Cellular and molecular aspects of cephalic neural crest development: workshop report

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Introduction

The neural crest (NC) is derived from the ectoderm at the lateral borders of the neural plate and is first distinct during the later stages of neurulation when prospective NC cells segregate from the surrounding epidermal ectoderm and neurectoderm along the entire dorsolateral aspect of the forming and newly formed neural tube. From this origin, NC cells migrate extensive distances throughout the embryo to give rise to a large number of differentiated cell types and contribute to a diverse series of organ systems. This unique cell population therefore poses fundamental questions of the mechanisms underlying morphogenesis and differentiation during embryonic development.

The developmental fate of NC cells reflects their point of origin along the anterior-posterior axis of the embryo. Of particular significance to the present volume is the major contribution of cells derived from the cephalic NC to the facial skeleton. In contrast, trunk NC cells do not give rise to skeletal structures at least in Aves, the best studied class. Regardless of their level of origin, NC cells apparently migrate along discrete pathways to specific destinations. The differentiated phenotype expressed by postmigratory cells reflects their ultimate location within the embryo and therefore, directly or indirectly, both the pathway along which they have migrated and their level of origin along the anterior-posterior axis. Consequently, the morphogenetic and differentiative programmes of NC cells must be regarded as inter-related events, although this inter-relation need not be obligatory.

Heterotopic grafts of NC between different levels of the anterior-posterior axis produce progenies of cells which migrate and localize according to the site of grafting rather than their site of origin, although there are important exceptions. Such experiments indicate that the local microenvironment is important in specifying the general sweep of morphogenesis of the NC population. The lines of differentiation of these heterotopically grafted NC cells was also in general appropriate for the site of grafting although again there were some appropriate cell types omitted and some inappropriate cell types produced. At least three extreme positions could be consistent with these observations. First, early NC cells might be all committed as to their subsequent fate and also protodifferentiated, the characters of which need not be directly related to the properties of the terminally differentiated state. Second, the cells might be uncommitted (within the broad range of NC potentialities) and therefore all possess equivalent characters not antecedent or related to any particular terminal state. Third, NC cells might be committed at an early stage, but not at this time express significant qualities of protodifferentiation other than those of NC cells as a whole. In the first case, the precise matching of position of localization with the line of terminal differentiation could be achieved by the appropriately protodifferentiated cells choosing one of a subset of morphogenetic pathways leading to the correct final position. In the second case, the matching of the final position and differentiated states could be achieved by cells following morphogenetic cues appropriate for NC cells in general, then being instructed as to the line of differentiation by the local microenvironment. In the third possibility, the cells could follow morphogenetic sequences of a general type, but the lineages that are maintained and expanded are selected by the microenvironment from those committed cells that happened to localize correctly.

More difficult to investigate would be the possibility that in the population of NC cells, the timing of commitment for any one line of terminal differentiation varies for cells that will become committed in that way. In addition, the different end states could become committed at different times if, for instance, commitment was achieved by a series of partial restrictions which remove single or groups of differentiated options from the potential differentiative programme. In this situation, experimental paradigms could be created which would confirm each one of the three extreme states: it would be important then to assess the relative importance of each at each axial level.

The question of the potential heterogeneity of NC cells was addressed by Jim Weston (Oregon) and complicates the analysis of their migration and differentiation. However, whether homogeneous or heterogeneous, NC cells must respond to environmental cues which promote their migration to an appropriate destination and arrest it once the cells have arrived there. Each of the cellular and molecular mechanisms used by NC cells undergoing morphogenesis are unlikely to be unique, but rather to be particular examples and sequences of events found in other systems in vivo and in vitro. Study of these events from first principles in any embryonic system including the NC is difficult, therefore powerful insights into NC development may be obtained from other simpler systems. To this end, Robert Pytel (Basel) discussed the molecular mechanisms underlying cell–matrix adhesion, Simon Goodman (Munich) demonstrated the significance of selective cell–matrix interactions to cell movement and Andreas Faisnnr (Heidelberg) described specific molecules mediating intercellular adhesion in the nervous system.

