Exogenous retinoic acid decreases *in vivo* and *in vitro* proliferative activity during the early migratory stage of neural crest cells

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Abstract. We have previously demonstrated that directional migration of neural crest cells (NCC) is associated with a high cell density, resulting from an active cell proliferation. It is also known that treatment with retinoic acid (RA) causes a dose-dependent inhibition of proliferation of some cell types, and that administration of RA during the early stages of embryonic development, induces cranio-facial abnormal patterns corresponding to NCC derivatives. In view of these findings, it was of interest to determine if exogenous RA is a potential modulator of the mitotic rate of NCC, and to explore the hypothesis of an inhibitory effect exerted by RA on the proliferative behaviour of NCC *in vivo* and *in vitro*.

Homogenates of RA-treated chick embryos showed a low [^3H]dT incorporation, indicating a generalized diminution of DNA synthesis. The labelling index (LI=number of labelled cells/total number of cells) revealed that NCC from RA-treated and control embryos had higher values of [^3H]dT incorporation than neural tube cells ($P<0.0001$). Autoradiographs of RA-treated chick embryos showed a significantly lower [^3H]dT incorporation in NCC at the prosencephalic and mesencephalic levels, as well as in the neural tube cells at the prosencephalic, mesencephalic and rhombencephalic levels, than in control chick embryos ($P<0.0001$). NCC cultures treated with 1 or 10 μM RA had a significantly lower LI than in cultures treated with 0.1 μM RA or control cultures ($P<0.04$). In chick embryos, the mitotic index of NCC was 0.026 for RA-treated and 0.033 for controls, while the duration of the cell cycle was significantly longer in the NCC of RA-treated embryos (~ 40 h) than in controls (~ 25 h). The length of the cell cycle phases of NCC was similar in both experimental conditions, except for G_1 phase, which was significantly longer in the RA-treated group than in controls.

These results show that RA blocks DNA synthesis and lengthens the proliferative behaviour of NCC both in early chick embryos and *in vitro*, effects that could modify the morphogenetic patterns of NCC distribution through a decreased cell population.

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Neural crest cells (NCC) segregate from the dorsal neural tube of vertebrate embryos, migrate along complex pathways and differentiate in a variety of cell populations (Le Douarin 1982). When NCC were cultured on stripes of fibronectin, mimicking the microenvironment of the narrow migratory pathways found *in vivo*, it was found that their directional migration was associated with a high cell density, probably resulting from an active cell proliferation (Rovasio *et al.* 1983, Rovasio & Thiery 1987). Recent reports from our laboratory have shown a high mitotic rate of NCC *in vivo* using the colchicine statokinetic method (Paglini & Rovasio 1994a), as well as a shortening of the cell cycle in comparison to other embryonic cell populations after autoradiography and [³H]dT incorporation (Paglini & Rovasio 1994b). We have also found that the *in vitro* high mitotic rate of NCC depends upon a fibronectin-containing substrate (Paglini & Rovasio, unpublished data).

On the other hand, a gradient of retinoic acid (RA), a metabolite of vitamin A, has been suggested as a regional specific morphogen for the homeobox-containing gene-dependent cephalo-caudal polarization of vertebrates (Chen *et al.* 1994). Accumulation of radiolabelled retinoids (Dencker *et al.* 1991), as well as cellular RA-binding proteins (Maden *et al.* 1991), have been detected in NCC. Moreover, it has been demonstrated that cell groups along the pathway of trunk NCC in the rat embryo appear to release retinoids (Wagner *et al.* 1992). It has been proposed that exogenous RA interferes with the development activity of NCC, inducing changes at the cell surface level (Pratt *et al.* 1987), decreasing their cell-substratum adhesion (Smith-Thomas *et al.* 1987), changing their capacity to extend and maintain lamellipodia (Thorogood *et al.* 1982), or disturbing extracellular matrix components (Moro-Balbás *et al.* 1993). These data strongly suggest the potential ontogenetic capacity of NCC to respond to microenvironmental RA.

During the early stages of embryo development, exogenous RA administered to experimental animals (Lohnes *et al.* 1994), or inadvertently ingested by pregnant women (Lammer *et al.* 1985), induced cranio-facial abnormal patterns corresponding to NCC derivatives. The basis for this phenomenon appears to reside in RA's ability – present as a cephalo-caudal concentration gradient (Chen *et al.* 1994) – to regulate the spatial expression of the homeobox-containing genes, thereby changing the phenotypic segmental characteristics of the cephalo-caudal axis (López *et al.* 1995) and affecting some aspects of the NCC dispersion from the neural tube (Gale *et al.* 1996).

