Retinoic acid modulation of the early development of the inner ear is associated with the control of c-fos expression

JUAN REPRESA1, ALBERTO SANCHEZ2, CRISTINA MINER2, JOHN LEWIS3 and FERNANDO GIRALDEZ2

1Departamento de Ciencias Morfológicas and 2Departamento de Bioquímica y Biología Molecular y Fisiología, Facultad de Medicina, Universidad de Valladolid, 47005-Valladolid, Spain
3Department of Anatomy & Cell Biology, State University of New York at Brooklyn, New York

Summary

The effects of retinoic acid (RA) on the early development of the inner ear were studied in vitro using isolated chick embryo vesicles. Low concentrations of RA (1–50 nM) inhibited vesicular growth in stage 18 otic vesicles that were made quiescent and then reactivated by either serum or bombesin. Growth inhibition was concentration-dependent and was paralleled by a reduction in the rate of DNA synthesis as measured by \[^{3}H\]thymidine incorporation. Half-inhibition occurred between 1 and 10 nM RA, and the full effect at 20 nM. Retinoic acid, in the presence of serum, induced the precocious differentiation of (1) secretory epithelium, the tegument vasculosum and endolymphatic sac and (2) early sensory and supporting epithelia. These structures were positioned in their corresponding normal presumptive areas. The overall direction of growth was reversed by RA and the ratio of the internal to the external vesicular surface area increased with RA concentration. The expression of the nuclear proto-oncogene c-fos in the developing otic vesicle was transient and stage-dependent. High levels of c-fos mRNA were positively correlated with cell proliferation. Incubation of growth-arrested otic vesicles with bombesin plus insulin at concentrations that induced cell proliferation produced a strong induction of c-fos. This mitogen-induced expression was suppressed by 25 nM RA. The results suggest (1) a role for retinoic acid in controlling the early development of the inner ear and (2) that this control is effected through the regulation of the proto-oncogene c-fos.

Key words: cell proliferation, oncogenes, retinoic acid, otic development, otic vesicle.

Introduction

Retinoic acid exerts a variety of biological effects. The teratogenic consequences of hypervitaminosis A and the administration of retinoids have been known for a long time and include alterations of growth and morphogenesis of the nervous system (Morriss, 1972; Geelen, 1979; Durston et al. 1989). A specific role for retinoic acid during normal development has been supported by experiments on the developing limb where it is thought to specify position across the limb anlage (Tickle et al. 1982; Thaller and Echelle, 1987). In vitro experiments, using cultured cells, have shown that retinoic acid inhibits cell proliferation and induces differentiation in transformed cells from embryonic origin (Jetten, 1986; Sidell et al. 1983). The mechanism by which retinoic acid exerts its actions is being extensively studied and the role of RA receptors as transcriptional factors is beginning to be understood (Petkovich et al. 1987; Evans, 1988). Modulation of gene expression by retinoic acid extends to a variety of genes including homeobox and proto-oncogenes (Adamson, 1987; Astigiano et al. 1989).

The development of the inner ear is an interesting example of organogenesis in the nervous system. At early developmental stages, it involves the formation of a transient structure, the otic vesicle, which undergoes a distinct period of cell proliferation that precedes the differentiation and histogenesis of various sensory, secretory and supporting elements (Van de Water, 1984; Giraldez et al. 1987; Swanson et al. 1990). This process can be reproduced in vitro and it can also be arrested, to then be reactivated by growth factors (Represa et al. 1988; Miner et al. 1988; Represa and Bernd, 1989). We exploit here this preparation to examine the effects of retinoic acid on the early development of the inner ear and its relation to the expression of the nuclear proto-oncogene c-fos. A possible association between RA and c-fos is relevant because the c-fos gene product acts also as a transcrip-
tional modulator whose expression is regulated by growth factors (see Curran, 1988). More generally, the possibility of regulatory roles for proto-oncogenes in normal growth during embryonic development is very attractive (Adamson, 1987).

The results show that physiological concentrations of RA (1–20 nm) produced a strong inhibition of cell proliferation along with a selective induction of cell tissue differentiation. These effects were associated with a rapid inhibition of mitogen-induced c-fos mRNA levels. They suggest a role for RA in controlling the early development of the inner ear and that this control is effected through the regulation of the proto-oncogene c-fos.