Potential heterogeneity in the early NC population

During the first 12 h of neural tube culture, a population of NC cells migrates onto the substratum. By 24 h, additional clusters of cells appear on the side of the neural tube and remain there. When these clusters are removed and plated, the cells disperse and assume a characteristic NC phenotype. However, even within about 10 h of detachment from the neural tube, a few
NC cells (1–2%) are postmitotic, morphologically similar to sensory neurones and stain with antibodies to neurofilaments and have a neurone-specific GQ ganglioside recognized by the antibody A2B5, suggesting that differentiation of some neurones occurs very early. Neurogenesis of clustered NC cells is only significantly expressed when they are plated out and the ability to give rise to neurones decreases with increasing time spent in the cluster prior to plating. Conversely, differentiation along the melanocytic pathway is augmented with increasing time prior to plating of the original cluster.

Neurones are postmitotic so their numbers can only increase by recruitment from an undifferentiated population. During the first day of culture from dispersed NC clusters, the percentage of A2B5-positive cells is relatively low but increases dramatically between day 2 and 3 to achieve a maximum of 15–30% of all cells on culture day 4. When cultures are labelled between day 1 and 2 with [3H]thymidine, 80% of the A2B5-positive cells are [3H]thymidine labelled on day 4. This number decreases to 50% when the cells are labelled between days 2 and 3, and 2–10% when the cells are labelled between days 3 and 4. Consequently, the majority of A2B5-positive cells become postmitotic during the first 2 days of culture.

The cells that give rise to neurones are derived from a neurogenic precursor population and not from a general, continually cycling, pool of undifferentiated NC cells. The addition of A2B5 and complement to day-2 cultures reduces the number of A2B5-positive cells to zero but has no detrimental effect on cells about to give rise to A2B5 immunoreactivity. However, the ability to give rise to A2B5-positive cells progressively decreases to virtually zero following immunocytoysis on culture days 3 through 7. These results suggest that there is an early commitment of developmental fate, particularly for the appearance of the neurogenic precursor population out of the general NC pool. There is also an important sequence of decisions, one of the first of which is to establish the primary sensory neurone population. The remaining cell population has melanogenic, gliogenic and neurogenic capability which subsequently segregates further to give rise to all other neurones.

When the neurogenic population has segregated out, the remaining NC cells possess only melanogenic and gliogenic potentialities. These remaining cells must select either melanogenic or gliogenic fates depending upon their ability to associate with differentiated neurones. If they associate with differentiated neurones they will undergo gliogenesis as opposed to melanogenesis. If they fail to do so they will undergo melanogenesis. If NC cells remain in clusters they lose neurogenic potential and acquire melanogenic potential. Relating this in vitro behaviour to the real situation, SEM studies show that when NC cells segregate from the neuroepithelium they initially cluster in a notch between the somite, the top of the neural tube and the overlying ectoderm. If this area of skin and associated NC is transplanted from an E10–11 mouse, its melanogenic potential is revealed. This notch therefore appears to be a staging area into which NC cells segregate before they are able to migrate over the dermomyotome and into the skin to produce pigment cells.

The very precocious acquisition of overt neuronal characters shows that, although not yet differentiated, a subpopulation of premigratory (or very early migratory) NC cells has undergone a decision to become neurogenic precursors. This is realized when these cells migrate to a position appropriate or permissive for neural differentiation. However, in some regions this may be more difficult: cells in the caudal portion of the somite cannot migrate ventrally or laterally and therefore remain longer in the notch. Cell death in the premigratory staging area has not yet been examined but it is possible that during the extra period in the notch the neurogenic precursors die leaving a population of cells with a melanogenic bias Consistent with this, 48h clusters in culture have lost cells with neurogenic potential and the proportion of dead cells increases. It has yet to be demonstrated that the cells that die are neurogenic precursors, but the timetable of restriction of potential is realistic. Heterogeneity within the NC population exists very early: it will be important to assess its overall importance and whether this has consequences for morphogenesis as well as differentiation, and whether selection (by, for example, cell death) in a committed population plays an important part in matching the NC population to its immediate environment.

