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Transforming growth factor-β control of cell-substratum adhesion during avian neural crest cell emigration in vitro

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Summary

It has been proposed that, in higher vertebrates, the onset of neural crest cell migration from the neural tube involves spatially and temporally coordinated changes in cellular adhesiveness that are under the control of external signals released in the extracellular milieu by neighboring tissues. In the present study, we have analyzed the dynamics of changes in cell-substratum adhesiveness during crest cell emigration and searched for regulatory cues using an in vitro model system. This model is based on the fact that, in vivo, crest cell dispersion occurs gradually along a rostrocaudal wave, allowing us to explant portions of the neural axis, termed migratory and premigratory levels, that differ in the time in culture at which neural crest cells initiate migration and in the locomotory behavior of the cells. We found that neural crest cell emigration is not triggered by the main extracellular matrix molecules present in the migratory pathways, as none of these molecules could abolish the intrinsic difference in the timing of emigration between the different axial levels. Using an in vitro adhesion assay, we found that presumptive neural crest cells from premigratory level explants gradually acquired the ability to respond to extracellular matrix material with time in culture, suggesting that acquisition of appropriate, functional integrin receptors was a necessary step for migration. Finally, we showed that members of the transforming growth factor-β family reduced in a dose-dependent manner the delay of neural crest cell emigration from premigratory level explants and were able to increase significantly the substratum-adhesion properties of crest cells. Our results suggest that acquisition of substratum adhesion by presumptive neural crest cells is a key event during their dispersion from the neural tube in vitro, and that members of the transforming growth factor-β family may act as potent inducers of crest cell emigration, possibly by increasing the substratum adhesion of the cells.

Key words: neural crest, cell adhesion, cell migration, transforming growth factor-β.

Introduction

In the vertebrate embryo, the neural crest originates from the dorsal region of the neural axis. This cell population subsequently becomes separated from the neural tube to disperse along defined pathways throughout the embryo and eventually to lodge in various locations where cells terminate differentiation (Le Douarin, 1982; Newgreen and Erickson, 1986). The process of crest cell separation from the neural epithelium is precisely regulated, both spatially and temporally. Neural crest cells first undergo migration from the most rostral levels of the neural tube in the forebrain and continue to separate in a rostrocaudal wave approximately parallel to the wave of segmentation of the axial mesoderm (reviewed in Newgreen and Erickson, 1986). Morphological studies of the trunk region in the avian embryo have permitted a detailed description of the main cellular events that accompany the onset of neural crest cell migration (Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985; Martins-Green and Erickson, 1986, 1987; Newgreen and Erickson, 1986; Levi et al., 1990). In this species, segregation of neural crest cells from the neural tube and dispersion occur simultaneously. During crest cell separation from the neural tube, the basal lamina that partially covers the dorsal part of the neural tube is completely disrupted, and cell-cell adhesion molecules of the cadherin family disappear from the surface of the emigrating neural crest cells, which acquire the ability to adhere and locomote on extracellular matrix material. However, these studies did not allow us to determine exactly the dynamics of crest cell dispersion, because it occurs in the embryo within a short period of time and in a restricted portion of the neural tube.

The underlying regulatory mechanisms of the onset of neural crest cell migration also remain to be established. It has been suggested that emigration is under the control of external signals released in the extracellular milieu by neighboring tissues. Using tissue grafting or transplantation

of extracellular matrix material adsorbed onto microcarriers, Löfberg et al. (1985) found that, in amphibians, neural crest cells are triggered to initiate rapid emigration by extracellular material originating from the dorsal epidermis of the rostral half of the embryo. However, which of the various components present in the extracellular matrix are responsible for inducing migration has not been determined yet. The search for such environmental cues is of crucial importance to elucidate the process of crest cell separation from the neural epithelium.

It is possible to reproduce, to the some extent, in an in vitro system the major events that accompany neural crest emigration in avians, taking advantage of the fact that it occurs gradually along a rostrocaudal wave (for details, see Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985). If the portion of the neural tube corresponding to the last somites of a 2.5 day-old embryo is explanted in culture, neural crest cells readily emigrate from the explant within less than 2 hours. In contrast, if the neural tube corresponding to the presomitic region is explanted in culture, neural crest cells are seen migrating only after a 6 to 7-hour delay. This observation would indicate that neural crest cells originating from the neural tube corresponding to the presomitic region have not entirely achieved the whole sequence of events necessary for initiation of emigration. Accordingly, these axial levels have been termed migratory (ML) and premigratory (PML) levels (Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985). Hence, this in vitro system is suitable to analyze sequentially the modulations of adhesive properties of neural crest cells originating from the two axial levels and to test various factors for their ability to control emigration of cells.

In the present study, using this in vitro model system, we have analyzed the modulations of cell-substratum interactions at the onset of crest cell emigration and have compared the migratory behavior of ML and PML crest cells in order to approach the acquisition of locomotory properties of cells. We find that PML neural crest cells have reduced capabilities of both adhesion and locomotion onto extracellular matrix molecules compared to ML crest cells. We also present evidence that members of the transforming growth factor-β (TGF-β) family can act as potent inducers of premature PML crest cell migration in vitro.

Materials and methods

Embryos

Japanese quail (*Coturnix coturnix japonica*) embryos were used throughout the study. Eggs were incubated at 38 ± 1°C and staged according to the number of somite pairs and to the duration of incubation.

Materials

Human plasma fibronectin was purified on a gelatin-Sepharose column as described previously (Rovasio et al., 1983). Mouse Engelbreth-Holm-Swarm sarcoma-derived laminin was purchased from Gibco Laboratories, and chicken tenascin was kindly provided by Dr M. Chiquet (Biozentrum der Universität Basel, Switzerland). Rat collagen I was prepared from tail tendon according to the method of Bornstein (1958). Polylysine was purchased from Sigma. A rat monoclonal antibody (ES46) to chicken β1-integrin subunit was kindly provided by Dr K.M. Yamada (NIH, Bethesda, MD, USA). Polyclonal antibodies to chicken vinculin and a mouse monoclonal antibody (ID-7.2.3) to the adherens junction-associated cell adhesion molecule (A-CAM) were a gift of Dr B. Geiger (The Weizmann Institute, Rehovot, Israel). A mouse monoclonal antibody, called NC-1, identical to HNK-1 and specifically recognizing neural crest cells at the time of their migration, was described elsewhere (Tucker et al., 1984; Vincent and Thiery, 1984). Human platelet-derived TGF-β1 and TGF-β2, biotinylated TGF-β1, and neutralizing rabbit antibodies to TGF-β1 recognizing both TGF-β1 and TGF-β2 were purchased from Research and Diagnostic Systems (British Biotechnology Ltd., Oxon, United Kingdom).

Cell cultures and assays for cellular migration

The trunk regions corresponding to the somitic and presomitic levels of embryos at 15-30-somite stages (see Fig. 1) were excised with a scalpel. The fragments were incubated for 30-60 minutes at room temperature in a 2.5 units/ml Dispase solution (Boehringer Mannhein, FRG), and neural tubes were separated manually from adjacent tissues with needles. In some experiments, fragments of notochords were left attached to the neural tubes to indicate the dorsoventral orientation of the explants. After dissociation, neural tubes were allowed to recover from enzyme treatment by an incubation in Dulbecco's modified Eagle's medium (DMEM) for 30 minutes and deposited in wells of Terasaki plates previously coated with fibronectin at 25 μg/ml, laminin at 50 μg/ml, collagen I at 10 μg/ml, tenascin at 50 μg/ml, or polylysine at 10 μg/ml in phosphate-buffered saline (PBS). Cultures were incubated in DMEM supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere and observed periodically with a Nikon phase contrast inverted microscope. In some experiments, the serum was depleted in fibronectin following the procedure described by Rovasio et al. (1983). For time-lapse video microscopy, cultures were observed with a Nikon Diaphot inverted microscope in a heated plexiglass chamber equipped with a video camera (Hamamatsu, Japan) connected to a TV monitor (Hitachi, Japan) and a time-lapse recorder (Mitsubishi, Japan). Migration paths were plotted on the TV monitor and copied onto transparent paper. The total distance of migration of selected cells was measured and the speed of locomotion calculated. The degree of persistence of movement was defined as the ratio between the linear distance and the total distance covered by the cells.

