Epithelial-mesenchymal conversion of dermatome progenitors requires neural tube-derived signals: characterization of the role of Neurotrophin-3

Gilat Brill¹, Nitza Kahane¹, Chana Carmeli¹, David von Schack², Yves-Alain Barde² and Chaya Kalcheim¹,*

¹Department of Anatomy and Embryology, Hebrew University-Hadassah Medical School, Jerusalem 91120, PO Box 12272, Israel
²Department of Neurobiochemistry, Max Planck Institute for Psychiatry, Am Klopferspitz 18a, 82152 Martinsried, Germany

*Author for correspondence

SUMMARY

Development of the somite-derived dermatome involves conversion of the epithelial dermatome progenitors into mesenchymal cells of the dermis. In chick embryos, neural tube-derived signals are required for this conversion, as the interposition of a membrane between neural tube and somites results in a failure of the dermatome to lose its epithelial arrangement. However, dermis formation can be completely rescued by coating the membranes with Neurotrophin-3, but not with the related molecule Nerve growth factor. Neurotrophin-3 was also found to be necessary for dermatome dissociation using in vitro explants or partially dissociated dermomyotomes. The functional relevance of these observations was investigated by neutralizing endogenous Neurotrophin-3 using a specific blocking antibody. Antibody-treated embryos revealed the presence of tightly aggregated cells between myotome and ectoderm instead of the loose dermal mesenchyme observed in embryos treated with control antibodies. As previous studies have demonstrated the presence of Neurotrophin-3 in the neural tube, these results suggest that it may be a necessary neural tube-derived signal required for early stages of dermis formation.

Key words: chick embryo, dermomyotome, dermis, dorsomedial lip, myotome, nerve growth factor, neurotrophin, sclerotome, somite, trkC

INTRODUCTION

The transformation of epithelial cells into mesenchyme is a basic process in embryonic morphogenesis. The paraxial mesoderm, composed of metamERICally arranged somites, provides a good model system to investigate these phenotypic conversions. The epithelial somites dissociate into a ventral mesenchymal part, the sclerotome, and a dorsal epithelial wall, the dermomyotome (DM). Whereas sclerotomal cells give rise to the vertebral cartilage, the DM develops into a two-layered structure, the dorsal epithelial dermatome that dissociates into the mesenchymal dermis and the underlying myotome that subsequently forms vertebral and limb muscles (Bellairs, 1963; Goulding et al., 1994; Williams and Ordahl, 1994).

The development of vertebral cartilage and muscle was shown to depend upon inductive signals arising from the axial structures (Christ et al., 1992; Strübel, 1955; Teillet and Le Douarin, 1983; Vivarelli and Cossu, 1986). Contact between neural tube and somites is required for muscle differentiation (Rong et al., 1992, Buffinger and Stockdale, 1994). In addition, the notochord has a ventralizing activity on somite derivatives, stimulating cartilage and inhibiting muscle formation (Pourquie et al., 1993). Recent experiments suggest that Sonic hedgehog mediates this effect, as it stimulates sclerotome formation (Fun and Lavigne, 1994; Johnson et al., 1994).

Virtually nothing is known about the cellular signals affecting development of the somite-derived dermatome. In this work, we have tested the hypothesis that cells of the epithelial dermatome require neural tube-derived cues in order to become mesenchyme that constitutes the dermis. Using an experimental paradigm that consisted of depriving the mesoderm of neural tube input, we could demonstrate that the conversion of dermatome to dermis is impaired without affecting either the survival or proliferation of dermal progenitors. Neurotrophin-3 (NT-3), a member of the neurotrophin family of growth factors, is known to play a role in neuronal survival during programmed neuronal death (reviewed by Korsching, 1993) and also has several activities earlier in ontogeny on distinct neural progenitors (Averbuch-Heller et al., 1994, Birren et al., 1993, Gaese et al., 1994, Kalcheim et al., 1992, Pinco et al., 1993). Both NT-3 mRNA and immunoreactive protein were shown to be present in the neural tube of early avian embryos (Pinco et al., 1993, Yao et al., 1993). Moreover, transcripts encoding trkC, the most selective NT-3 receptor (Lamballe et al., 1991), were detected not only in the early nervous system but also in the dermatome of avian embryos by in situ hybridization (Kahane and Kalcheim, 1994, Williams et al., 1993, Zhang et al., 1994). These results suggest that NT-3 may also be instrumental in mediating the effect of the neural tube on early stages of dermis formation.
MATERIALS AND METHODS

Embryos
Chick (Gallus gallus) and quail (Coturnix coturnix japonica) eggs from commercial sources were used for this study.