Other workers found that exogenous retinoic acid causes a dose-dependent inhibition of proliferation of some normal (Zouboulis *et al.* 1991) and tumoral (Lotan *et al.* 1981) cell types, as well as a prolongation of the cell cycle time due to a lengthening of G₁ phase (Russel & Haddox 1981, Bakke 1986). Besides which, cultures of trunk NCC treated with RA showed a total cell number noticeably reduced when compared with control values (Dupin & Le Douarin 1995, Ito & Morita 1995).

Reduction in the NCC proliferative activity could be involved in the mechanism of their early dispersion through a decreased cell population (Twitty & Niu 1954). Hence, the purpose of this study was to explore the hypothesis of a possible inhibitory effect of exogenous RA on the proliferative behaviour of NCC both *in vivo* and *in vitro*. RA induced a diminution of the proliferation of NCC in both experimental conditions, as well as a lengthening of their *in vivo* mitotic cycle.

MATERIALS AND METHODS

Treatment of cultured chick embryo

Fertile chick eggs (Cobb line) were incubated for 30 h at 38°C in an humidified atmosphere up to stage 9 of Hamburger & Hamilton (HH) (1951). Afterwards, the whole egg was

transferred to a modified 'shell-less' culture system; a flat ring of 2% agarose in phosphate buffered saline (PBS) (20 mm outer diameter, 10 mm inner diameter and 2 mm thick) was placed over each blastoderm (Battiatto *et al.* 1996). A container was thus formed around the embryo, where 50 μ l of 10 μ M retinoic acid (RA) (all-*trans*; Sigma) dissolved in 40 μ M dimethylsulfoxide (DMSO, Sigma) and prepared in PBS were placed. Control embryos were treated with DMSO-PBS alone. After post-incubation for 3.5 h at 38°C, 50 μ l of 0.37 MBq/ml tritiated thymidine ($[^3\text{H}]\text{dT}$) solution (248 GBq/mmol, New England, Boston, MA) prepared in PBS were added and the chick embryos were incubated for 30 min, reaching stage 10 of HH. In a parallel series of 'cold' control embryos, PBS solution alone was added.

Neural crest cells culture

Cultures of NCC from the trunk region were prepared according to the technique of Cohen & Konigsberg (1975). Briefly, fertile chick eggs were incubated for 48 h at 38°C in a humidified atmosphere up to stage 13–14. Blastoderms and the surrounding membranes were excised and washed with PBS, and a trunk explant corresponding to the last 5 somite pairs was cut away. The block of tissues was incubated in 200 μ g/ml Dispase (Calbiochem Corp., La Jolla, CA) in Dulbecco's Modified Eagle's Medium (DMEM, Sigma) without serum for 15 min at room temperature, and the explant of neural tube was carefully isolated from the surrounding tissues using tungsten microneedles and transferred to a Petri dish (35 mm) containing 900 μ l DMEM with 10% fetal calf serum (FCS, Notocor, Cba., Argentina).

Cultures were grown for 24 h afterwards the medium (450 μ l) was changed by the same volume of RA-containing medium to give a final concentration of 0.1, 1 or 10 μ M. Control cultures received an equal volume of DMSO-DMEM. After 24 h of treatment, 100 μ l of 222 KBq/ml $[^3\text{H}]\text{dT}$ solution (248 GBq/ml, New England) prepared in PBS were added and the cultures were incubated for 30 min. In a parallel series of 'cold' cultures, PBS alone was added.

Evaluation of DNA synthesis

Groups of 19 RA-treated and 14 control chick embryos were treated with $[^3\text{H}]\text{dT}$ in the same manner than that described above ('Treatment of cultured chick embryos'). Afterwards, they were washed to remove the free $[^3\text{H}]\text{dT}$ and then homogenized in 400 μ l ethanol. Each homogenized embryo was mixed with 2000 μ l of a scintillation cocktail (Aquasol Universal LSC) and counted by means of a scintillation counter (LKB 1214-Rackbeta, 54% $[^3\text{H}]$ -efficiency) for 5 min/embryo. Data were statistically evaluated with the Anova test (Steel & Torrie 1987).