Assays for cellular adhesion

Cellular adhesion assays were performed in 10 cm bacteriological Petri dishes. The use of Petri dishes designed for cell culture was avoided because they gave high non-specific cell adhesion. Small areas of the dishes were incubated at 37°C for 1 hour with 20 μl of fibronectin at 25 μg/ml, laminin at 50 μg/ml, collagen at 10 μg/ml, tenascin at 50 μg/ml, or polylysine at 10 μg/ml in PBS, followed by incubation with bovine serum albumin (BSA) in PBS at 10 mg/ml for 30 minutes and extensive washes in PBS. ML and PML neural tubes used for the assays were dissected manually into two portions, a dorsal fragment containing presumptive neural crest cells and a ventral fragment assumed to be composed exclusively of neural tube cells. The fragments were incubated in a 1 mM EDTA solution for 15 minutes and resuspended in single cell suspension by gentle pipetting. Neural crest cells were collected from neural tubes cultured on fibronectin for 18 hours and detached from the dish after incubation in a 1 mM EDTA solution. Cells were collected and sedimented at 1,000 rpm for 10 minutes, then resuspended in DMEM containing 10% fibronectin-depleted serum. Cells were counted with a Coulter Counter (Coultronic), and a 25 μl aliquot of cell suspension containing approx-

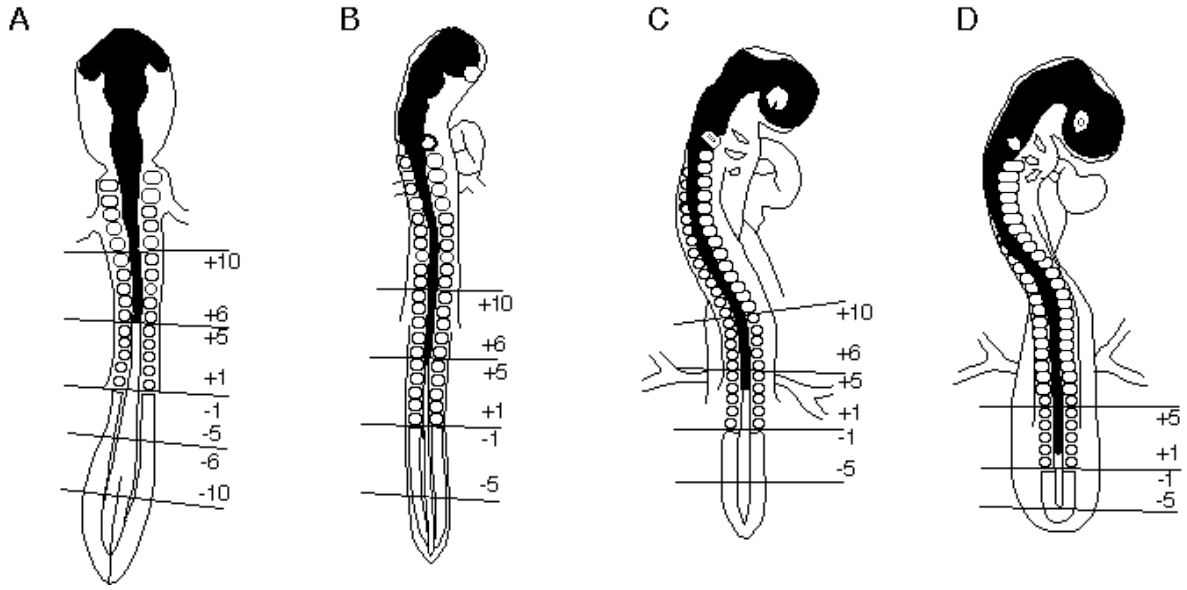

Fig. 1. Schematic drawing of embryos at the 15-somite stage (A), 20-somite (B), 25-somite (C) and 30-somite (D), showing the axial levels at which initiation of crest cell migration has already occurred in vivo (the corresponding neural tube is shown in black) and the trunk fragments that were dissected out and explanted in culture for determination of timing of crest emigration in vitro. Somites are numbered positively from the last formed one and presumptive somites are numbered negatively from the most rostral aspect of the segmental plate. Note that neural crest cell emigration does not exactly parallel somite segmentation, but is slightly faster.

imately 2.5×10^3 cells was deposited on the areas precoated with the proteins to be tested. The dishes were then incubated at 37°C for 1 hour, rinsed in PBS to remove the non-adherent cells and fixed in a 3.7% formaldehyde solution in PBS. Attached and spread cells were counted under a Nikon inverted phase contrast microscope.

Immunofluorescence staining of cell cultures

For immunofluorescent labeling, neural tubes were explanted onto fibronectin-coated glass coverslips in Petri dishes. Cells were grown for varying periods of time up to 24 hours in DMEM supplemented with 10% serum. After washes in serum-free DMEM, cultures were fixed in 3.7% formaldehyde solution in PBS for 5-10 minutes at room temperature, rinsed in PBS, and subjected to permeabilization with 0.5% Triton X-100 in 10 mM morpholinoethanesulfonic acid solution for 5 minutes for A-CAM and vinculin immunodetection. No permeabilization was performed for NC-1 and β1-integrin immunostaining. Cultures were then subjected to immunofluorescent staining essentially as described previously (Duband et al., 1988). For control experiments, non-immune rabbit antibodies or unrelated monoclonals were substituted for each primary antibody. Preparations were observed and photographed with a Leica epifluorescence microscope equipped for microphotography.

Detection of TGF-β-binding activity

The presence of TGF-β-binding activity on the cell surface was revealed by flow cytometry using the TGF-β-fluorokine kit from Research and Diagnostic Systems. Briefly, cells were dissociated and collected as described above for cell adhesion assays. This procedure of cell dissociation was found to preserve TGF-β-binding activity on cells. After washes in DMEM, cells were counted with a Coulter Counter and 20 μl aliquots of cell suspension containing approximately 2×10^5 cells were deposited in wells of microtiter plates. Biotinylated TGF-β1 was subsequently added according to the protocol provided with the fluorokine kit, and the plate was incubated for 1 hour at 4°C on ice to prevent possible internalization of receptors. After washes inthe washing buffer of the kit, fluorescein-conjugated avidin was applied to the cell suspension and the plate was further incubated for 30 minutes at 4°C on ice. After intervening washes, the cell suspension was analyzed by flow cytometry with a FACScan (Becton Dickinson). Forward scatter was measured with linear amplification and fluorescence intensity was measured with logarithmic amplification. Data collected from $2\text{-}4 \times 10^3$ cells were analyzed with Lysis software in the form of fluorescence histograms. For control experiments, biotinylated TGF-β was either omitted or preincubated with neutralizing antibodies to TGF-β1 at 200 μg/ml prior to incubation with cells.

Detection of TGF-β in embryonic tissues

The presence of TGF-β in embryonic tissues was detected by radioimmunoassay. Fragments of embryos of 18-25-somite stage were dissected out and homogenized in Tris-buffered saline (TBS; 0.02 M Tris, 0.5 M NaCl, pH 7.5). A macromolecular fraction was precipitated in cold acetone (9 volumes), centrifuged at 8,000 ***g*** for 15 minutes, and rinsed in cold acetone. After three washes in cold acetone, the material was resuspended in TBS, and proteins were quantified by the method of Bradford (1976). Aliquots of extracts containing 0.05-2.5 μg of proteins were deposited in wells of radioimmunoassay plates. The wells were then saturated with 5% (weight/volume) nonfat dry milk in PBS for 2 hours at 37°C and incubated with rabbit anti-TGF-β antibodies in PBS-5% milk for 12 hours at 4°C. After several extensive washes in PBS-0.2% Tween 20, the wells were incubated with iodinated protein A (Amersham; 2×10^5 cts/minute per well) for about 5 hours at 4°C. After rinsing in PBS-0.2% Tween 20, the wells were dried, and the radioactivity was determined with a gamma counter.