Materials and methods

Experiments were done on chick embryo otic vesicles that were isolated by microdissection at stage 18 and cultured according to procedures described previously (Represa et al. 1988; Represa and Bernd, 1989) but with the following modifications. The culture chamber contained 250 μl of antibiotic-free culture medium, vesicles were incubated at 37°C in an atmosphere of 5% CO2 and the standard culture medium consisted of serum-free M-199 medium with Earle’s salts (Flow laboratories).

For morphometry, otic vesicles were processed as previously described (Represa et al. 1986; Giraldez et al. 1987). Briefly, isolated otic vesicles were fixed with Bouin’s solution, dehydrated and embedded in paraffin. Serial frontal sections (8 μm) were then stained with haematoxylin–cosin. Morphometric measurements were made using graphical reconstructions in camera lucida. Some specimens (Fig. 5) were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, dehydrated in a graded series of acetones and propylene oxide and embedded in Epon resin. Frontal or sagittal sections (1 μm) were cut and stained with 1% toluidine blue. Light microscopy autoradiography was performed as described elsewhere (Bernd and Represa, 1989).

DNA synthesis was measured as acid-precipitable [3H]thymidine incorporation. Vesicles were placed in standard incubation medium containing 0.4 μm (10 μCi ml−1) [3H]thymidine for periods of 24 h. Vesicles were then washed in cold Ringer, extracted with 10% trichloroacetic acid and the radioactivity incorporated into acid-precipitable material was counted in Triton X-100–toluene using a scintillation counter.

For Northern Blot Analysis, total RNA was extracted by lysing isolated otic vesicles in 3 μm guanidinium isothiocyanate and centrifugation over a CsCl gradient. RNA was quantitated by measuring OD260 and analysed by electrophoresis in formaldehyde 1.3% agarose gels. After transfer to nylon membranes (Nytran, Schleicher and Schuell), the Northern Blots were hybridized with a random-priming labelled avian c-fos probe (2×10⁶ cts min⁻¹ μg⁻¹) in 5×SSPE, 50% formamide, 0.1% SDS for 20 h at 55°C, washed and autoradiographed. The c-fos probe (Molders et al. 1987) was generously provided by Drs Rolf Muller and Martin Zenke.

Results

Retinoic acid inhibits cell proliferation

The early development of the vertebrate inner ear involves the thickening and invagination of the ectoderm and the formation of the otic vesicle. At developmental stage 18 (Hamburger and Hamilton, 1951), it consists of a fluid-filled cavity lined by a transporting epithelium (Represa et al. 1986; Giraldez et al. 1987). In about 48 h the otocyst goes through a period of intense cell proliferation and evolves to form a more complex structure with signs of growth and morphogenesis. Cell division in the otic vesicle can be arrested in vitro by incubation in serum-free media and then reactivated by growth factors and mitogens (Represa et al. 1988; Miner et al. 1988). Fig. 1 shows a typical experiment where the effects of retinoic acid on this growth-factor-reactivated growth were assayed. The photograph on the left shows an otic vesicle that was isolated at stage 18 and arrested for 24 h (OS). Two other vesicles reactivated to grow with 10% serum (10S) or with 100 nm bombesin plus 5 μg ml⁻¹ insulin (B+I) are shown. They displayed the characteristic signs of growth and were taken as control conditions. The presence of RA in the incubation medium produced a concentration-dependent inhibition of vesicular growth whether induced by serum or bombesin. The numbers in Fig. 1 indicate RA concentrations (nm) and it can be seen that the inhibitory effect was detectable at 1 nm RA and increased up to 50 nm. The incorporation of [3H]thymidine into acid-precipitable material in cultures corresponding to the above experiments are illustrated in Fig. 2. DNA synthesis was reduced in parallel with the inhibition of growth. Inhibition of [3H]thymidine incorporation was already detectable at 1 nm RA and concentration and a maximal effect occurred between 10 and 25 nm RA in vesicles reactivated by bombesin and serum, respectively. Fig. 3 compares autoradiographic sections of otic vesicles incubated with serum (10%) in the absence (Fig. 3A, C) or in the presence (Fig. 3B, D, E) of retinoic acid. An example of an arrested vesicle, incubated in the normal medium but in the absence of additives (Fig. 3F), is shown for comparison. The reduced uptake of labelled thymidine with retinoic acid was associated with the loss of labelling of nuclei in the epithelium. It should be noted that the regional pattern of cell proliferation in the otic vesicle, ventral and medial, was preserved in the presence of RA.