Microsurgery
Preparation of silastic membranes
Silicone rubber membranes were prepared by adding 1 part of MDX-4210 catalyst (Dow Corning Corp. Midland, Mich.) to 10 parts of silastic MDX-4210 base (Dow Corning Corp., Michigan.). The mixture was applied thinly to a microscope slide and allowed to cure at 150°C for one hour. Rectangles of the membrane were cut to a length corresponding to 3-6 somites. Membranes were then incubated overnight at 4°C in phosphate-buffered saline (PBS) pH 7.4 containing 100 ng/ml of purified human recombinant NT-3, NGF or no factor before implantation.

Implantation of silastic membranes
Silastic membrane insertion was performed as previously described (Kalcheim and Le Douarin, 1986). Chick embryos were at the 23- to 32-somite stage. A unilateral slit, 3 to 6 somites in length was performed to separate the neural tube from the adjacent somites either at the brachial level or at the level corresponding to newly segmented epithelial somites. The membrane was inserted into the slit, thus separating the neural tube from the somites. Membranes projected in height well beyond the superficial ectoderm to prevent regeneration of the contact between dermomyotome and dorsal neural tube until the time of fixation. Embryos were killed either 6 or 24 hours after surgery.

In vitro assays
Isolation of dermomyotomes
Chick embryos (25 somites) were pinned onto sylgard-coated dishes and covered with PBS. Strips containing seven somites were excised from the level rostral to the 10th caudal segments. In some experiments, strips containing the attached neural tube were taken. In other experiments, a slit between the neural tube and adjacent mesoderm was performed on both sides of the embryos. The isolated fragments containing ectoderm, dermomyotomes and sclerotomes were grown in three-dimensional (3-D) collagen gels as described below. Alternatively, the isolated strips without neural tube were subjected to digestion in 50% pancreatin to detach dermomyotomes from ectoderm and sclerotome.

Dermomyotomes grown in 3-D collagen gels
Collagen gels were prepared as described previously (Gvirtzman et al., 1992). Explants were placed in a slit made in the collagen, and were incubated for either 6 or 16 hours in serum-free medium (SFRI). After digestion in 50% pancreatin to detach dermomyotomes from ectoderm and sclerotome.

Dermomyotomes grown on fibronectin-coated dishes
Isolated dermomyotomes were subjected to partial mechanical dissociation with the aid of a glass micropipette (tip opening of 30-50 μm). The dissociated dermomyotomes were cultured in 70 μl SFRI in the center of 35 mm culture dishes precoated for 2 hours with 50 μg/ml fibronectin. Each dish received an equivalent of 10 dermomyotomes. Cell aggregates were allowed to attach to the substratum and growth factor was added 2 hours after seeding. Cultures were further incubated for 6 or 16 hours and then fixed.

Treatment of embryos with anti-NT-3 antibodies
The production and specificity of anti-NT-3 monoclonal antibodies were previously reported (Gaese et al., 1994). E2 chick embryos received on the chorioallantoic membrane 2·5×10⁶ anti-NT-3 hybridoma-secreting cells in combination with 30 μl of anti-NT-3 ascites fluid. Control embryos received a similar number of hybridoma cells that secrete an antibody directed against a sugar residue of a cell surface adhesion protein of Dictyostelium. All the antibodies used belong to the IgG1 subclass. Levels of antibody were measured in the heads of the embryos using a 2-site enzyme immunoperoxidase assay, as described (Gaese et al., 1994). Embryos were fixed on E4 in Bouin’s fluid and processed for serial sectioning and 13F4 immunolabeling.

Immunocytochemistry
Embryos and tissue explants
Experimental embryos and tissue explants were fixed in Bouin’s fluid and embedded in Paraplast. Serial transverse 7 μm sections were stained with the 13F4 antibody (Rong et al., 1987) to visualize myotomes. Antibody binding was revealed by a goat anti-mouse secondary antibody coupled to horse-radish peroxidase (1:50). Sigma followed by aminoethylbenzidine treatment. Sections were counterstained with Harris hematoxylin.

Cultured cells
All cultures were stained with the Hoechst nuclear stain (Serva; 1 μg/ml) for 15 minutes at room temperature.

[3H]Thymidine incorporation and autoradiography
In vivo
Embryos that received untreated membranes were removed from the egg 6 hours after surgery. The operated area was isolated and transferred to an Eppendorf tube containing 100 μl of 12.5 μCi/ml labeled thymidine (specific activity 55-57 Ci/mmol; Amersham) in PBS. At the end of 15 minutes incubation at 37°C, embryo fragments were washed in PBS and fixed in Bouin’s fluid. Histochemical processing was performed as described above, slides were then coated with NTB-2 emulsion and exposed for 4 days. Sections were counterstained with hematoxylin.

In vitro
1.5 μCi/ml of labeled thymidine was added to the cultures for 1 hour. After extensive washing with PBS, cultures were fixed with Bouin’s fluid and stained with the Hoechst nuclear stain. Culture dishes were coated with NBT2 emulsion for 4 days.