The Interaction of cells with their environment

(i) RGD-mediated cell–matrix interactions

In addition to fibronectin, a number of extracellular matrix (ECM) glycoproteins such as the collagens, laminin, vitronectin, fibrinogen and von Willebrand factor can be found in various tissues. Cloning of the fibronectin gene has demonstrated twenty or more forms generated by differential splicing of a single gene. These different forms of fibronectin may have different functional properties with respect to their interaction with the cell surface and could have different cell surface receptors. Collagen is equally complicated comprising a large number of different types. The cell attachment site of fibronectin has been identified as a tripeptide sequence -Arg-Gly-Asp (RGD), now considered to be a major recognition signal used in the interaction of many ECM proteins, including collagen, with cell surfaces. Unlike fibronectin and collagen, although it is an adhesive ECM component laminin does not contain an RGD sequence. However, it is possible that a strict RGD sequence is not always required: for example, in fibronectin, KQAGG present in the C-terminal part of the gamma chain inhibits its interaction with blood platelets. An asp (D) shortly preceded by a positive charge could be the sole invariant feature of this interaction system. Flexibility or variability would not be expected with a short sequence such as RGD. However, the cell responses and hence receptors to ECM proteins containing RGD are distinct, indicating the importance of flanking or more distant sequences in dictating the specificity of the key RGD region.

Receptors for ECM components have been identified using two approaches First, polyclonal or monoclonal antibodies were prepared against the cell surface or membrane fractions and screened for specific inhibition of cell attachment to a matrix component. Two independently characterized but equivalent monoclonal antibodies, CSAT and JG22, inhibit the interaction of avian cells with not only fibronectin but also with laminin. Similar antibodies specifically inhibiting attachment to fibronectin have been generated against mammalian cells. In each case, the interaction could be perturbed by short synthetic peptides containing the RGD sequence. The second approach employed affinity chromatography using
the ECM component, fragments or synthetic peptides as a ligand to isolate receptors from a cell solubilize.

Surface-labelled osteosarcoma cells were applied to a fibronectin affinity column and putative receptors eluted with a hexapeptide containing the RGD sequence. Further purification by wheat germ agglutinin affinity chromatography resulted in the isolation of a heterodimeric receptor of subunit $M_1$, $140 \times 10^3$ (140K). Reassuringly, these were the same as those identified by the above-mentioned antibody approach. When vitronectin was used as ligand, a different heterodimeric receptor was obtained. Yet another receptor was isolated by peptide affinity chromatography of platelets. When incorporated into lipid vesicles these distinct receptors have different binding specificities for extracellular ligands. The receptor isolated from platelets is identical to a previously identified platelet membrane protein (gp110/11a) which serves as a fibronogen receptor but also interacts with fibronectin, vitronectin and von Willebrand factor. Although similar to the platelet fibrinogen receptor, the vitronectin receptor is very specific recognizing neither fibrinogen nor fibronectin. The mammalian cellular fibronectin receptor is equally specific for fibronectin. None of these receptors bind laminin which distinguishes them from the avian CSAT protein complex. Although the specificities are quite different, in all cases the binding of each of these receptors to the appropriate ligand can be completely or partially inhibited by RGD-containing peptides.

The biochemical properties of these RGD receptors ally them with a larger family of heterodimeric cell surface glycoproteins which have diverse receptor functions. The electrophoretic mobility of the two subunits, termed alpha and beta, in this family changes characteristically under reducing conditions. The alpha chain of these receptors is cleaved after biosynthesis generating a 25K fragment released only under reducing conditions. Consequently, under nonreducing conditions, the alpha chain is significantly larger. The beta chain contains a tightly folded cysteine-rich domain and migrates faster under nonreducing conditions. There is no apparent homology between the alpha and beta chains but all the beta chains are related with ~45% homology at the peptide level. Both chains of the fibronectin and vitronectin receptor have been cloned and sequenced; they are different gene products but closely related to one another and this is likely to be true of other members of this group. Because they are integral membrane proteins this family of receptors has been termed the integrins.

