an interesting inverse relationship between the trophic signals for pluripotent cells and committed melanogenic cells. The presence of SCF alone appears to be lethal for committed melanogenic cells, but trophic for pluripotent cells. In contrast, the SCF/neurotrophin combination seems to be lethal for pluripotent cells, but supports the survival of committed melanogenic cells. These may be mechanisms for enrichment of pertinent subsets of neural crest cells within the different migratory pathways.

MITOGENIC AND ANTIPROLIFERATIVE EFFECTS

Basic fibroblast growth factor (bFGF) is a known mitogen in the nervous system. Contrary to expectation, colony assays in the presence of bFGF did not result in an increase in the size of neural crest cell colonies (Zhang et al. 1997*a*). BrdU incorporation studies, however, indicated an increase in the portion of S-phase nuclei per colony in the presence of bFGF, both in colonies that are formed by pluripotent cells and in colonies that are formed by committed melanogenic cells. This observation indicates that bFGF triggers mitosis, but that another factor is required either for cell division or for the survival of proliferating cells. When bFGF was combined with a neurotrophin, NGF, BDNF or NT-3, colony size increased significantly, but only in colonies that were formed by pluripotent cells (Zhang et al. 1997*a*). Sequential addition of the factors indicated that bFGF is required prior to the neurotrophin, suggesting that bFGF-primed neural crest cells become dependent on another factor. For pluripotent cells, neurotrophins can fulfill this rescuing role, whereas the factor acting in the melanogenic lineage remains to be determined. Interestingly, all 3 neurotrophins tested were able to support proliferation of pluripotent neural crest cells (and/or their immediate progeny). Again, this may indicate a role of the nonselective neurotrophin receptor, p75NTR.

In contrast, transforming growth factor-beta 1 (TGF-β1) blocked proliferation of all neural crest cells, pluripotent as well as committed melanogenic cells (Zhang et al. 1997*a*). This strong antiproliferative effect of TGF-β1 was dominant over the mitogenic action of the bFGF/neurotrophin combination (Zhang et al. 1997*a*). Neural crest cells are known to synthesise and release the latent form of TGF-β1 (Brauer & Yee, 1993) and plasminogen activator (Valinsky & Le Douarin, 1985; Erickson & Isseroff, 1989). Plasminogen activator is a serine protease that converts the inactive precursor, plasminogen, into the active protease, plasmin. Since plasminogen is abundant in the embryo at sites of tissue remodelling, it is conceivable that neural crest cells have an autocrine mechanism that stops them from proliferating when they enter a plasminogen-rich area.

THE NEUROGENIC ACTION OF TGF-β1

In addition to its strong antiproliferative action, TGF-β1 promoted neurogenesis in neural crest cell cultures (Zhang et al. 1997*a*). Even when corrected for the decrease in colony size, the number of sympathetic neuroblasts and sensory neuron precursors per colony

sharply increased in the presence of TGF-β. Since the portion of both types of neuroblasts increased within the same colony, it is believed that TGF-β acts on a common neurogenic precursor, which has been postulated by Marusich & Weston (1992) and which may be present in sympathetic and dorsal root ganglia in the young embryo (Sieber-Blum et al. 1993).

SENSORY NEUROGENESIS

In causing an increase in the number of colonies that contained sensory neuron precursor cells, SCF showed neurotrophin-like activity (Langtimm et al. 1996). Sensory neuron precursors were identified with an antibody against the stage specific embryonic antigen-1 (SSEA-1), which we have found to be specific for the sensory neuron lineage among neural crest derivatives of the quail (Sieber-Blum, 1989*b*). SCF and its receptor, *c-kit*, are expressed in embryonic dorsal root ganglia in the chick and mouse (Matsui et al. 1990; Keshet et al. 1991; Motro et al. 1991; Orr-Urtreger et al. 1991). Moreover, Carnahan et al. (1994) have found that SCF selectively supports survival of small DRG neurons. It is therefore conceivable that the SCF-mediated increase in SSEA-1-immunoreactive cells observed in our cultures indicates a role of SCF in cell type specification of nociceptive and thermoreceptive DRG neurons, in addition to its trophic role in more mature small DRG neuroblasts.

The SCF-mediated increase in the generation of SSEA-1-immunoreactive neuroblasts was neutralised by NGF and NT-3, but not by BDNF. We have shown previously that BDNF is involved in sensory neuron specification (Sieber-Blum, 1991). Our present data indicate that the promoting activities of SCF and BDNF are neither additive nor synergistic (Langtimm et al. 1996). This may indicate that in addition to p75NTR, SSEA-1-immunoreactive sensory neuron precursors also express TrkB, the BDNF-selective neurotrophin receptor. Thus NGF and NT-3, but not BDNF, may activate p75NTR and lead to apoptosis of SSEA-1-positive cells. This may be an additional embryonic mechanism to eliminate site-inappropriate cells and enrich site-appropriate cells.