In the two systems described above, thymidine incorporation was measured as the proportion of cells with more than ten grains/nucleus of the total cells. Precursors of dermis were scored as 13F4-negative cells located ventral to the ectoderm. These dermatome cells were scored in every second section of embryos in the operated as compared to the intact contralateral side. In cultures of dermomyotome cells, cell counts were performed in approximately 20 microscopic fields/dish which comprised a total of 500-1600 cells. Results are expressed as the mean ± s.d. of triplicate cultures.

In situ hybridization with an avian-specific trkC probe
A fragment encoding 270 bp of the extracellular domain of chicken trkC that corresponds to amino acids 283-373 of the porcine molecule (Lamballe et al., 1991) was cloned into a pBC KS vector (Stratagene) suitable for transcribing antisense and sense RNA. In situ hybridization was performed as previously described (Averbuch-Heller et al., 1994).

RESULTS
Dermatome-dermis transition is dependent upon neural tube-derived signals
To test for a possible role of the neural tube in the development of somite derivatives, a non-permeable silastic membrane was
inserted between the neural tube and the adjacent somites in E2.5 chick embryos. Fig. 1A shows a transverse section through an embryo 24 hours after the operation. At this stage of development, the control intact dermatome has lost its epithelial structure and has dissociated into dermis, except for the dorso-medial lip, which retained an epithelial conformation (Fig. 1A, control side). In contrast, the dermatome on the operated side remained epithelial and no dermis formed. The membrane implantation at this time of development selectively affected dermatome dissociation, as normal muscle can be observed in

Fig. 1. Impaired dermis formation in embryos that received silastic grafts between neural tube and somites: Reversal by Neurotrophin-3. Transverse sections through the operated area of E4 embryos implanted on E2.5 with control silastic (Sil) membrane (A, control and operated sides), silastic membrane pretreated with NT-3 (B, only operated side) or with NGF (C, only operated side). Grafting of intact silastic membranes results in the presence of an epithelial dermatome (DT) in the operated side (A, right) as opposed to a well developed dermis (D) in the contralateral intact side (A, left). In contrast, note that grafting of NT-3-treated membranes completely rescued a normal dermis with no epithelial cells remaining distal to the implants (B), and in the presence of NGF-treated membranes an extensive dermatome remains although few dermal cells (arrows in C) have dissociated from the epithelium. Note as well that the extent of mesenchymalization in the presence of NT-3 (B) even surpassed that seen in the control side of parallel embryos (A). Serial section analysis revealed no apparent effect on the 13F4 myotomes or sclerotomes. Abbreviations: Ao, dorsal aorta, DML, dorsomedial lip of the dermatome, M, myotome, Mn, mesonephros, No, notochord, NT, neural tube, S, sclerotome, Sil, silastic membrane. Bar=40 μm.
the operated side of experimental embryos (revealed by expression of the 13F4 epitope). In agreement with previous observations (Kalcheim and Le Douarin, 1986), no dorsal root ganglia developed distal to the site of membrane implantation (not shown). This effect of neural tube deprivation on dermis development was found in 75% of the embryos analyzed (n=20). Comparable results were obtained upon grafting the membranes either at epithelial somite levels or at a more rostral (brachial) level corresponding to a recently formed epithelial dermomyotome. These results indicate that there is a relatively broad time window in which the neural tube is required for dermis formation, well beyond that reported previously for muscle development (Rong et al., 1992).

The lack of dermis formation in the operated embryos could be caused by death of dermatome progenitors or by an impairment in their proliferation. To test for these possibilities, three embryos received untreated silastic membranes and were further incubated in ovo for additional 6 hours, a period during which dermatomes on both sides remain epithelial and could therefore still be compared. At the end of the incubation, a 15 minute pulse of radiolabeled thymidine was delivered to the embryos and total dermatome cell number, as well as the number of dermatome cells that incorporated the label, were monitored in alternate histological sections. Table 1 summarizes the results obtained. Separation of somites from the neural tube did not affect the total number of dermatome cells present in the operated as compared to the intact side of each experimental embryo. In line with this result, no significant pyknosis could be seen in either the experimental or control dermatomes at 6 hours, nor in the epithelial dermatomes of another series of embryos incubated for 24 hours after surgery (not shown). Furthermore, this type of operation did not affect the ability of the dermatome cells to incorporate radiolabeled thymidine when compared to their normal counterparts, as a similar percentage of thymidine-incorporating cells was therefore still be compared. At the end of the incubation, a 15 minute pulse with radiolabeled thymidine.