Results

In order to avoid any possible confusion in distinguishing

neural crest cells prior and during migration as well as their areas of origin in the embryo, the following terminology will be used. Neural crest cells that have undergone migration will be termed migrating neural crest cells or, simply, neural crest cells. Conversely, neural crest cells that are still integrated in the neural epithelium prior to departure will be termed presumptive neural crest cells. The term migratory level (ML) will be used for the axial levels at which neural crest cell migration is starting, whereas the term premigratory level (PML) will be applied to regions where separation of crest cells from the neural tube has not yet occurred.

Time table of trunk neural crest cell emigration in vitro

The timing of avian neural crest cell emigration in vitro has been analyzed previously (Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985). We have reexamined this process in greater detail in order to define precisely the axial levels corresponding to the ML and PML regions in embryos at various developmental stages (see Fig. 1). Neural tubes obtained from somitic and presomitic regions of embryos at stages between 15 and 30 somites were explanted onto fibronectin substrata, and the appearance of a layer of spread neural crest cells along the rostral and mid-part of the neural tubes was scored every hour during the first day of culture. Fig. 2A shows the percentage of neural tube explants corresponding to somitic and presomitic regions of a 20-somite stage embryo that produced neural crest cells as a function of the duration of culture. In contrast to the somitic region in which release of crest cells occurred in 100% of the explants within 2 to 3 hours, the proportion of presomitic explants that were surrounded by crest cells remained low during the first 5 hours of culture and increased sharply between 6 and 8 hours to reach about 50% of the explants and 100% after 18 hours in culture. The timing of neural crest cell emigration at each axial level was defined as the time in culture at which neural crest cells had emigrated in 50% of the explants. As shown in Fig. 2B, neural tubes from all somitic levels tested produced migrating neural crest cells within 1-2 hours in culture whatever the developmental stage considered. In contrast, the timing of crest cell emigration from presomitic regions varied with the developmental stages. In embryos earlier than 25 somites, the timing of emigration was 6-8 hours whereas, at stages later than 25 somites, it dropped to about 3 hours. The timing of crest cell emigration determined in vitro was found to correlate approximately with the pattern of initiation of migration observed in vivo, except at the 15-somite stage where the lag phase for level −6 to −10 was only one hour longer than for level −1 to −5 (for details, see Newgreen and Erickson, 1986). The so-called ML and PML regions coincided with the embryonic somitic and presomitic regions, except in late stages in which the PML region was restricted to the caudal portion of the presomitic area.

In the subsequent experiments, ML and PML explants were obtained from regions corresponding to the last five somites and to presumptive somites −2 to −6, respectively, from 18-22-somite stage embryos. The use of embryos older than 25 somites was avoided because of the reduced time lag between the onset of neural crest cell migration in ML and PML regions.

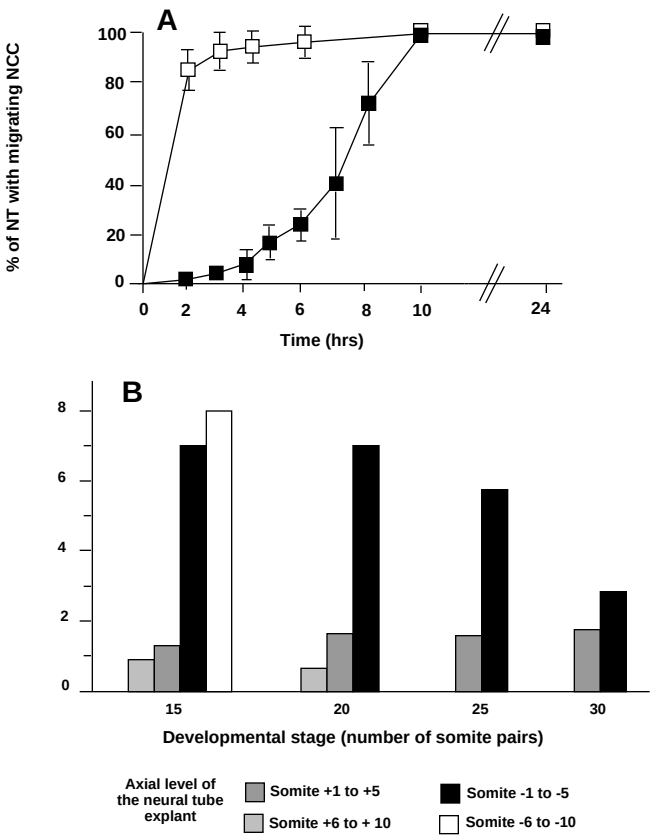

Fig. 2. Timetable of in vitro emigration of neural crest cells. (A) Somitic (open squares) and pre-somitic (solid squares) regions of embryos at the 20-somite stage were explanted onto fibronectin substrata and the presence of spread neural crest cells (NCC) along the rostral and mid portions of each neural tube explant (NT) was recorded every hour. Results are expressed as the percentage of explants that produced crest cells as a function of the duration of culture. Routinely, a mimimum of 15 different explants at each axial level were analyzed for every experiment. Values represent the mean ± s.d. of at least 10 different experiments. Timing of emigration of crest cells was defined as the time in culture at which neural crest cells have emigrated in 50% of the neural tube explants. (B) Timing of crest cell emigration in vitro as a function of the developmental stage of the embryo and of the axial level explanted. Somitic regions generally produce crest cells within 2 hours, in contrast to presomitic regions which yield crest cells after 6-8 hours in culture. Note that the delay of crest cell emigration from presomitic regions is decreased in embryos older than 25 somites, due to the fact that, at these stages, the axial level where crest cell emigration occurs has almost reached the pre-somitic region (see Fig. 1D).

Comparative migratory behavior of ML and PML neural crest cells

The morphology and migratory behavior of ML and PML neural crest cells were analyzed on fibronectin substrata using light and time-lapse video microscopy. ML neural crest cells separated from the dorsal side of the neural tube explants within 1-2 hours and readily spread onto the substratum to form a monolayer. Migration was intense during

the subsequent hours in culture, producing a rapid extension of the area of cell outgrowth (Fig. 3A, B). By 15 hours in culture (Fig. 3C), the neural crest population organized into a large halo around the neural tube. On PML explants, no crest cells either round or spread were detected along the neural tube until 6-7 hours in culture (Fig. 3D). Neural crest cells became separated from the neural tube, first in its rostral end, then progressively along its mid portion (Fig.