The effect of retinoic acid on cell proliferation was also estimated by measuring the volume of the epithelial tissue of the otic vesicle. This was obtained morphometrically from serial reconstructions of otic vesicles that were incubated in the presence of 10% serum and with increasing concentrations of retinoic acid (Fig. 4). These experiments were done in parallel with those used for DNA incorporation measurements. Cell density values remained fairly constant at different RA concentrations. For example, cell density values (×10⁸ cells cm⁻³) were 95±26 for 10S in the absence of RA, 80±22 in 17 nm RA and 89±8 in 0S (42 fields per vesicle, 2 vesicles per condition). This indicates that the measurements of epithelial tissue volume should faithfully reflect the absolute number of epithelial cells in each condition. For the cell densities given above and the corresponding values of epithelial volume from
Fig. 1. Retinoic Acid (RA) inhibits growth of explanted otic vesicles. Otic vesicles were isolated at stage 18, made quiescent by incubation for 24 h in the absence of serum and then reactivated for another 24 h in the appropriate media. Reactivation was carried out either by the addition of 100 nm bombesin plus 5 μg ml⁻¹ insulin (B+I) (upper row) or 10% foetal calf serum (10S) (lower row), in the presence of RA (nM) at the concentrations indicated by the numbers. Calibration bar=100 μm.
Retinoic acid inhibits mitogen-induced c-fos expression

Finally, we examined the possibility that the effects of RA were related to c-fos expression in the otic vesicle. Fig. 9A shows a Northern Blot analysis of otic vesicles at different developmental stages (indicated by the numbers). Levels of c-fos mRNA were undetectable in stage 12, they were just measurable in stage 18, increased in stage 21 and were again undetectable in stages 22 and 27. Values of optical density are plotted in Fig. 9B along with the cumulative cell number taken
Retinoic acid and c-fos in the otic vesicle

from Giraldez et al. (1987) and normalized to the value in stage 21. Two other experiments gave a similar stage-dependent increase in the mRNA level of c-fos. The expression of c-fos appeared, therefore, transient and correlated to the proliferative phase of the development of the otic vesicle.

Incubation of growth-arrested vesicles with bombesin plus insulin for 20 min at concentrations that induced cell proliferation and vesicular growth, produced a strong induction of c-fos (Fig. 10A, lane B+I). RNA extracted from stage 21 otic vesicles was run in this particular experiment as a positive control (lane 21). In view of the anti-proliferative effect of retinoic acid on bombesin-induced cell proliferation, we examined the effect of RA on c-fos induction. Growth-arrested otic vesicles were incubated in the presence of bombesin plus insulin for 20 min in the presence of RA (25 nM). The result is shown in Fig. 10A (lane B+I+RA) and

Fig. 3. Photomicrographs of 8 μm sections of otic vesicles processed for autoradiography. Quiescent vesicles were cultured for 24 h in 10% FCS in the absence (A and C) and in the presence of 10 nM RA (B, D) and 25 nM RA (E). One vesicle incubated in serum-free is shown in F. Photographs at the bottom are enlargements of the vesicular wall corresponding to the vesicles shown above. Magnification: 75× for A and B, and 920× for the others.
Fig. 4. Retinoic acid effects on epithelial tissue volume. Otic vesicles were cultured in the presence of 10% FCS in the presence of different concentrations of RA. Values of epithelial tissue volume were obtained morphometrically and plotted against the concentration of RA in the incubation medium. Values are mean±s.e. of complete reconstructions of 3 different vesicles per condition.

Fig. 5. Retinoic acid induction of the tegumentum vasculosum. Micrographs of 8 μm frontal sections of otic vesicles processed for conventional light microscopy. (A) Quiescent vesicles were incubated for 24 h with 10% FCS in the presence of 10 nM RA (magn.=150×). An enlargement of the upper part is shown in B (520×). To the right, C (100×) and D (460×) show the appearance of the cochlear duct after 5 days in culture with 10% FCS.

demonstrates that the mitogen-induced increase in c-fos mRNA level was suppressed in the presence of retinoic acid even below the control value (0S). A semiquantitative analysis of these results is given in Fig. 10B, where normalized values of optical density are displayed.