**Results represent mean ± s.d. of the number of dermatome cells in 36-56 sections counted in each embryo. Dermatome cells were counted in transverse sections as hematoxylin-positive nuclei located between the surface ectoderm and the 13F4-positive myotomes.**

**Table 1. Separation of somites from the neural tube does not affect the number of dermatome cells nor their proliferative capacity**

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Number of dermatome cells/section*</th>
<th>[3H]Thymidine-positive cells/total dermatome cells (%)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control side</td>
<td>operated side</td>
</tr>
<tr>
<td>64</td>
<td>53.0±117</td>
<td>51.7±13.4</td>
</tr>
<tr>
<td>66</td>
<td>74.2±16.1</td>
<td>72.9±18.5</td>
</tr>
<tr>
<td>67</td>
<td>86.5±18.6</td>
<td>82.8±26.9</td>
</tr>
</tbody>
</table>

Embryos received untreated silastic membranes at the brachial level and were incubated for 6 hours. Incubation with [3H]thymidine was performed as described under Methods. *Results represent mean ± s.d. of the percentage of dermatome cells bearing thymidine grains over nuclei after a 15 minute pulse with radiolabeled thymidine.

As NT-3 is present in the neural tube of early avian embryos (Pinco et al., 1994; Yao et al., 1993), we then tested whether this molecule could overcome the effects of neural tube deprivation on dissociation of dermatome cells in vivo. In striking contrast with the results obtained with control membranes, pretreatment of the membranes with NT-3 completely restored the normal situation and, in 100% of the cases examined (n=7), the presence of a well-developed dermis between the ectoderm and the 13F4-positive muscle was observed (Fig. 1B). Dermis formation was evident in all embryos along the entire length of the grafts to an extent virtually indistinguishable from that observed in the normal contralateral sides (Fig. 1, compare panels A control side and B).

Implantation of membranes treated with NGF, a molecule highly related to NT-3, revealed that in 5 out of the 6 cases examined, an extensive epithelial dermomyotome remained on the operated side, though in 3 of the above, a few dermal cells also dissociated from the epithelium and populated the region underlying the ectoderm (Fig. 1C, arrows). In all these cases, complete dissociation of the dermomyotome was evident on the control side (not shown; see also Fig. 1A).

**Expression of trkC mRNA in somite derivatives**

Since treatment of chick embryos with NT-3 was found to profoundly influence dermatome development, we next examined the distribution of transcripts coding for trkC, a tyrosine kinase receptor that selectively binds NT-3. While no expression of trkC mRNA was found in the segmental plate or epithelial somites in embryos with 8-35 somite pairs, a signal was first detected in the dorsal portion of somites undergoing dissociation into sclerotome and dermomyotome (Fig. 2A,B). Significantly, the sclerotome and myotome remained negative at all stages examined (Fig. 2) and an apparent dorsoventral gradient of expression of trkC mRNA was evident in the developing dermomyotome (Fig. 2C,D arrows). In rostral segments at E4 and in E5 along the axis, where the epithelial dermomyotome gave rise to muscle and dermis, positive hybridization signals remained confined to the region of the dorsomedial lip, the last epithelial remnant (Fig. 2E,F). These results suggest that the role played by NT-3 on dermis development in ovo may be mediated by the trkC receptor, whose mRNA is present at the appropriate time on dermis precursor cells.

**NT-3 stimulates epithelial-mesenchymal transition of dermatome cells in vitro**

**Expalnts of paraxial mesoderm in 3-D collagen gels**

Paraxial mesoderm strips containing sclerotomes, epithelial dermomyotomes and the overlying ectoderm were excised from E2.5 embryos and grown for 1 day in 3-D collagen gels in the absence or presence of the neural tube or of NT-3. As shown in Fig. 3A, in the presence of the neural tube, dermomyotome dissociation is complete in 60.8% of the explants (n=23), and a well-developed mesenchymal dermis is observed between myotome and ectoderm, similar to the in vivo normal situation. In contrast, incubation of mesodermal strips each composed of 4 to 7 consecutive segments without added factor resulted, in all cases (n=7), in the maintenance of the epithelial dermomyotome while impairing its dissociation into dermal cells (see in Fig. 3B).
each 13F4-positive myotome). However, in tissue strips incubated with NT-3 (10 ng/ml, from the time of explantation), mesenchymal cells populating the dermal anlagen between myotome and ectoderm were detected one day later in 83% of the cases examined (n=6), and no aggregated cells were present in the vicinity of the myotomes (Fig. 3C). Thus, similar to the results obtained in the silastic-treated embryos, NT-3 can mimic the effect of the neural tube on dermatome-dermis conversion. As a control, strips with NGF (10 ng/ml) were incubated. In 6 strips, no dermis formed, in one case dermis developed and in the remaining 2 cases an intermediate phenotype consisting both of aggregated cells and of some dissociated precursors was observed (not shown). Interestingly, these results are very similar to those obtained for NGF-treated grafts (see Fig. 1C).