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The integrin family comprises three groups, each defined by a common beta chain combined with a number of different alpha chains. The first group includes the fibronectin receptor together with five proteins, one of which may be the laminin receptor (at least in Aves) and others which are expressed mainly on leucocytes. The second group includes the vitronectin receptor and the platelet glycoprotein IIb/IIIa. Based on apparent molecular weight, subunit composition, chemical structure and sequence homology, this second group also includes the position-specific antigens of Drosophila. These antigens were identified by monoclonal antibodies and their expression is related to the position of cells within the Drosophila embryo. The third group is expressed exclusively on leucocytes and includes surface proteins which function as receptors for complement and are involved in the recognition and cell-cell adhesion of cytotoxic T-lymphocytes.

The identification of sequences on ECM molecules recognized by cells, and the cell surface receptors carrying out this recognition point to a complex system, are highly specific yet bear strong areas of structural similarity at both target and receptor levels. The involvement of these in the central developmental mechanism of cell adhesion indicates their importance to the NC system. This is born out by the findings that perturbation techniques developed in model systems in vitro using receptor-specific antibodies and key recognition peptides both disturb cranial NC cell migration.

(ii) The interaction of cells with laminin

Myoblasts are a particularly useful system with which to examine the molecular mechanism by which cells interact with and respond to specific components of their extracellular environment. To establish the musculature associated with the appendicular skeleton, myoblasts migrate from the somite into the limb bud where they differentiate. Myoblasts can be readily isolated from murine skeletal muscle and are capable of proliferating, locomoting and differentiating in vitro up to a stage of syncytial contractile myotubes which assemble a basement membrane with components derived from fibroblastic cells. Established myoblast cell lines also exhibit the same kinetic and molecular differentiation pathway as cells in vivo.

Laminin is a $M_1$, 900K cross-shaped protein exclusive to basement membranes. Primary myoblasts rapidly extend on laminin substrates to become polarized and proliferate rapidly. After 4 days of culture the population is extremely dense and contains many differentiated myotubes. This programme requires no exogenous growth or serum factors. In contrast, myoblasts exhibit a rounded or stellate morphology when cultured on fibronectin and proliferate and differentiate poorly. This is not owing to a difference in cell adhesion since they attach equally well on laminin or fibronectin substrates. In contrast, they are unable to attach to plastic or plastic coated with either collagen type I or many other ECM molecules. The behaviour of myoblasts on fibronectin and laminin can be compared directly by assay on side-by-side tracks of fibronectin and laminin. Under these conditions, the cells attach and extend lamellipodia and filopodia on both substrates. However, they are highly motile on laminin but move extremely slowly, if at all, on fibronectin. The effect of fibronectin was not due to the cells being anchored too securely or not securely enough because variation of concentration and therefore of cell adhesion did not promote locomotion. Mixtures of laminin and fibronectin on the substrate result in the cells still being migratory, unlike on fibronectin alone, suggesting that the interaction of laminin with its receptor is a positive signal for motility. Clearly, laminin stimulates the motile system of myoblasts independently of its effect on cell adhesion per se. Nevertheless, at low concentrations of laminin the cells were highly motile while at very high concentration the cells were flatter and less motile.

Treatment of laminin with elastase generated a number of discrete fragments, two of which (namely, E8, the end of the long arm and E1-4, all of the cross region) are of particular significance for cell attachment. Tumour cells attach to laminin, its E8 fragment...
or its E1-4 fragment. In contrast, primary myoblasts attach to and locomote on the E8, but not E1-4, fragment and are not inhibited by GRGDSP. Myoblasts possess approximately 15x10^3 receptors with an affinity of 1 ns while E1-4 receptors are not measurable. Analysis of a myoblast cell line demonstrates that as the cells differentiate the number of E8 receptors remains constant but the number of E1-4 receptors increases. If the cells are allowed to proliferate to very high densities and differentiate into myotubes, they eventually downregulate both receptors to about 5x10^2 per cell. The affinity for E8 remains the same as at earlier stages but the affinity for E1-4 is an order of magnitude higher. This implies that there are distinct receptors for two regions of laminin and that these are individually regulated in relation to the differentiation programme.