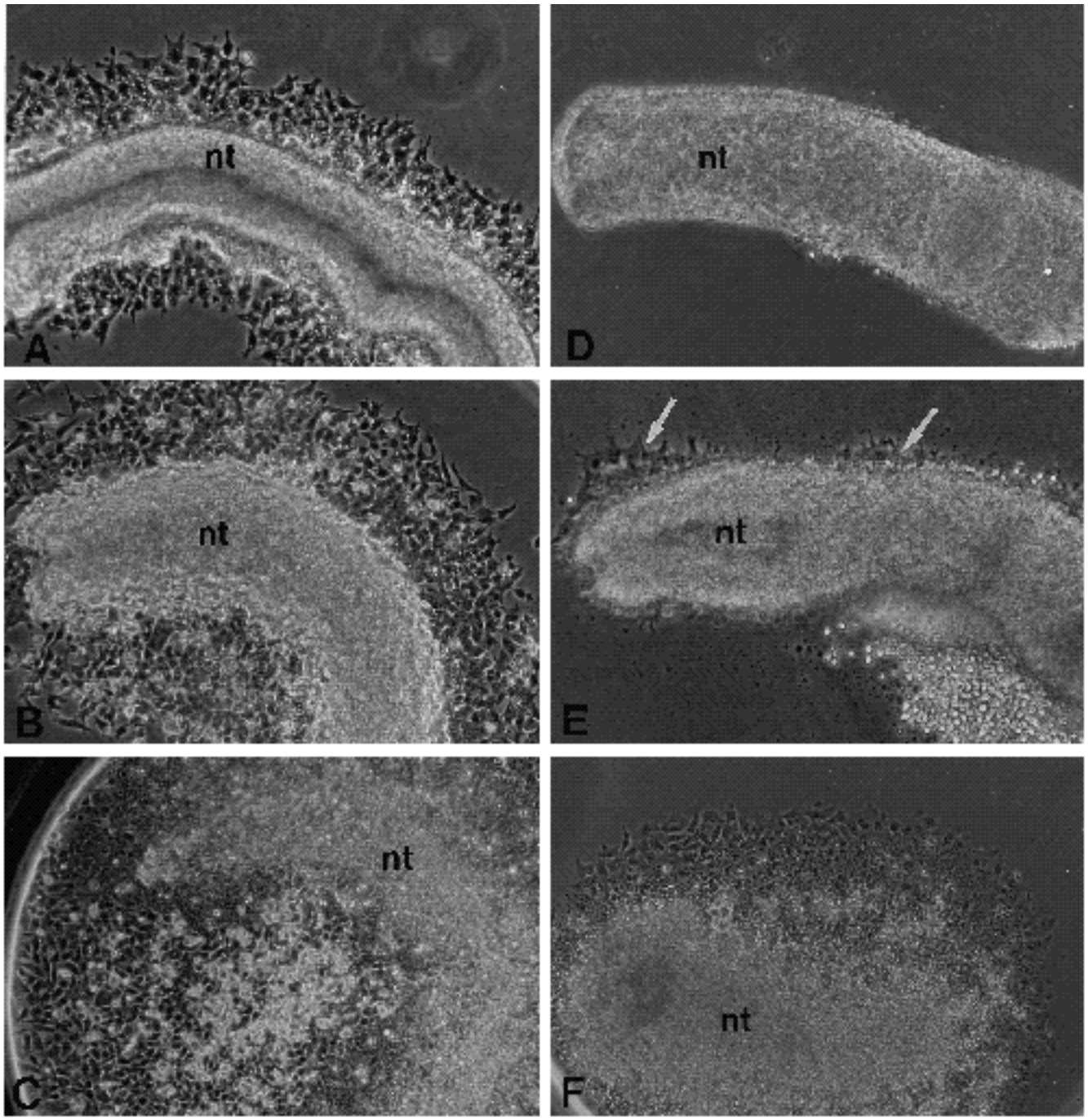

Fig. 3. Comparative migration of neural crest cells from ML (A-C) and PML (D-F) axial levels. Neural tubes were deposited on fibronectin substrates in Terasaki wells, and the eventual presence of spread neural crest cells along the explant was examined periodically after 3 hours (A, D), 7 hours (B, E), and 18 hours (C, F) in culture. Neural crest cells can be seen migrating out of ML explants within 2 hours and, by 3 hours, they form a several cell layer-wide outgrowth along the entire border of the neural tube. The outgrowth expands very rapidly and, by 18 hours in culture, the crest population totally covers the surface of the Terasaki well. In PML explants, crest cells are detected only after 6-8 hours in culture, first along the rostral and mid portions of the neural tube explants. Expansion of the outgrowth is slow and cells remain tighly cohesive. By 18 hours of culture, only part of the well is covered by cells. Arrows point to early emigrating cells from PML explants. nt, neural tube.

3E). Cell spreading also occurred occasionally in the extreme caudal portion of the neural tube explants. These cells could be easily distinguished from neural crest cells by their morphology and were interpreted as undifferentiated cells deriving from the area where the mesoderm is still fused to the neurectoderm. As neural crest cells initiated migration, they formed a dense population of highly cohesive and flattened cells. By 15 hours in culture, the crest population has expanded out of the explant, but less extensively that ML crest cells. The apparent important development of the outgrowth resulted chiefly from the neural tube disaggregation (Fig. 3F).

The neural crest phenotype of cells emigrating from PML explants was assessed using immunofluorescent labeling for the NC-1/HNK-1 epitope. Only a few cells exhibited NC-1 immunoreactivity at the onset of emigration. The proportion of NC-1-positive cells increased gradually in the outgrowth and, by 24 hours in culture, the majority of spread cells were labelled (data not shown). A similar observation was made for ML crest cells (not shown, but see Newgreen et al., 1990). Although this result does not exclude the presence of some non-crest contaminating cells, it indicates that most cells migrating out of ML and PML neural tubes were of crest origin.

Using time-lapse microcinematography, it appeared that ML and PML crest cells differed noticeably in their locomotory behavior. ML neural crest cells exhibited an intense lobopodial activity and changed shape very rapidly from flattened and multipolar to bipolar and elongated. In addition, cells frequently exchanged neighbors. This motile behavior resulted in a rapid displacement of cells (mean value for speed of locomotion being 60 μm/hour) with a moderate persistence of movement, particularly among cells located at the periphery of the outgrowth (0.65 ± 005). PML neural crest cells showed, in contrast, a less active locomotory behavior; emission of lobopodia was slow and changes in cell shape less frequent. In addition, cells remained in contact with their neighbors for long periods of time. Consequently, the speed of locomotion of cells was reduced (30 ± 6 μm/hour), and the persistence of movement was high (0.75 ± 0.1).

In order to exclude the possibility that the differences observed in the morphology and motile behavior of ML and PML neural crest cells might have resulted from local axial specificities in the properties of crest cells, we have analyzed neural crest cells originating from a defined axial level (i.e. somite pairs 18-23) at two distinct stages of development (17-somite and 23-somite stages). The level considered corresponded to the PML region at the 17-somite stage and to the ML region at the 23-somite stage. It was found that neural crest cells migrating out of these explants exhibited essentially the same morphology and motility as crest cells obtained from ML and PML regions from the same embryo at the 23-somite stage (not shown).

Comparative organization of adhesion sites of ML and PML neural crest cells

ML and PML neural crest cells were cultured on fibronectin substrata for varying periods of time and were examined for cell-substratum and cell-cell adhesion sites using immunofluorescence labeling for the β1-integrin subunit, vinculin, and the cell adhesion molecule A-CAM. As described extensively elsewhere (Duband et al., 1986, 1991), ML neural crest cells displayed relatively uniform, diffuse cell surface staining for β1 integrins (Fig. 4A). Vinculin was in focal contact sites at the tips of cell processes (Fig. 4C). In PML neural crest cells, β1 integrin was also evenly distributed on the cell surface, but was enriched in areas of cell-cell contact (Fig. 4B). Vinculin was located in the focal contact sites of cells situated at the periphery of the outgrowth, but was essentially found in the areas of cell-cell contact (Fig. 4D). Finally, while A-CAM was not detectable on the surface of ML neural crest cells (Fig. 4E), it was found at high levels in areas of intercellular contacts of PML cells (Fig. 4F). Identical patterns for β1 integrin, vinculin and A-CAM were observed at various periods of time during neural crest cell outgrowth.

The role of extracellular matrix molecules in the triggering of neural crest cell emigration

The emigration-inducing activity has been shown to be contained in the extracellular milieu of neural crest cells (Löfberg et al., 1985). Several extracellular matrix molecules were therefore investigated for possible inducing activity of crest cell emigration. PML neural tubes were explanted onto fibronectin, laminin, collagen I, and tenascin substrata, and the timing of neural crest cell emigration was recorded and compared with that obtained with ML explants on the same substratum molecules. Polylysine was used as a control. As mentioned above, fibronectin permitted rapid emigration of ML crest cells; the timing of emigration being 1.5 hour. Laminin also produced rapid emigration of ML crest cells at rates slightly lower than fibronectin (timing of emigration was 3 hours; Fig. 5A). Collagen I appeared significantly less efficient than fibronectin and laminin in promoting crest cell emigration; timing of emigration dropped to at least 6 hours (Fig. 5A). In addition, crest cells were rarely spread, as opposed to what was observed on fibronectin and laminin. The timing of emigration of PML crest cells was considerably delayed on laminin and collagen as compared with fibronectin (at least 12 hours for both substrata; Fig. 5B). A substantial proportion of PML neural tube on collagen never produced crest cells. Polylysine and tenascin were poor substrates for migration of both ML and PML neural crest cells (Fig. 5A, B). No spread cells were seen around the neural tube explant which often did not adhere to the dish. Neural crest cells remained as aggregates attached to the neural tube, indicating that they were able to separate from the neural tube but could not migrate on the substratum.