Explants of isolated epithelial dermomyotomes in 3-D collagen gels

To test whether NT-3 can directly act on epithelial dermomyotomes to promote their conversion into dermis, isolated dermomyotomes were grown in collagen gels with or without NT-3 for 6 hours. Similar to the results obtained in the previous systems, numerous mesenchymal cells were seen around the explants in 70% of the cases in the presence of NT-3 (10 ng/ml, n=17, Fig. 4B). These cells were 13F4-negative, suggesting that they are of dermal phenotype rather than myoblasts leaving the myotome. In striking contrast, 73% of control dermomyotomes (n=11) remained epithelial and virtually no cells were seen leaving the explants (Fig. 4A). A similar picture was observed 24 hours after explantation (n=3 and 4 for control and NT-3-treated explants, respectively). The number of dissociated cells was monitored in each of the explants grown for 6 hours. 10 to 38 serial sections were scored per dermomyotome and an average of 10.05±5.6 cells/section in the NT-3-treated samples were found to leave the dermomyotomes compared with only 3.5±3.1 cells/section in the controls.

Fig. 2. Expression of trkC mRNA in the dermatome of normal chick embryos. (A,B) Transverse section through an E2.5 embryo showing trkC mRNA by in situ hybridization to the dermomyotome (DM) of the dissociating somite. (C,D) Transverse section through an E3 embryo showing the expression of trkC transcripts to the somite-derived dermatome (DT) layer (arrows). Sclerotome and myotome show background levels of hybridization. (E,F) Transverse section through an E5 embryo showing expression of trkC mRNA to the dorsomedial lip of the dermatome (DML, arrowheads). Sclerotome (S) and myotome (M) are negative, as well as the mesenchymal dermis. Note in A, C and E the distribution of trkC transcripts to the dorsal and marginal zones of the neural tube (NT), respectively, and to dorsal root ganglia (E,F) as described previously (Kahane and Kalcheim, 1994). A, C and E, dark field; B, D and F, bright field. Abbreviations: No, notochord. Bar=100 μm.
NT-3 promotes dissociation of epithelial dermomyotome cells without stimulating their survival or proliferation

The effects of NT-3 were further tested on dermomyotomes consisting of small epithelial clusters grown on tissue culture dishes. Consistent with the findings described above, control cultures and cultures treated with NGF (10 ng/ml) remained predominantly composed of small cell aggregates, as assessed by the packed appearance of the Hoechst-positive nuclei (Fig. 5A,B). Few if any cells left the aggregates to migrate onto the substratum. This picture was observed 6 hours after initial seeding as well as after overnight incubation. In striking contrast, in the presence of NT-3 (10 ng/ml), partial dissociation of most clusters was observed as early as 6 hours after seeding (not shown), followed by complete dissociation of the aggregates at 16 hours (Fig. 5C,D). In this system, NT-3 specifically promoted a conformational change in cell shape without regulating cell number. This is because in parallel cultures treated with NT-3, there was no change in the total number of cells, nor was any change measured in the proportion of cells that incorporated radiolabeled thymidine into nuclei when compared to cultures grown in the absence of added factor (Table 2).

Endogenous NT-3 plays a role in the early stages of dermis formation

To assess the physiological significance of NT-3 on dermatome-dermis conversion, we used an antibody specifically neutralizing the activity of NT-3 (Gaese et al., 1994). Embryos were treated with the antibodies starting at E2 and were fixed in E4. In intact E2 embryos, dissociation of the epithelial somite is already underway at cervical levels, but not at more caudal levels where epithelial somites and unsegmented paraxial mesoderm are present. At E4, a rostrocaudal gradient of dermis development was observed in untreated embryos (n=3). These embryos showed a well-dissociated dermis composed of loose mesenchymal cells embedded in extracellular matrix. This picture was evident along the rostrocaudal extent of the axis up to the level of the hindlimbs (Figs 6A, 7A). From this region caudal, dermal cells had a more compact appearance and occupied a smaller space between myotome and ectoderm (Fig. 6, compare panel B with A), probably as a result of a less-developed matrix in which these cells become progressively embedded. Finally, in the tail region of the E4 embryos, no dissociation was apparent and an epithe-
lial dermatome was observed overlying the 13F4-positive myotome (Fig. 6C).

Treatment of the embryos with anti-NT-3 antibodies strikingly inhibited dermis formation. In 7 out of 13 treated embryos, cell clumps were observed with disrupted matrix fibrils between the ectoderm and myotome (compare panels C and A in Fig. 7). This picture prevailed at cervical levels of the axis, whereas from the brachial region caudad, compact, aggregated cells predominated, similar to the situation observed at caudal (lumbosacral) levels of control embryos (compare panels B in Figs 7 and 6). Similar to the results obtained in embryos with silastic implants, embryos that received anti-NT-3 antibodies revealed morphologically normal sclerotomes and myotomes, suggesting that NT-3 selectively influences dermatome formation (Fig. 7). To assess for the specificity of the NT-3 antibodies used, 7 embryos were injected with a control monoclonal antibody (see Methods). While comparable antibody levels were reached, dermis development was normal in all cases and was not different from that seen with untreated embryos.