DIFFERENTIATION OF SYMPATHETIC NEUROBLASTS

Sympathetic neuroblasts are generated by pluripotent neural crest cells, since in clones they appear together with other phenotypes, such as pigment cells, sensory neuron precursors and possibly other cell types (Sieber-Blum & Cohen, 1980; Sieber-Blum, 1989*a*). One of the earliest, if not the first, sympathetic neuron marker expressed during embryogenesis is the norepinephrine (noradrenaline) transporter (NET; Rothman et al. 1978; Zhang & Sieber-Blum, 1992). The NET is expressed by all adrenergic neurons (Pacholczyk et al. 1991; Amara & Kuhar, 1993; Blakely et al. 1994). Its function is thought to eliminate norepinephrine (NE) from the synaptic cleft in order to terminate the transsynaptic signal (Axelrod, 1965). Our data indicate that NET has an additional role during the commitment of pluripotent neural crest cells into sympathetic neuroblasts.

Not much is known about the mechanisms that initiate NET expression during embryogenesis. We have found that NT-3, in particular when combined with bFGF, causes an increase in the number of colonies containing cells capable of NE uptake (Zhang et al. 1997*b*). This effect is neurotrophin-specific as there were no significant increases observed with NGF or BDNF. The level at which NT-3 regulates NET expression remains to be determined.

NT-3 alone, or combined with bFGF, did not affect terminal adrenergic differentiation, as measured by expression of 2 enzymes in the biosynthetic pathway of NE, dopamine-β-hydroxylase (DBH) and tyrosine hydroxylase (Zhang et al. 1997*b*). However, when NE itself was added in the presence of NT-3, more colonies contained DBH-positive sympathetic neuroblasts. This finding indicates that NE uptake plays a key role in sympathetic neurogenesis. We interpret our results as follows. Neural crest cells, which are known to express the NT-3 selective receptor (Lamballe et al. 1991; Barbacid, 1994) TrkC (Tessarollo et al. 1993), upregulate NET when they encounter NT-3. NT-3 is present in the early embryo in several tissues, including the neural tube (Maisonpierre et al. 1990; Pinco et al. 1993; Scarisbrick et al. 1993; Yao et al. 1994; Zhang et al. 1994). Once the cells express NET, they can internalise NE. The source of NE in the avian embryo is the notochord, which synthesises NE and other neurotransmitters before any functioning neurons have developed (Strudel et al. 1977). In other species and/or other adrenergic tissues, additional mechanisms are conceivable. At high sodium concentrations, NET can reverse and release NE. Leakage of NE is yet another possibility (Schwartz, 1991).

Cocaine and some tricyclic antidepressants, such as desipramine, block NE uptake (Coyle & Snyder, 1981; Goodman Gilman et al. 1980). Both drugs block the NE-mediated increase of adrenergic differentiation in neural crest cell colony assays (Sieber-Blum, 1989*c*; Zhang et al. 1997*b*), supporting our notion that NE uptake plays a critical role in adrenergic