Substratum-adhesion properties of presumptive and migratory neural crest cells

The substratum-adhesion properties of presumptive and migrating neural crest cells (i.e. prior to and during migration) from ML and PML regions were measured using an in vitro adhesion assay (Fig. 6). Migrating neural crest cells obtained from ML regions were found to spread onto fibronectin and laminin with high efficiency. Likewise, neural crest cells migrating out of PML explants were able to spread onto these substrates at rates very similar to those

attained by their ML counterparts. Presumptive neural crest cells originating from ML regions also exhibited a significant ability to spread onto fibronectin and laminin. In contrast, spreading of PML presumptive crest cells was poor on both fibronectin and laminin; less than 10% of the cells could flatten onto either substrate. Neural tube cells obtained from ventral portions of ML or PML explants also showed weak capacity to spread on fibronectin and laminin. Polylysine, collagen I, and tenascin did not permit adhesion of all cells tested. These results indicate that neural

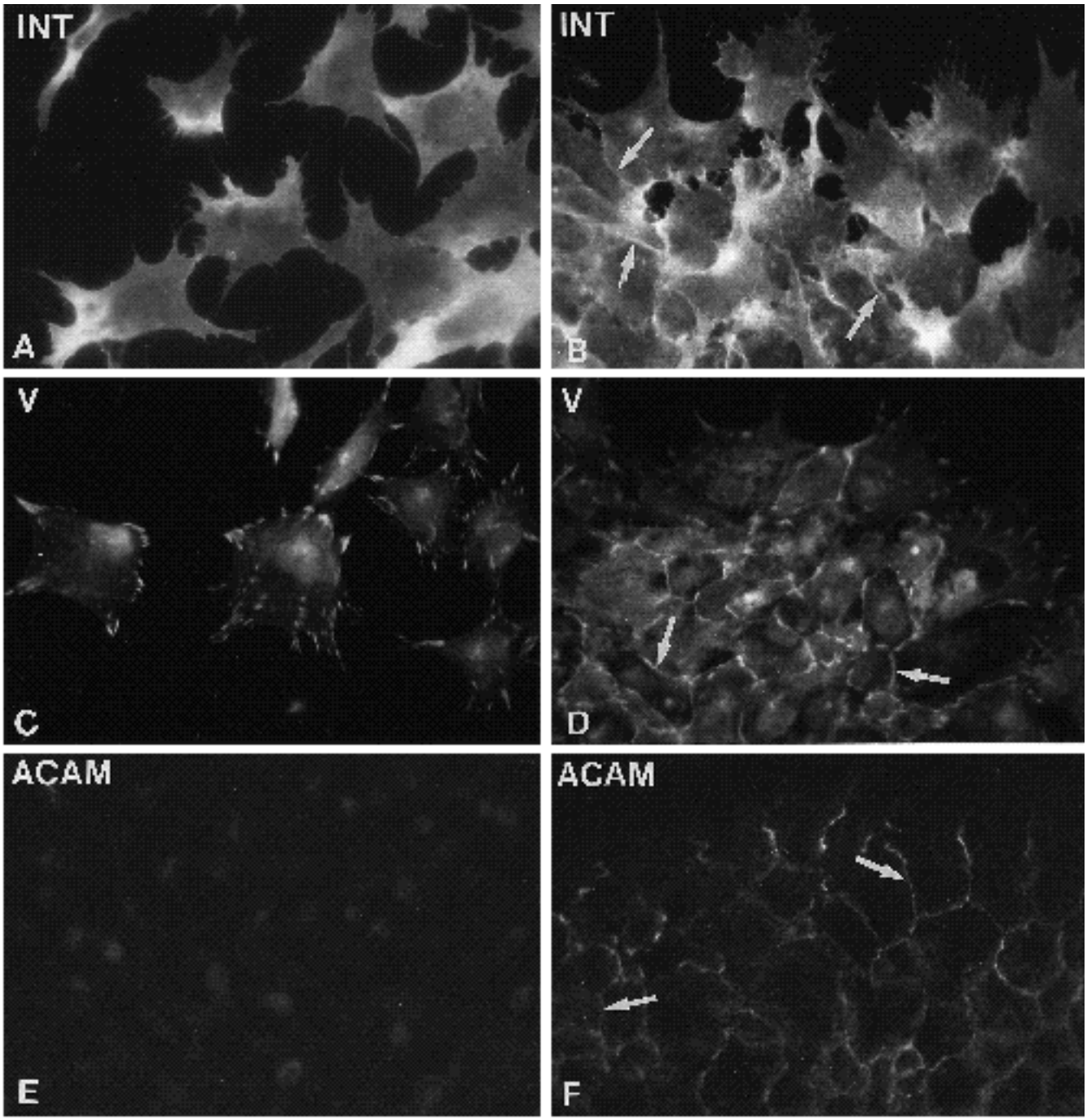

Fig. 4. Immunofluorescence detection of the β subunit of integrin receptors (INT; A, B), vinculin (V; C, D), and A-CAM (E, F) on ML (left panels) and PML (right panels) neural crest cells cultured on fibronectin. Cells were cultured for 18 hours, fixed in formaldehyde and permeabilized with Triton X-100, except in A and B where no permeabilization was performed. ML crest cells exhibit integrin receptors with a uniform pattern of staining on their surface. Vinculin is concentrated in focal contacts at the tips of cell processes. These cells are devoid of A-CAM on their surface. PML neural crest cells also show diffuse integrin staining, but with a particular enrichment in the areas of cell-cell contacts where vinculin and A-CAM are also concentrated. Arrows point to intercellular adhesion sites on PML crest cells.

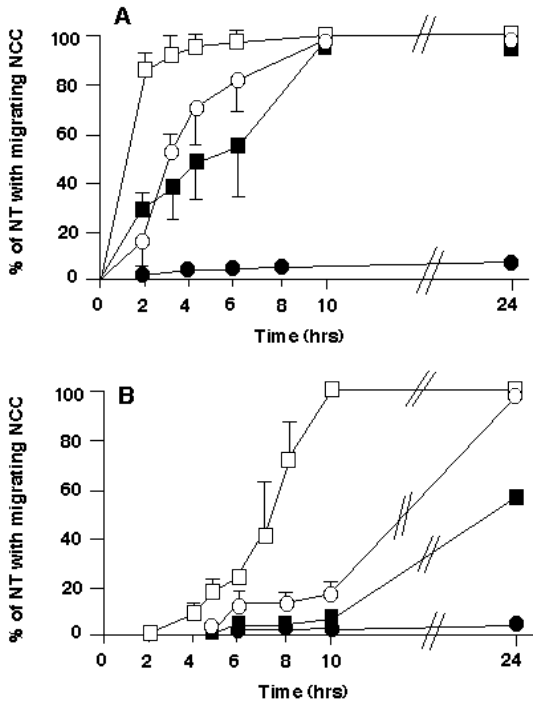

Fig. 5. Timetable of crest cell emigration from ML (A) and PML (B) explants on various extracellular matrix components. Neural tube explants (NT) were deposited on fibronectin coated at 25 μg/ml (open squares), laminin at 50 μg/ml (open circles), collagen at 10 μg/ml (solid squares) and polylysine at 10 μg/ml or tenascin at 50 μg/ml (solid circles). Values represent the mean ± s.d. of at least 5 different experiments. A minimum of 15 different explants at each axial level were analyzed for every experiment. Note that neural crest cell (NCC) emigration from ML and PML explants on laminin and collagen is delayed compared to fibronectin and that tenascin and polylysine do not permit crest cell migration for either ML or PML explants at all.

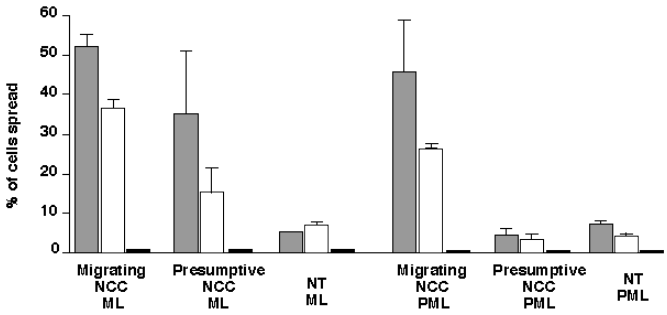

Fig. 6. Comparative substratum-adhesion properties of presumptive and migrating neural crest cells (NCC) and of neural tube cells (NT) from ML and PML regions. ML and PML neural tubes were explanted and dissected into a dorsal portion enriched in presumptive crest cells and a ventral portion composed essentially of neural tube cells. Fragments were then dissociated into single-cell suspensions and processed for adhesion assay on fibronectin at 25 μg/ml (stippled bars), laminin at 50 μg/ml (open bars), and collagen at 10 μg/ml, polylysine at 10 μg/ml or tenascin at 50 μg/ml (solid bars). Migrating neural crest cells were collected from neural tubes cultured on fibronectin for 18 hours and detached from the dish prior to adhesion assay. Results are expressed as the percentage of cells spread onto each substrate. Note that collagen, tenascin and polylysine do not support adhesion of any cells tested. Values represent the mean ± s.d. of at least 5 different experiments.

crest cells from PML regions acquire substratum-adhesion properties during transition from premigratory to migratory state.