Interestingly, epithelial dermatomes were also observed in the tail region of all the anti-NT-3 treated embryos, much like in the control embryos. Because at the onset of antibody treatment (E2), tail segments corresponded to a region of unsegmented mesoderm, we infer that this factor is not involved in either somite segmentation, or somite dissociation into sclerotome and dermomyotome. NT-3 is, therefore, likely to be acting on cells of the already formed dermomyotome, a contention supported by the expression pattern of the trkC receptor transcripts.

DISCUSSION

This study shows that, in avian embryos, the formation of dermis from the epithelial dermatome depends upon neural tube-derived signals, in the absence of which an epithelial dermatome remains. This lack of conversion can be observed both in vivo and in vitro. The lack of dermis formation in the absence of neural tube signals seems to result from a specific failure in mesenchymalization of epithelial cells rather than a deleterious effect on their survival or proliferation. Indeed, no significant cell death is detected over a 24 hour period following implantation of the membrane. It also seems unlikely that the absence of dermis results from decreased proliferation of progenitor cells of the dermatome, since no bilateral differences in the percentage of dermatome cells incorporating thymidine are measured.

Axial organs such as the neural tube may have diverse effects on paraxial structures differing in their state of commitment and differentiation. For example, the survival of neural crest cells is absolutely dependent upon the neural tube at an early postmigratory stage, but only partially dependent at later stages during gangliogenesis (Kalcheim and Le Douarin, 1986). Likewise, somite cells require a time window of several hours of contact with the neural tube in order to differentiate normally into vertebral muscle (Rong et al., 1992). At later stages, such as those investigated in this study, neural tube deprivation has no effect on myogenesis. Past this sensitive period for muscle development, separation from the neural tube only affects mesenchymalization of dermatome cells.

In two previous works, the effect of axial structures on muscle and sclerotome development was tested. Christ et al. (1992) performed neural tube removal experiments at the level of the unsegmented mesoderm and, under these conditions, the presence of medially fused somite derivatives, including a dermatome, were observed. Most interestingly, although not discussed by the authors, an epithelial dermatome apparently remains 2 days after surgery (corresponding to E4, a time when a well-dissociated dermis is expected to be present, see Fig. 2e,f in Christ et al., 1992). Similarly, Rong et al. (1992), have
reported that, in neuralectomized embryos, from E9 onward, the dorsal skin is transparent and becomes inflated with an abnormal pattern of feathers, particularly in the dorsolateral region. Since feather development from ectoderm is induced by underlying dermal condensations, this result would be suggestive of the presence of possible dermal abnormalities. Together with the present study, the above data would suggest a role for the neural tube in mesenchymalization of the epithelial dermatome, similar to the notion proposing that the notochord expresses a mesenchymalizing factor for cartilage progenitors (Christ et al., 1972, Pourquie et al., 1993).

The present study also indicates that NT-3 supports the formation of a normal dermis under conditions otherwise impairing dermatome dissociation. Thus, NT-3 can replace the requirement for the neural tube in the dermatome-to-dermis conversion both in vivo and in vitro. Two possible local sources of NT-3 for the developing dermatome could be envisaged: the neural tube and the somite itself. If the second possibility is considered, then the role of the neural tube would be to regulate the level of NT-3 in somites or somite-derived structures, such as the myotome, where the factor could act locally. Although we cannot exclude this possibility, it seems less likely because, if present in the mesoderm, NT-3 must exist at levels below detection by immunocytochemistry or in situ hybridization. The most likely source of NT-3 at this stage would be the neural tube that contacts the dorsomedial aspect of the dermatomyotome at early stages of somite dissociation. Indeed, the presence of NT-3 immunoreactivity as well as mRNA was documented in the early neural tube (Pinco et al., 1993, Yao et al., 1993), where the factor can function locally to promote the differentiation of motoneurons (Averbuch-Heller et al., 1994) and of neural crest progenitors (Kalcheim et al., 1992; Pinco et al., 1993).

**Table 2. NT-3 does not stimulate the number of cultured dermatomyotome cells that incorporate thymidine into nuclei**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Field density</th>
<th>[3H] Thymidine-positive cells/field</th>
<th>[3H] Thymidine-positive cells/total cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>57.3±0.20</td>
<td>17.0±0.5</td>
<td>29.5±0.7</td>
</tr>
<tr>
<td>10 ng/ml NT-3</td>
<td>56.7±11.5</td>
<td>17.6±3.7</td>
<td>30.9±0.3</td>
</tr>
</tbody>
</table>

Partially dissociated dermatomyotomes were grown on fibronectin-coated dishes for 6 hours. A pulse of radiolabeled thymidine was given for an additional hour, as described under Methods. Field density represents the number of cells per microscopic field. 20-27 fields were quantified in control cultures and 15-25 in NT-3-treated dishes. Results represent the mean ± s.d. of triplicate cultures.