Synthesis and secretion of laminin and collagen type IV by myoblasts increases as the cells differentiate. Addition of laminin to differentiating myoblasts stimulates them to secrete more basement membrane components, so this could act as an autocrine development factor. Thus, myoblasts move out of the somite provided they can adhere to a motility-promoting laminin substrate. They then begin to secrete laminin which stimulates the proliferation and differentiation programme. As the cells move they secrete even more laminin until it reaches such a concentration that the cells are unable to move and then accumulate a basement membrane. This could provide a mechanism by which the cells gather themselves in the muscle anlage. This system therefore demonstrates an entire developmental programme based on cell adhesion and extending through locomotion and adhesion, and differentiation and localization. Uniquely, this is independent of exogenous growth factors, but depends on the precise molecular nature of the ECM component to which the cells attach, and on the type and number of receptors on the cell surface.

(iii) Molecular mechanisms of intercellular adhesion

Selective intercellular adhesion is a subclass of intercellular membrane interactions. The concept of selective intercellular adhesion suggests that cell surfaces might possess specialized molecular structures which would confer preferential adhesivity to another surface. This type of selective intercellular adhesion could generate hierarchies of adhesivity between cell populations and thereby contribute to multicellular structure formation. The establishment of connectivity patterns in the CNS as well as for tissue organization elsewhere. Immunological approaches have led to the identification of the neural cell adhesion molecule N-CAM. Similar strategies have resulted in the production and characterization of two monoclonal antibodies, L1 and L2, both of which are directed against neural antigens. L1 recognizes two main constituents from the CNS with M, of 200 and 140x10^2. L2 recognizes 160K and 100K components, the smaller of which has been identified as the myelin-associated glycoprotein (MAG). The L2 epitope is identical to the sulphated glucuronid acid residue recognized by the HNK-1 monoclonal antibody. This epitope is present on some, but not all, L1, N-CAM, or MAG molecules; for example, only 30-35 % of the L1 molecules and 20% of the N-CAM population express this epitope. This indicates that the addition of the carbohydrate epitope during post-translational modification is independent of the glycoprotein backbone. Despite the common L2 epitope these proteins have unrelated protein backbones. The L2 epitope may be indicative of a functional classification (i.e. adhesion) for glycoproteins of different structure.

An additional adhesive glycoprotein, termed J1, was identified on the basis of its possession of the L2 epitope. Polyclonal antisera prepared against purified J1 glycoprotein react with the cell surface of astrocytes and oligodendrocytes in vitro. At early stages of cerebellar development this antibody reacts with high molecular weight membrane species of 220K and 200K. With subsequent development these two components disappear and components with molecular weights of 160 and 180K predominate. In a cell suspension to cell monolayer adhesion assay, monovalent J1 antibodies specifically interfere with neurone-astrocyte adhesion but not with neurone-neurone or astrocyte-astrocyte adhesion. In contrast, L1 antibodies perturb only neurone-neurone and not neurone-astrocyte adhesion, while N-CAM antibodies interfere with both neurone-neurone and neurone-astrocyte adhesion.

Pulse-chase labelling followed by immunoprecipitation of primary glial cultures demonstrates that the main J1 glycoprotein synthetic products are the 220K and 200K forms. Both components are present in the cell solubilizate and the culture supernatant. In addition, there is a minor amount of 160K synthesized. The half-life of these components is approximately 30min in the cell layer, after which they can be identified in the culture supernatant, consistent with the hypothesis that J1 glycoproteins are constitutive secretion products. This is confirmed by the inhibition of J1 secretion by monensin.