We then determined their timing of acquisition of substratum-adhesion properties in vitro (Table 1). Neural tube explants were incubated for varying periods of time in DMEM supplemented with fibronectin-depleted serum in bacteriological Petri dishes in order to avoid explant attachment to the bottom of the dish and subsequent cell emigration. The explants were then dissociated into single cell suspensions and substratum-adhesion properties of cells were determined. PML presumptive crest cells acquired the ability to spread onto fibronectin after a period of 4 hours, reaching values very similar to those obtained with PML migratory crest cells.

TGF-β as a possible inducer of neural crest cell emigration in vitro

Because extracellular matrix molecules failed to induce premature emigration of PML crest cells, we have searched among the various known soluble factors that are released in the extracellular environment as to which of them may contain the emigration-inducing activity. TGF-β is a leading candidate for several reasons: (i) it is expressed precociously during embryogenesis; (ii) it is involved in a variety of morphogenetic processes; and (iii) it regulates integrin expression (Heine et al., 1987; Sporn et al., 1987; Mercola and Stiles, 1988; Potts and Runyan, 1989; Massagué, 1990; Sporn and Roberts, 1990; Godin and Wylie, 1991).

PML and ML neural tubes were explanted onto fibronectin substrata in the presence of TGF-β at varying concentrations, and the timing of neural crest cell emigration was recorded. As shown in Fig. 7, both TGF-β1 and TGF-β2 showed a strong ability to induce a rapid, premature emigration of PML neural crest cells. After 5 hours in culture, about 40% of the neural tube explants produced neural crest cells in the presence of TGF-β1 at 20 ng/ml and of TGF-β2 at 10 ng/ml, compared with the 15%

Table 1. *Effect of TGF-β1 on the spreading of PML presumptive crest cells onto fibronectin substrates as a function of the duration of culture*

Duration of incubation (hours)	Cell spreading (%)	
	−TGF-β	+TGF-β
2	8±2	7±3
3	7±3	9±2
4	26±4*	49±9*

PML neural tube explants were incubated for 1-4 hrs in DMEM supplemented with serum in the presence of TGF-β1 at 20 ng/ml and subsequently dissociated into single cell suspension before processing for adhesion assay on fibronectin at 25 μg/ml. Results are expressed as the percentage of spread cells as a function of the incubation time prior to the adhesion assay. Values represent the mean ± s.d. of at least 4 different experiments.

*These values were found to be statistically significantly different both one from the other and from the other values (P<0.001).

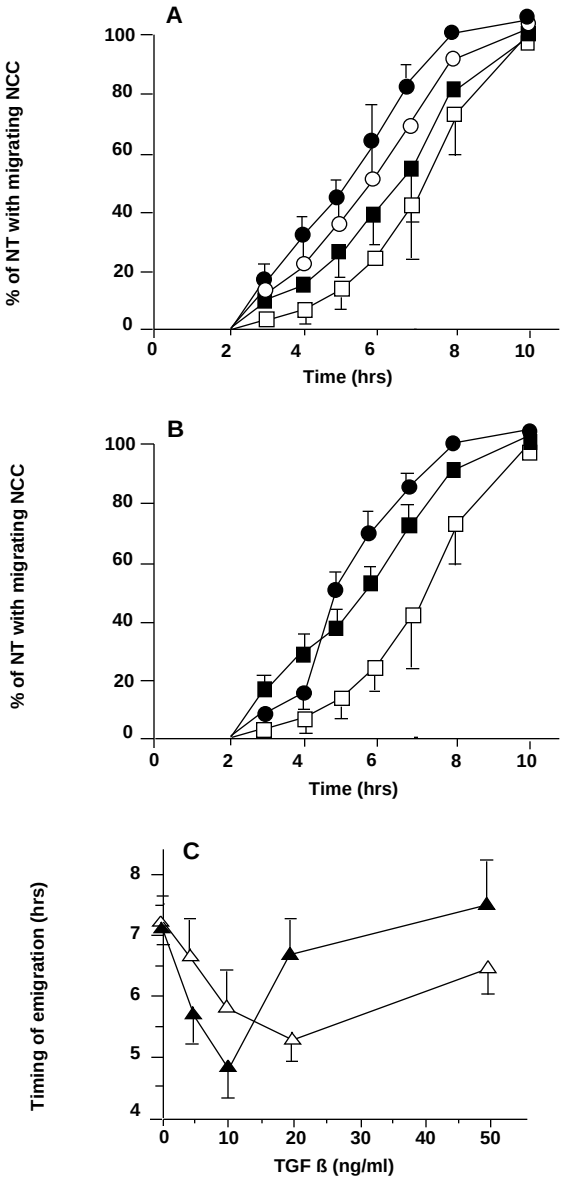

Fig. 7. Effect of TGF-β1 and TGF-β2 on the timing of neural crest cells from PML explants. PML explants were deposited on fibronectin substrates in Terasaki wells in the presence of varying amounts of TGF-β1 or TGF-β2 and the timing of neural crest cell (NCC) emigration was recorded. (A) Percentage of neural tube explants producing migrating crest cells as a function of the duration of culture and of the concentration of TGF-β1 added. Open squares, no TGF-β1; solid squares, TGF-β1 at 5 ng/ml; open circles, TGF-β1 at 10 ng/ml; solid circles, TGF-β1 at 20 ng/ml. (B) Percentage of neural tube explants producing migrating crest cells as a function of the duration of culture and of the concentration of TGF-β2 added. Open squares, no TGF-β2; solid squares, TGF-β2 at 5 ng/ml; solid circles, TGF-β2 at 10 ng/ml. (C) Dose response curve for the timing of emigration of PM neural crest cells in the presence of TGF-β1 (open triangles) and TGF-β2 (solid triangles). Values represent the mean ± s.d. of at least 5 different experiments. A minimum of 15 different explants were analyzed for every measurement. Values for timings of emigration of crest cells in the presence of TGF-β1 at concentrations above 5 ng/ml and of TGF-β2 at 5 and 10 ng/ml were found to be statistically significantly different from the controls ($P < 0.01$).

observed in the control experiments (Fig. 7A, B). No effects of TGF-β1 on crest emigration were detected at concentrations lower than 5 ng/ml and stimulation was maximal at concentrations higher than 20 ng/ml (Fig. 7C). The dose-response curve for TGF-β2 was different. It was inactive at concentrations below 2 ng/ml and above 15 ng/ml, and maximal activity was reached by 10 ng/ml of the factor (Fig. 7C). It should be noted that TGF-β1 and TGF-β2 never totally abolished the delay in PML crest cell emigration; the maximal reduction in the timing of emigration could be estimated at 2-3 hrs. The number of migrating cells, their morphology and motility were also examined in the presence of TGF-β. They were not found to be modified by TGF-β1 or TGF-β2 (not shown).

In order to confirm further the effect of TGF-β on neural crest cell emigration, PML neural tube explants were incubated with TGF-β1 at 20 ng/ml in the presence of neutralizing antibodies to TGF-β at 100 μg/ml. It was found that, under these conditions, the effect of TGF-β1 was totally abolished; timing of emigration dropped from 5 hours in the absence of antibodies to 7.5 hours in the presence of the neutralizing anti-TGF-β antibodies.