![Fig. 5](image-url) Neurotrophin-3 stimulates the dissociation of dermatomyotome cells cultured on fibronectin-coated dishes. Isolated dermatomyotomes were partially dissociated and cultured for 16 hours as described in Experimental Procedures. The experiment was performed twice in triplicate samples each (see Table 2 for details on cell numbers). Note the complete dissociation of the aggregates in the presence of NT-3 (C,D) and, in contrast, aggregated cells remaining in the absence of factor (A,B). (A,C) Fluorescence micrographs showing Hoechst-positive nuclei; (B,D) phase contrast. Bar: 40 μm.
Neither in vivo nor in vitro do we observe any effects of exogenous NT-3 or of NT-3 deprivation on muscle development. However, this selectivity towards dermatome cells was less apparent in the partially dissociated dermomyotomes cultured on planar fibronectin substrata (Fig. 5). A time-dependent dissociation of single cells from the central clusters was observed in cultures treated with the factor, that culminated after 24 hours with their complete dissociation apparently also involving the myogenic cells. In view of the lack of effect of NT-3 on myogenic cells in the other systems, we interpret this putative myoblast disaggregation as secondary to the primary effect of NT-3 on dermatome precursors. An attempt to identify myoblasts with the 13F4 antibody failed under the culture conditions used, because it revealed a non-specific perinuclear product in all the cells present in the cultures. Nevertheless, staining for desmin revealed under our culture conditions a faint staining of myogenic cells. In 6 hour cultures, desmin-immunoreactivity was confined to the clusters, but was not found among the cells that had individualized on the substratum (unpublished observations). Inasmuch as myoblasts, like dermal fibroblasts, have the ability to spread onto planar substrata of extracellular matrix glycoproteins, we suggest that, in this system, NT-3-induced dissociation of dermal progenitors facilitated the migration of myogenic cells.

While the response of dermatome cells to NT-3 was observed in all systems tested in the present work, NGF was ineffective in stimulating dermatome dissociation when dermomyotomes isolated from ectoderm and sclerotome were employed. In embryos and whole mesoderm explants, the qualitative picture obtained with NGF was that of partial cell dissociation while a still significant epithelial dermatome remained in all cases. A possible interpretation of these results is that the superficial ectoderm lying immediately dorsal to the dermatome mediates a response of dermatome cells to NGF. The superficial ectoderm could also be a potential source of NGF (see for example Davies et al., 1987). In addition, a transient expression of NGF mRNA was detected in somites of rodent embryos (Wheeler and Bothwell, 1992).

Of all neurotrophin receptors described so far, the NT-3 receptor TrkC is the most selective one. It was thus of particular interest to follow the pattern of trkC mRNA expression during dermatome development. trkC mRNA was first detected along the epithelial dermatome, but once mesenchymal cells of the dermis have formed, trkC transcripts become restricted to the dorsomedial lip of the dermatome. This area is of particular interest because it also expresses qm1, a myogenic regulatory gene (de la Brousse and Emerson, 1990). Thus, the dorsomedial lip of the dermatome might be a site containing...
bipotential progenitors both of the myogenic and dermogenic lineages (see also de la Brousse and Emerson, 1990). An additional marker for dermatome and dermis is the homeodomain protein gMHox. It would be interesting to assess whether the neural tube and neural tube-derived signals have any effect on gMHox expression, as this molecule has been proposed to participate in the maintenance of mesenchymal cell lineages derived from the somites and the neural crest (Kuratani et al., 1994).

Two additional DNA-binding proteins have been reported to localize to the dermatomes, among other embryonic structures. The first is engrailed-1, shown to localize to the medial part of dermatomes and subsequently to myotomes and dermis (Gardner and Barald, 1992). The second is PAX-3, shown to localize predominantly to the lateral half of the dermomyotome that contains limb muscle precursors, whereas its expression is low in the medial half that contains axial muscle and dermis progenitors (Williams and Ordahl, 1994). Subsequently, PAX-3 becomes localized to the lateral part of the dermatome (Johnson et al., 1994) but never to the dermis (Williams and Ordahl, 1994).

In this study, the process of dermatome-to-dermis conversion in embryo sections and explants was followed using morphological and histological criteria, as well as by 13F4 immunostaining that clearly delineated the myotomes. The availability of a differential marker that defines the transition between an epithelial dermatome and mesenchymal dermal cells would be useful, mainly for work with cultures where the topographical relations between the different cell types may be altered. Unfortunately, both engrailed-1 and gMHox stain the cells in both conformations and PAX-3 stains mostly a sub-population of lateral dermome precursors, less relevant to dermis formation. In addition, while trkC mRNA is expressed in dermatome under normal conditions, it is down regulated from the residual dermatome that has been deprived from neural tube input (Brill and Kalcheim, unpublished results).