J1 polypeptides are independent simultaneous synthesis products and there is no interconversion between the different forms. J1 160K and 180K are relatively more minor components. J1 160K isolated from adult brain and J1 220K isolated from astrocyte cultures were Western blotted with J1 antibodies. The antibodies were eluted from each of the bands and reapplied to the blots. J1/160 antibodies cross-react with the 160K and 180K components but only weakly with the 220K, indicating that the homology between 160K and 220K is rather low. Antibodies against the 180K component cross-react with the 160K and, although weaker, the 220K. Antibodies against the 220K component cross-react with the 160K. Thus, there are two high molecular weight forms with a rather high degree of homology and two low molecular weight forms also with a high degree of homology. These two groups share a common epitope but homology between them is relatively low. At the present time, little is known about the J1 180 and 160K components. J1 200/220 represent members of a group of closely related or equivalent glycoproteins which are indistinguishable from tenascin on an immunological basis and distribute identically in non-CNS tissues. In addition, J1/tenascin is likely to be identical to cytotactin. The distribution of immunoreactivities indicates that the J1 glycoproteins can also be found in the ECM where its spatiotemporal distribution is related to patterns of cell interaction and disorganization in ventral and lateral development. It has been proposed that glial cells in the CNS form a spatial scaffold which directs the pattern of neurite extension. The heterotypic cell interaction mediated by J1 provides an opportunity to examine the role of specific intercellular adhesion in the establishment of the cellular architecture of the CNS and other tissues.

Conclusion

The morphogenetic and differentiative histories of NC cells are very complex
but they consist of events that are in themselves comparatively simple. In order to identify the underlying processes it is necessary to be able to dissect the system into components of restricted degrees of freedom. Therefore it is of clear importance to recognize the degree of pre-existing heterogeneity in populations of NC cells. The usual experimental approach, for example, is to obtain populations of NC cells in vitro. Results from these cells are then interpreted as pertaining to cells which are uniform in terms of capabilities and commitment. That this is not necessarily the case has been suggested by Weston. Future experimentation on the nature of differentiative controls must more clearly distinguish between selective mechanisms (whose importance may have been underestimated) and instructive mechanisms (the timing of which may have been placed too late in the NC development timetable).

Likewise the possibility of precocious commitment carries the possibility that the various morphogenetic capabilities of the cells in the population can be separated into those appropriate for some cells protodifferentiated for one line versus those appropriate for other lines of protodifferentiation. It is therefore crucial to identify at each stage and for each axial level, the degree of commitment of the cells, the degree to which this is expressed functionally at the time, and the timetable with which it will become expressed as development proceeds.

The morphogenetic behaviour of NC cells as a whole is different from other embryonic cells, and undoubtedly rests on their cell adhesive repertoire. In particular the receptors of the integrin family which recognize RGD-sequence targets obviously play an important role. Methods of analysis developed and described by R. Pytela in model systems in vitro will help to unravel the precise contributions of this to cranial NC morphogenesis. However, the degree of relationship between the members of this family of receptors, and the similarity of their ligands means that a lack of specificity may occur especially when the very complex situation in vivo is probed.

Building from this ECM adhesion system, results are often extended more or less directly to complex responses: cell morphology, locomotion, migration, morphogenesis and differentiation. S. Goodman’s results with the myoblast system indicate that this extension must only be made with caution; two ECM molecules (fibronectin and laminin) promote cell adhesion similarly and both encourage cell spreading, but higher order functions are quite different – in particular the locomotory behaviour of the cells and their differentiation is radically different on the two molecules. There are also important lessons here for those seeking to explain the morphogenesis and differentiation of NC cells.

Cell–ECM interactions are very important for development, but cells also interact with other cells of the same or different types. A programme for studying the adhesive nature of these interactions was described by A. Faisser, using as a model the CNS where cell–ECM interactions are less important. These studies showed that heterotypic and homotypic cell–cell adhesive functions could be assigned to specific cell surface molecules and that various arrays of these molecules could act in concert. Moreover, the distinction between cell adhesive molecules and ECM adhesive molecules can become blurred in some cases. The phenomenon of differential cell aggregation and disaggregation is a feature of NC development and must rest on cell–cell adhesive mechanisms. Avenues are now open to investigate these at the molecular level, using approaches and reagents developed in these model systems.

In considering the control of early NC cell development, knowledge of the mechanisms involved has advanced dramatically, but for both molecular markers of differentiation and for functional molecules this has depended upon the identification in the NC systems of molecules isolated and characterized elsewhere, in older embryonic or adult tissues or in transformed cells. This means that any molecules not characterized in these conditions will be ignored; these may well be the most important in defining the special properties of the NC. The application of molecular biological approaches may in the future lay even these mechanisms open to study, so that the NC (and other early vertebrate developmental systems) will not be restricted to the limits set by more accessible systems.

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