Presumptive and migrating neural crest cells express surface receptors for TGF-β

It is possible to reveal TGF-β-binding activity on the surface of cells using biotinylated TGF-β. Presumptive neural crest cells obtained from dorsal portions of ML or PML neural tubes and migrating crest cells were dissociated into single-cell suspensions and incubated successively with biotinylated TGF-β and fluorescein-conjugated avidin as described in Materials and methods. Fluorescent stainings of the cell populations were quantitated and analyzed by flow cytometry. In control experiments in which TGF-β was omitted, the overall fluorescence emitted by each cell type was faint. In the presence of TGF-β, the peak of fluorescence was significantly displaced by one order of magnitude for presumptive crest cells from PML regions (Fig. 8A) or from ML regions (not shown) and for migrating neural crest cells (Fig. 8B). The curves of relative fluorescence intensity exhibited a single peak, suggesting that all cells bound TGF-β at similar levels. As a control for the specificity of binding of TGF-β on the surface of crest cells, neutralizing antibodies were incubated at 200 μg/ml with TGF-β prior to incubation with the cells. Under these conditions, the mean value for fluorescence intensity emitted by the cells was significantly reduced but was not entirely abolished due to the large amount of biotinylated TGF-β necessary for the labeling (not shown).

Detection of TGF-β in embryo extracts at the time of crest cell emigration

To demonstrate the presence of TGF-β-like molecules in the embryo at the time of neural crest cell emigration, proteins from 18-25-somite stage embryos were extracted and processed for radioimmunoassay using purified polyclonal antibodies to TGF-β. Heart extracts were used as a positive control (Potts and Runyan, 1989). Fig. 8C shows the binding curve of the antibodies as a function of the concentration of proteins adsorbed to the wells. Antibodies to

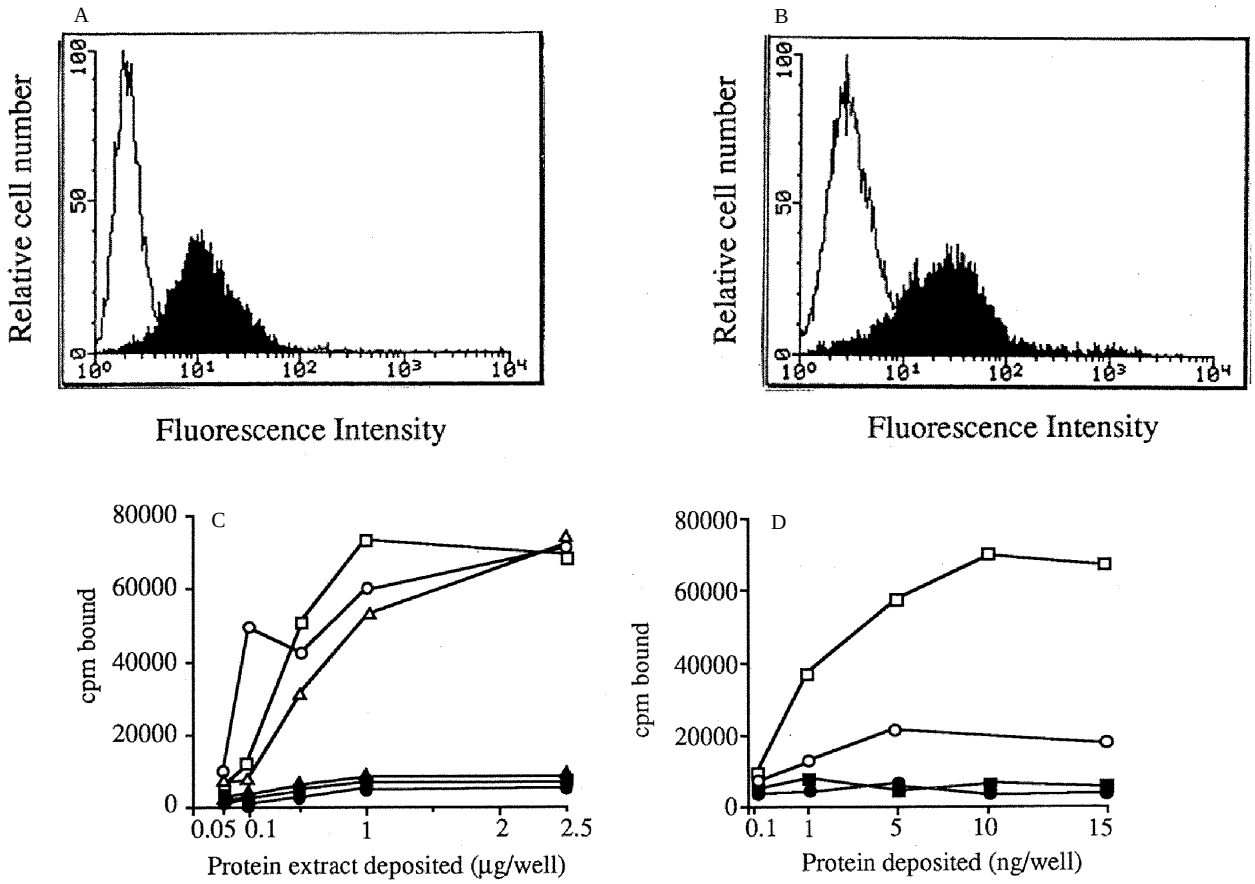

Fig. 8. (A, B) Evidence for TGF-β-binding activity on the surface of presumptive and migrating neural crest cells from PML explants. Cells were dissociated into single-cell suspensions, collected, and incubated first with biotinylated TGF-β1 for 1 hour at 4°C then with fluorescein-avidin. Immunofluorescence emitted by cells was analyzed by flow cytometry. Routinely, 2-4 × 10^3 cells were analyzed in each sample. Results are expressed as histograms of the relative number of cells as a function of the fluorescence intensity measured with logarithmic amplification. (A) and (B) show fluorescence histograms for PML presumptive and PML migrating neural crest cells, respectively, in the presence (filled curves) or absence (open curves) of TGF-β. (C, D) Immunodetection of TGF-β-like molecules in extracts of trunk regions of 18- to 22-somite-stage embryos. (C) Trunk regions corresponding to PML (triangles) and to ML (squares) axial levels and hearts (circles) were extracted with acetone and processed for radioimmunoassay using antibodies directed against TGF-β1 at 10 µg/ml and iodinated protein A (2 × 10^5 cts/minute per well) (D) Radioimmunoassay with purified TGF-β (squares) and basic FGF (circles) at defined concentrations coated to the wells. Wells incubated with anti-TGF-β antibodies are represented by open symbols and controls with a non-immune antibody by solid symbols. Results are expressed in number of cts/minute bound to the wells as a function of the quantity of proteins deposited into the wells. Values represent the mean of at least 4 different measurements in at least 2 experiments.

TGF-β reacted specifically with heart and ML and PML extracts. No radioactivity was found in the absence of the primary anti-TGF-β antibody or with a non-immune antibody. Antibodies to TGF-β did not cross react with purified basic fibroblast growth factor (Fig. 8C).

TGF-β increases substratum adhesion of neural crest cells

To evaluate the possible functional role of TGF-β in the crest cell emigration process, we have analyzed the substratum-adhesion properties of PML presumptive crest cells in the presence of TGF-β (Table 1). PML neural tubes were incubated in bacteriological Petri dishes in the presence of TGF-β1 at 20 ng/ml. After varying periods of time, the neural tubes were dissociated into single-cell suspensions and the fibronectin-adhesion properties of cells were measured. In the absence of TGF-β, fibronectin adhesion of PML presumptive crest cells increased from about 8% to about 25% after 4 hours of incubation. In the presence of TGF-β, fibronectin adhesion was not significantly altered during the first three hours of incubation as compared to the control but, after 4 hours, it was dramatically increased to about 50%.

Discussion

A number of previous morphological studies have proposed that the time-dependent modulations of the adhesive properties of presumptive neural crest cells are critical events for their dispersion from the neural tube (for a review, see Newgreen and Erickson, 1986). In the present study, we have analyzed the changes in cell-substratum adhesion during crest cell emigration using an in vitro model system originally designed by Newgreen (Newgreen and Gibbins,

1982; Newgreen and Gooday, 1985). This system is suitable for examining sequentially the different cellular events that accompany crest cell emigration and to search for possible regulatory factors. Our major findings are: (i) well before migration, neural crest cells show restricted ability to adhere to extracellular matrix molecules; cells acquire the ability to adhere to the substratum only gradually as they undergo separation from the neural tube; (ii) neural crest cells migrating out of PML neural tubes have incomplete locomotory competence; and (iii) TGF-β is able to induce premature emigration of neural crest cells in vitro, possibly by increasing substratum adhesiveness of cells.