Autoradiographic procedure

Groups of RA-treated and control chick embryos without vitelline membrane or agar ring were fixed in Carnoy's fluid (ethanol/acetic acid, 3:1) for 12 h, dehydrated in an ethanol series and embedded in paraffin. Series transversal sections of 5- μ m thickness were mounted on acid-cleaned glass slides previously coated with chromic gelatin. Treated and control cell cultures were fixed with 4% formaldehyde in PBS for 30 min, then both histological sections and cell cultures were processed for autoradiography using NTB-2 emulsion (Kodak) diluted 1:1 with distilled water at 45°C (Baserga & Malamud 1969). Autoradiographs and slides of 'cold' controls were stored in a black sealed plastic box at 4°C for 10 days. Standard slides carrying histological sections of several organs from $[^3\text{H}]\text{dT}$ -injected Balb-c mice (85.2 KBq/g body weight) were included as 'hot' controls. Afterwards, the slides and dishes were

developed with PQ7 (Romek) for 3 min at 18°C and counterstained with haematoxylin and eosin.

Cell counts

In preliminary experiments, the background values were established for [³H]dT-treated chick embryos and cell cultures. Labelling was considered to be positive when six or more silver grains for chick embryos, and fifteen or more silver grains for cell cultures over the nucleus were found (Baserga & Malamud 1969). Semi-serial sections of comparable embryos and cell cultures were counted at a magnification of $\times 1000$ and $\times 800$, respectively, by the same person, in order to diminish subjective errors.

In five RA-treated and in four control chick embryos, cell counts of all NCC and neural tube cells were performed at the prosencephalic, mesencephalic and rhombencephalic levels. An average of 18 sections in each level was counted (about 3200 NCC and 14800 neural tube cells for embryo). NCC were counted from the onset of emigration, when they detach from the dorsal neural tube, up to the stage of their arrival to the vicinity of mesodermic cells. At these early migratory stages, the morphological identification of NCC is easily made, since they are not yet intermixed with cephalic mesodermal cells. These cell counts were performed to express the LI at each axial level, and the cell cycle parameters of a pool of all cephalic segments. On *in vitro* NCC, an average of 1500 cells/explant were counted for each culture repeated in triplicate in each experimental condition.

The labelling index (LI) was determined in both *in vivo* and *in vitro* experiments, and statistical comparisons were made employing the Kruskal–Wallis and Mann–Whitney non-parametric tests to check for significant differences ($P < 0.05$). Previously, the minimum sample test was applied to obtain data about the sufficient number of embryos and sections through each axial level studied (Steel & Torrie 1987).

Assessment of cell cycle parameters

The duration of the S phase of chick embryo NCC has been previously established in our laboratory in the same experimental conditions (Paglini & Rovasio 1994b) and the present data of LI allowed us to obtain the duration of the cell cycle or generation time (T_c) (Quastler & Sherman 1959).

The length of mitosis (M phase) was calculated according to the expression $M/T_c = MI/Ln2$ where MI is the mitotic index representing the number of mitosis over the total number of cells, and Ln2 is a factor representing the exponential growing of early embryo cell populations (Smith & Schoenwolf 1987).

The duration of $G_2 + \frac{1}{2}M$, corresponding to the curve of labelled mitosis (Paglini & Rovasio 1994b), was considered as the time required to arrive to 50% of labelled mitosis (Baserga & Malamud 1969). Consequently, knowing the length of the M phase, the value of the G_2 phase was calculated. The remaining G_1 phase was obtained as a difference, using the known values: $G_1 = T_c - (S + G_2 + M)$.

RESULTS

Proliferative parameters were determined at the prosencephalic, mesencephalic and rhombencephalic levels of chick embryos, as well as in the NCC cultures on both RA-treated and control experimental conditions. Autoradiographs of RA-treated chick embryo sections showed lower [³H]dT incorporating in the NCC at the prosencephalic and mesencephalic levels, and in the neural tube cells at all the axial levels studied, than in the control chick

embryos. Cultures of NCC treated with RA had a significantly lower [^3H]dT incorporation than the control cultures. Likewise, measurements on triplicate independent experiments of homogenates of RA-treated chick embryos revealed significantly lower values of [^3H]dT incorporation (6269 dpm) than in the controls (11567 dpm; $P < 0.0001$), indicating a generalized diminution of DNA synthesis in the RA-treated embryo tissues.

Labelling index (LI) in chick embryos

The NCC had the highest values of [^3H]dT incorporation with respect to the neural tube cells in both RA-treated and control embryos ($P < 0.0001$) (Figure 1). However, the LI of NCC at both the prosencephalic and mesencephalic levels, as well as the neural tube cells at all the levels studied, were significantly lower in RA-treated embryos than in control chick embryos ($P < 0.001$) (Figure 1). On the other hand, the LI of NCC and neural tube cells of control embryos were similar at the three axial levels studied. For the RA-treated embryos the LI of NCC was decreased at the mesencephalic level when compared to the rhombencephalic levels ($P < 0.02$). In the neural tube cells of RA-treated embryos the LI was significantly higher at the prosencephalic level than in mesencephalic and rhombencephalic segments ($P < 0.0001$) (Figure 1).