In this study, the physiological relevance of NT-3 in the process of dermatome-dermis conversion is demonstrated using an antibody specifically neutralizing the biological activity of NT-3 and not that of the related molecules NGF, NT-4 or BDNF (Gaese et al., 1994). The selective deprivation of NT-3 in developing embryos with this antibody perturbs the formation of a dermal mesenchyme otherwise observed in age-matched controls. Interestingly, other aspects of somite development, like segmentation and subsequent dissociation into dermomyotome and sclerotome were not affected. Neither were sclerotome and myotome appearance disturbed in the treated embryos. The significance of these observations is twofold: first, that the activity of NT-3 is selective towards the somite-derived dermatome and, second, that NT-3 is not

Fig. 7. Early dermis formation is disrupted in chick embryos treated with anti-NT-3 antibodies. Transverse sections through E4 embryos immunolabeled with the 13F4 antibody and counterstained with Hematoxylin to reveal nuclei. (A) Normal E4 embryo at the cervical level of the axis showing a well-dissociated mesenchymal dermis (D). (B,C) Anti-NT-3-treated embryo. (B) brachial level of the axis showing tightly aggregated nuclei between myotome and ectoderm (arrows), and a more severe phenotype in the cervical region (C) revealing the presence of clumped cells embedded in an apparently disrupted extracellular matrix (arrows in C). Abbreviations: DRG, dorsal root ganglia; M, myotome; S, sclerotome. Bar: 30 μm.
involved in initial somite patterning. As recently demonstrated, initial dorsal-ventral patterning of the somites is determined by ventral axial structures, the floor plate and the notochord (Pourquin et al., 1993), which may stimulate sclerotome formation via the Sonic hedgehog gene product (Fan and Lavigne, 1994; Johnson et al., 1994). NT-3 is, thus, the second defined midline-derived signal playing a role in differentiation of somite derivatives.

In the experiments involving grafting of silastic membranes to disconnect neural tube and somites, we clearly observed a complete lack of dermatome-dermis conversion and the presence of a residual epithelial dermatome in a distal position with respect to the implant. Although in the anti-NT-3-treated embryos, the presence of a dissociated dermis was never observed, the dermatome did not remain completely epithelial; instead, cells remained tightly packed and clumped, resembling a transition state between dermatome and dermis observed in intact E4 embryos at caudal areas of the axis only. These observations may suggest either that complete deprivation of endogenous NT-3 was not achieved, or more likely, that additional neural tube-derived factors may be involved in driving this epithelial-mesenchymal conversion. Indeed, identical results were obtained with embryos in which the measured levels of anti-NT-3 antibody varied over a range of concentrations of more than ten, suggesting that the antibody concentration was well above saturation.

The deprivation of endogenous NT-3 will serve as a useful approach in future experiments to determine the significance of dermatome-to-dermis transition to the subsequent development of dermal derivatives, as well as to assess the capacity of the defective cells or of neighboring fibroblasts to compensate for local absence of dermis. In this context, it is worth pointing out that no mention of possible defects in dermal derivatives was reported for mice with targeted deletions of the NT-3 or the trkC genes (Ernfors et al., 1994; Klein et al., 1994; Tesarollo et al., 1995). Subtle early dermal defects might not have been looked for in these animals, epithelial-mesenchymal transition could be delayed but eventually take place; finally, dermal defects might never be present in these mice.

The biological activity of NT-3 and the expression of its cognate receptor trkC in the dermatome suggest that the NT-3-trkC ligand-receptor interactions may activate a signal transduction cascade involved in epithelial-mesenchymal conversion of specific primary epithelia. Another identified molecule, the scatter factor/hepatocyte growth factor can mediate, in a paracrine manner, conversions between mesenchyma and epithelia, and also has potent mitogenic and morphogenetic effects on epithelial cells in vitro. Similar to NT-3, this factor also acts through a cell surface receptor with tyrosine kinase activity, the C-met proto-oncogene (Sonnenberg et al., 1993; Tsarfaty et al., 1994). Other receptors with tyrosine kinase activity, such as pp60^{src}, were shown to mediate the loss of epithelial characteristics and the acquisition instead of invasive properties in specific cells (Behrens et al., 1993). Further experiments are required to unravel the identity of the molecules involved in the NT-3-mediated loss of epithelial properties of dermatome progenitors.

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REFERENCES


Lamballe, F., Klein, R. and Baracabid, M. (1991) trkC, a new member of the NT-3 and dermis development
trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell 66, 967-979.


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