Previous studies on the spatiotemporal distribution of extracellular matrix components during early embryonic development did not reveal extensive changes in the macromolecular composition of the matrix prior to and during onset of crest cell migration, thus indicating that the direct extracellular environment of presumptive crest cells is permissive for emigration long before its occurrence (Thiery et al., 1982; Sternberg and Kimber, 1986). In addition, a complete basal lamina which would potentially constitute a physical barrier preventing the release of cells is never observed along the dorsal side of the neural tube prior to crest cell emigration (Tosney, 1978; Newgreen and Gibbins, 1982; Martins-Green and Erickson, 1986, 1987; Duband and Thiery, 1987). These in vivo observations correlate with our present in vitro data showing that none of the extracellular matrix components known to promote neural crest cell locomotion are able to trigger individually or in combination premature neural crest cell emigration. A similar observation has been made using three-dimensional extracellular matrices synthesized by cultured embryonic fibroblasts, instead of purified molecules (Newgreen and Gibbins, 1982). It seems that, although a favorable extracellular matrix must be a prerequisite, changes in the extracellular matrix composition probably do not initiate migration. The inability of neural crest cells to separate rapidly from the neural tube and undergo migration would then possibly reside in the inability of cells to adhere to the matrix. Using an in vitro adhesion assay, we found that, in contrast to ML presumptive neural crest cells, PML presumptive crest cells lacked the capacity to spread onto extracellular matrix components. This finding indicates that during transition from the premigratory to the migratory state, neural crest cells gradually acquire substratum adhesion. We demonstrated this progressive acquisition of substratum adhesion in vitro and found that it occurs only a few hours preceding the onset of migration. These results strongly suggest that the lack of appropriate or functional receptors for extracellular matrix material can constitute a major restraint for crest cell emigration. However, whether acquisition of matrix adhesion is sufficient to permit migration remains to be determined.

Most receptors of extracellular matrix molecules belong to the family of integrins which are heterodimers of noncovalently bound α and β subunits (for a review, see Hynes, 1992). Both in vivo and in vitro studies have provided evidence of expression of the β1-integrin subunit on the surface of neural crest cells both prior to and during migration (Duband et al., 1986; Krotoski et al., 1986). Quantitation of β1-integrin by immunoprecipitation and immunofluorescent staining using flow cytometry did not reveal extensive changes in the level of expression of the β1 subunit on the surface of crest cells at the onset of migration (our unpublished data). The acquisition of fibronectin adhesion would possibly result from changes in the pattern of integrin α subunits on the surface of presumptive neural crest cells. Alternatively, integrin function may be modified on the surface of neural crest cells during transition from the premigratory to the migratory state. Indeed, there are numerous examples of activation of integrin function, including the αIIbβ3 integrin on platelets and β2 integrins on leukocytes (see for examples, Kieffer and Phillips, 1990; Larson and Springer, 1990) and, recently, it has been shown that, during terminal differentiation of keratinocytes, the α5β1 integrin is inactivated prior to disappearance from the cell surface (Adams and Watt, 1990).

Because of its biological effects and its tissular distribution, TGF-β appeared as a leading candidate among compounds that are released in the extracellular matrix to play an important role in the control of crest cell emigration. Like fibroblast growth factors, TGF-β can be trapped by extracellular matrix materials, which in turn regulate its biological activities (Yamaguchi et al., 1990; Paralkar et al., 1991). TGF-β has been implicated in a variety of biological events involving cell dispersion. For example, it stimulates invasion and metastatic potential of certain cells (Welch et al., 1990) and, during embryonic development, it has been found to promote in vitro phenotypic change of epiblastic cells into mesoderm during gastrulation and migration of cardiac cushion cells from the endocardium (Potts and Runyan, 1989; Choy et al., 1990; Potts et al., 1991; Sanders and Prasad, 1991). TGF-β has also been shown to exert a chemotropic effect on mouse primordial germ cells in vitro (Godin and Wylie, 1991). At the cellular level, besides its regulatory role in cell division, TGF-β promotes the synthesis of extracellular matrix molecules, including fibronectin and collagens, and causes marked alterations in the repertoire of integrins (for a review, see Massagué, 1990).

The effect of TGF-β in inducing premature emigration of PML neural crest cells and in enhancing fibronectin adhesion of these cells is in complete agreement with the known effects of this growth factor in other systems. We propose that TGF-β may stimulate the onset of neural crest cell emigration by modifying either the expression pattern or the binding specificities of integrins on the surface of these cells. Because of the multiplicity of the functions listed for TGF-β, other effects of this growth factor on presumptive neural crest cells are likely to occur. TGF-β, however, was found not to affect the cell-cell adhesion properties, the proliferation, or the locomotory behavior of crest cells, indicating that other factors may act in concert with it, each with precise functional specificities, to permit the coordinated occurrence of the different cellular events that accompany crest cell emigration.

Although we provided evidence of the presence of TGF-β-like molecules in the area of crest cell emergence using radioimmunoassay analyses, we do not know, at the present time, the exact tissue origin of TGF-β and which of the

various members of the TGF-β family is responsible for the inducing activity. The patterns of distribution and expression of various members of the TGF-β family during embryonic development have been analyzed in various species using immunofluorescence and in situ hybridization techniques. TGF-β1, β2 and β3 and activin were all found in multiple sites in the embryo, predominantly in mesodermal tissues and at sites where cell dispersions are likely to occur (Heine et al., 1987; Lehnert and Akhurst, 1988; Akhurst et al., 1990; Gatherer et al., 1990; Mitrani et al., 1990; Pelton et al., 1989, 1990; Potts and Runyan, 1989; Godin and Wylie, 1991; Millan et al., 1991; Potts et al., 1991; Sanders and Prasad, 1991; Schmid et al., 1991). Various reports have described the presence of different members of the TGF-β family in migrating trunk neural crest cells (Schmid et al., 1991), in the neural tube at the time of neural crest cell migration (Millan et al., 1991; Flanders et al., 1991) and in the somitic epithelium but not in the segmental plate, correlating with the spatiotemporal pattern of crest cell emigration (Heine et al., 1987). It is therefore possible that TGF-β is released by the neural epithelium and somitic cells and acts via a paracrine mechanism on presumptive neural crest cells. TGF-β may also be produced by presumptive crest cells themselves and induce migration by an autocrine mechanism. Another possibility is a concomitant synthesis of TGF-β by the somitic mesoderm, the neural tube and presumptive crest cells, resulting in the local increase of TGF-β, necessary for its action.

The action of TGF-β on neural crest cell emigration can be related to the process of induction during early embryonic development. In the avian embryo, the neural crest arises from epiblastic cells at the boundary between the presumptive territories of the epidermis and the neural tube (Rosenquist, 1981), suggesting that commitment of crest cells occurs precociously during neural induction. In *Xenopus* embryos, both FGF, TGF-β and activin are capable of mesodermalizing blastula-derived animal caps (Kimelman and Kirchner, 1987; Slack et al., 1987; Rosa et al., 1988; Smith et al., 1990). Another activin-related factor, PIF, obtained from a mouse macrophage cell line, possesses little mesoderm-inducing activity in amphibians, but is a potent inducer of anterior structures and organizes the body axis (Sokol et al., 1990). In particular, its action leads to formation of dorsal mesoderm (somites and notochord) and neural tissues (Thomsen et al., 1990). Thus, in amphibians, it seems that two distinct waves of morphogens belonging to the TGF-β family are necessary to control first mesodermal induction, then establishment of rostrocaudal and mediolateral polarities. In avians, activin and PIF have also been found to induce formation of axial structures, including notochord, somites and neural epithelium (Mitrani et al., 1990). It can be proposed that, in avians, various members of the TGF-β family may act sequentially as morphogens, possibly in combination with other growth factors, and induce first in a polarized manner differentiation of both mesodermal and neural structures, and secondarily segregation and dispersion of neural crest cells from the neural tube.

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