Cell cycle parameters in chick embryos

The length of the cell cycle phases was established on NCC of RA-treated and control embryos (Table 1). Whereas the total duration of the cell cycle was significantly longer in the NCC of RA-treated embryos (~ 40 h) than in controls (~ 25 h), the mitotic index was 0.026 for RA-treated embryos and 0.033 for control embryos. On the other hand, the lengths of the

Figure 1. Labelling index (LI) of neural crest cells (NCC) and neural tube cells (NT) corresponding to prosencephalic (PRO), mesencephalic (MES) and rhombencephalic (RHO) segments of RA-treated (RA) and control chick embryos. Values are expressed as the average of 18 sections in each segment. An average of 3200 NCC and 14800 NT cells were computed for each of five RA-treated and four control embryos. Kruskal-Wallis and Mann-Whitney's non-parametric tests were performed. a v c, $P < 0.0001$; b v d, $P < 0.0001$; e v g, $P < 0.0001$; f v h, $P < 0.0001$; i v k, $P < 0.0001$; j v l, $P < 0.0001$; a v b, $P < 0.001$; c v d, $P < 0.0001$; e v f, $P < 0.0001$; g v h, $P < 0.0001$; i v j, NS; k v l, $P < 0.0001$; a v e, NS; a v i, NS; e v i, NS; c v g, NS; c v k, NS; g v k, NS; b v f, NS; f v j, $P < 0.02$; b v f, NS; d v h, $P < 0.0001$; d v l, $P < 0.0001$; h v l, NS. NS = not significant. □, control; ■, RA.

Table 1. Cell cycle duration of NCC of control and RA-treated embryos*

Cell cycle	Control†	RA-treated‡
S phase	3.20	3.20
G ₂ phase	0.67	0.52
M phase	1.20	1.50
G ₁ phase	20.12	34.70
T _c	25.19	39.92

*Values in hours.

†Mean of 3200 NCC per each of the five RA treated and four control embryos.

cell cycle phases of NCC were similar in both experimental conditions, except for the G₁ phase, which was significantly longer in the RA-treated group than in controls (Table 1).

Labelling index (LI) in NCC culture

A dose-dependent inhibition of cell proliferation was evident in RA-treated NCC cultures. Although NCC cultures treated with RA 1 μM and 10 μM had a significantly lower LI than in the control condition ($P < 0.04$), no statistically significant differences between NCC cultures treated with 0.1 μM RA and controls were found (Figure 2).

DISCUSSION

The observations of NCC moving along narrow pathways at a high cell density (Rovasio *et al.* 1983, Rovasio & Thiery 1987), as well as previous (Paglini & Rovasio 1994a,b) and present data, support the concept of a high mitotic activity of NCC *in vivo* and *in vitro*. Furthermore, it is known that the morphogenetic distribution of NCC may be modulated by extrinsic

Figure 2. Labelling index (LI) of neural crest cell cultures treated with 0 (control), 0.1, 1 or 10 μM of RA. Data are expressed as mean values of 1500 cells/explant from triplicate experiments. Mann-Whitney's non-parametric test was performed. a v. b, NS; c v. d, NS; a v. c, $P < 0.04$; a v. d, $P < 0.04$; b v. c, NS; b v. d, NS. NS=not significant.

factors involving microenvironmental signals (Rovasio *et al.* 1983, Rovasio & Thiery 1987). An important developmental active molecule from the embryonic milieu is RA (Thaller & Eichele 1987), which can control the expression of homeobox-containing genes along the body axis (Chen *et al.* 1994, López *et al.* 1995). However, how this control is exerted and what part it plays in normal development are as yet open questions. Likewise, it is known that exogenous RA and its receptor(s) participate in epigenetic malformations related to NCC development (Pratt *et al.* 1987, Moro-Balbás *et al.* 1993, Lohnes *et al.* 1994, Gale *et al.* 1996), although the mechanism(s) involved has (have) not yet been ascertained.