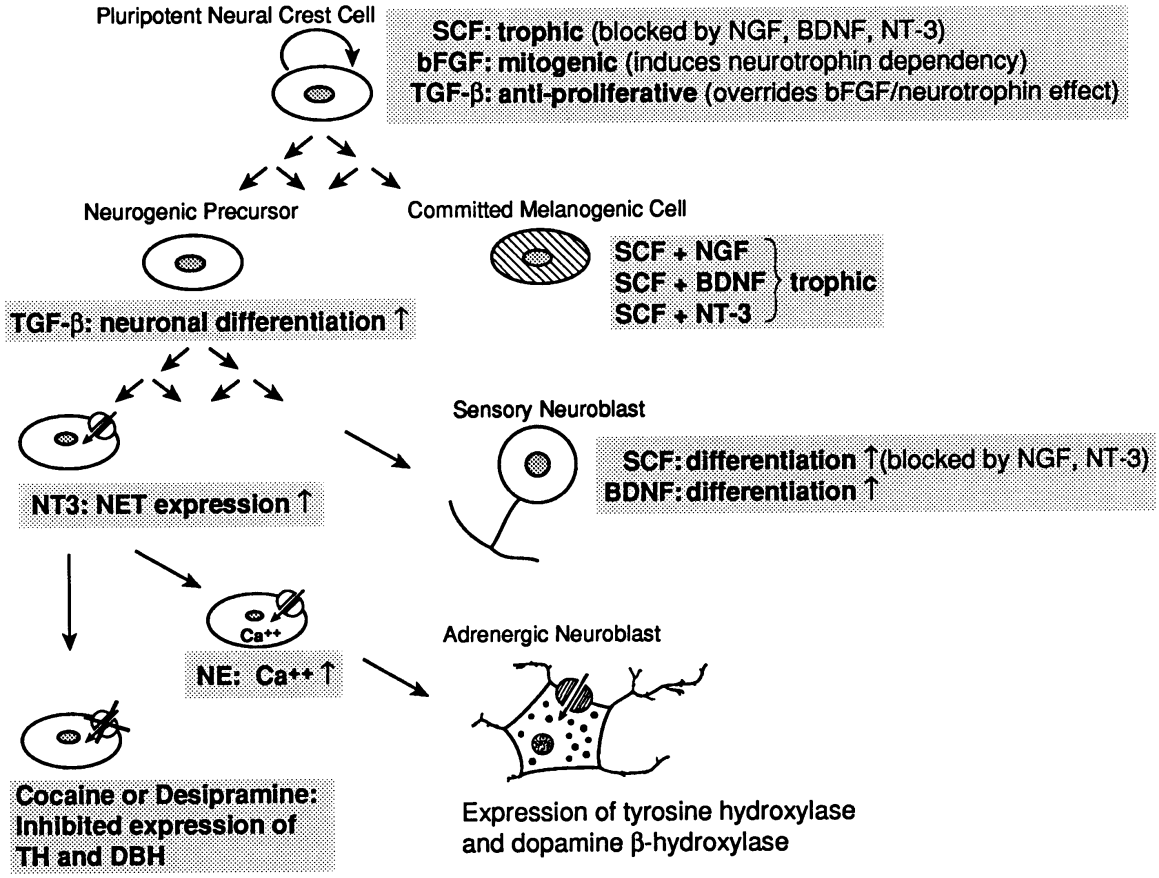

Fig. Differentiation pathways for pluripotent neural crest cells.

differentiation. Our result also emphasises the teratogenic potential of these drugs. Inhibition of adrenergic differentiation by cocaine may for instance explain some of the symptoms exhibited by neonates who have been exposed to cocaine or desipramine in utero. These infants have difficulties regulating respiration and show a higher incidence of the sudden infant death syndrome (Hill & Tennyson, 1986; Gingras & Weese-Mayer, 1990; Gingras et al. 1990). Functions of respiration and arousal are controlled by the locus ceruleus, an adrenergic nucleus in the brainstem. It may well be possible that the differentiation of locus ceruleus neurons is also inhibited by NE uptake inhibitors.

Conclusions

Our data indicate that neurotrophins, SCF and other pertinent growth factors affect neural crest cell proliferation, survival and differentiation in several lineages. They also emphasise the importance of the concerted action of growth factor combinations, rather than of individual factors. SCF is a survival factor for pluripotent neural crest cells, whereas bFGF is a mitogen and TGF-β acts as an antiproliferative signal. Basic FGF-primed cells subsequently become dependent on another growth factor, such as neurotrophins in the case of pluripotent neural crest cells (Fig. 1). The mechanism that controls segregation of committed melanogenic cells remains to be determined. It is likely to be a signal from the ectoderm because early emigrating neural crest cells contain a significant portion of pluripotent cells (Richardson & Sieber-Blum, 1993), whereas late emigrating neural crest cells contain a larger portion of committed melanogenic cells (Artinger & Bronner-Fraser, 1992). Our previous observations suggest that the mechanism is likely to involve protein kinase C (PKC). When neural crest cells were exposed long-term to the tumour promoters, presumptive neuronal cells became pigmented. Since tumour promoters are known to influence PKC activity (Nishizuka, 1984), our observation suggests that changes in the activity of a PKC is involved in melanocyte cell type specification. The survival of committed melanogenic cells is supported by the combination of SCF and a neurotrophin, rather than by SCF alone as commonly has been thought. Neurogenic precursors, capable of generating autonomic and sensory neuroblasts, are likely to be generated by a loss of melanogenic and Schwann cell potentials. Some early emigrating neural crest cells already contain pluripotent cells that have

lost the capacity to generate pigment cells (Sieber-Blum & Cohen, 1980). Moreover, the potential of generating Schwann cells is lost soon thereafter (Duff, Richardson & Sieber-Blum, unpublished). Presumably, this loss of options leads to a neurogenic precursor cell, capable of generating both autonomic and sensory neurons. The mechanisms that direct segregation into autonomic and sensory neuron lineages also remain to be elucidated. Our data indicate that SCF and BDNF are involved in directing neural crest cells into the sensory neuron lineage (see Fig.). In contrast, NT-3 appears to be important for sympathetic neuron development by upregulating high affinity NE uptake. NE uptake is likely to result in a calcium-mediated signalling mechanism, since ratio imaging indicated an increase in intracellular free calcium after NE treatment (Zhang et al. 1997*b*). Cotransport of sodium accompanies NE uptake, which is therefore electrogenic, conceivably leading to membrane depolarisation and the opening of calcium channels. Intracellular free calcium may then act as a signal transduction mediator and possibly activate the neurotrophin signalling pathway, which directly or indirectly is thought to lead to expression of TH. NE uptake inhibitors block the NE uptake and may thus inhibit the activation of the signal transduction pathway that promotes adrenergic expression (Fig.).

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