In this study, we utilized the developmental active form of all-*trans*-RA (Creech Kraft 1992), to look for its direct effect on the chick embryo and NCC cultures. We have shown a low proliferation of NCC and neural tube cells, and a generalized diminution of DNA synthesis *in vivo*, as well as a decrement of the proliferation of NCC *in vitro* after treatment with exogenous RA. Additionally, the homogeneous proliferative behaviour of NCC and neural tube cells along the cephalic segments during the early development of the chick embryo was altered by exogenous RA at a higher dose than that of the presumptive endogenous gradient of RA (Chen *et al.* 1994, Thaller & Eichele 1987). Hence, our present results may be related to recent experimental data about morphogenetic polarization of the vertebrate embryos, which may be modulated by an interaction between the expression of homeobox-containing genes and a cephalo-caudal concentration gradient of RA (Chen *et al.* 1994, López *et al.* 1995).

The striking fall of DNA synthesis in early chick embryo tissues and the decrease of [³H]dT incorporation by *in vivo* and *in vitro* early migratory NCC seen in this study after a brief pulse of exogenous all-*trans* RA, are in disagreement with the results reported by other investigators, such as Pratt *et al.* (1987). However, the absence of any effect of RA on DNA synthesis and NCC proliferation reported in that paper was obtained on purely morphological and qualitative bases, inasmuch as counts of the labelled nuclei or other specific estimation of [³H]dT incorporation were not performed. On the other hand, while Henion & Weston (1994) reported that RA selectively increases the neurogenic lineage of NCC, other authors showed that the total number of trunk avian NCC *in vitro* was noticeably reduced after treatment with 10 µM RA (Dupin & Le Douarin 1995). Furthermore, Rockwood & Maxwell (1996) reported no effects on avian NCC proliferation of all-*trans* retinoic acid treatment at lower doses than those used in our work, while a significant drop in the total number of NCC at RA doses of 10⁻⁸ M was observed. In these last two papers, it was proposed that the fall in the number of NCC could be due to a toxic effect; unfortunately, the authors did not describe nor showed clear evidence to sustain their conclusions (Ito & Morita 1995, Rockwood & Maxwell 1996). Abnormal development was observed on early stage embryos treated with 10⁻³ to 10⁻⁵ M of RA pipetted over the blastoderm for 20 h (Modak *et al.* 1993), as well as by the use of localized RA doses of 5 to 100 mg/ml (Gale *et al.* 1996). It might be expected that such a treatment is very hard for the young embryo. On the contrary, the single dose of our short treatment with RA 10 µM was not high enough to cause malformations but only functional changes at cellular level. Thus, under our experimental conditions, we saw no abnormal morphological evidences of toxic changes, either on the whole chick embryo or in NCC cultures. Comparisons between those papers and our work would be only speculative due to the substantial differences that exist between both experimental approaches.

At present, it is not possible for us to interpret the quantitative differences between *in vivo* (cephalic) and *in vitro* (trunk) NCC proliferation. Results from Ito & Morita (1995), and preliminary results from our laboratory suggest a difference in cell behaviour with respect to

several morphometric and dynamic parameters between cephalic and trunk levels of NCC (unpublished data).

The site of action of RA on the cell cycle is apparently dependent on the cell type and on the experimental conditions. In this context, it has been reported that RA prevented murine and human melanoma cell lines — a NCC derived population — from entering into the S phase (Lotan *et al.* 1981), and that a RA-treated CHO cell line remained restrained in the G₁ phase of the cell cycle (Russell & Haddox 1981). In the RA-treated human cell line NHIK-3025, a dose-dependent inhibition of growth in the range of 1 mM–10 μ M with cells accumulated in G₁ was shown (Bakke 1986) and, in other cell models, it was observed that RA blocks the cell cycle at the S or G₂ phases (Marth *et al.* 1985). In our experiments, NCC from RA-treated embryos had a significantly longer generation time with a prolonged G₁ phase as compared to the control embryos. Inhibition of ornithine decarboxylase (ODC), normally increased during the G₁ phase and essential for cell cycle progression, could be one possibility to explain the mechanism(s) by which RA inhibits the proliferation of different cell types, since it has been demonstrated that retinoids inhibit ODC activity and consequently arrest cells in the G₁ phase (Russell & Haddox 1981).

The results of the present work extend previous data supporting the high proliferative rate of NCC (Paglini & Rovasio 1994b). Likewise, we demonstrated that RA has a blocking effect on DNA synthesis in both *in vivo* and *in vitro* early migratory NCC, and that it lengthens their cell cycle by extending the G₁ phase for longer period than in controls. It remains to be elucidated whether the effects of the proliferative blockage presented in this work could bear some key relationship with the complex mechanism(s) involved in the regulatory activity of the intrinsic microenvironmental gradient of RA.

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