Discussion

The results reported here show that retinoic acid inhibits cell proliferation, induces differentiation and positional changes, and suppresses c-fos induction. A crucial point is that the observed effects of retinoic acid on the otic vesicle are produced at concentrations ranging between 1 and 25 nM. These can be considered as 'physiological' concentrations since (1) these figures are within the range of concentrations measured in chick embryos by HPLC, 10–50 nM, (Thaller and Eichelle, 1987), and (2) they are close to the affinity of RA-receptors for RA, 0.6–30 nM (Giguere et al. 1987; Petkovich et al. 1987). This quantitative correspondence is important because it sets a constraint for any possible role of retinoic acid in normal development.
Evidence has accumulated during recent years that retinoic acid can operate as a morphogen during embryogenesis and that concentration gradients of RA modulate tissue pattern formation in the developing limb (see Summerbell and Maden, 1990 for review). The effects of retinoic acid on the otic vesicle can be taken as a more general extension of this property. Retinoic acid induced in the otic vesicle differentiated tissue organizations such as the *segmentum vasculosum*, early sensory epithelium and endolymphatic sac, which normally appear much later in development. In view of the results presented here, the search for early
Fig. 7. Retinoic acid induction of a differentiated endolymphatic sac exhibiting reversed polarity. The photographs correspond to 8 μm paraffin sections of an otic vesicle cultured for 24 h in the presence of serum (10%) and 10 nM RA. Magnification = 100× and 800×.

molecular markers of differentiation for the specific cell types becomes imperative and current work is in progress in that direction. Our results show that, morphologically, the differentiation of secretory structures can be accelerated by retinoic acid to reach very advanced stages of differentiation in the 24 h incubation period of observation. This effect, although present, was less pronounced in the case of sensory epithelia where fully developed receptors were not detected. The origin of this apparent differential sensitivity to retinoic acid remains unknown. The induction of differentiation had not only the property of being physiological in terms of the appearance of the tissue, but also it was located in the 'correct' presumptive areas of the otic vesicle, i.e. those from which they would originate during normal development (Li et al. 1978). Whether this is related to the regional distribution of RA receptors or to the selective activation of cellular programme is unknown. The effect of RA reversing the normal polarity of the growth of the otic vesicle is perhaps more difficult to interpret, but may well reflect the ability of RA or RA-gradients to organize growth in three dimensions. It has to be considered that, although non-stationary, a gradient of RA was established in the cultures, which was normal to the plane of the epithelium and directed towards the vesicular cavity.

The effects of retinoic acid on the differentiation of vesicular tissue were associated with a strong inhibition of cell proliferation. That cells inhibited to proliferate were those of the vesicular epithelium with little contribution, if any, from the surrounding mesenchymal cells was confirmed by the parallel evolution of epithelial tissue volume and DNA synthesis measurements. Thus, as in normal development, RA-induced differentiation is associated with the interruption of cell division. The precocious appearance of differentiated structures could either be due to a direct activation of specific transcriptional programme by RA or to the induction of such activity as a result of the inhibition of cell proliferation. Differentiation may normally be incompatible with proliferative phases and the transition to differentiative periods could be then controlled by inhibition of cell proliferation by RA. These observations on the effects of RA on the early development of the inner ear, taken together, suggest a
Northern blot analysis of otic vesicles

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2.1 kb

2.2 kb

Stage 12 17 18 21 22 27 c+ c-

Fig. 9. (A) Northern blot analysis of isolated otic vesicles. Total RNA was purified from 50 otic vesicles for each stage and 20 μg of total RNA were loaded per lane. The numbers indicate the developmental stage. Lane labelled c+ was a positive control that consisted of RNA from extraembryonic membranes (Müller et al. 1982) and lane c− was RNA from L929 cells. (B) Densitometric values of different stages (bars) are plotted along with the cumulative increase in cell number (○) taken from Giraldez et al. (1987), against time and the corresponding developmental stage. Values were expressed as a percentage of the stage 21 value.

Physiological role for this molecule in the regulation of the development of the otic vesicle. Retinoic acid could, therefore, represent a putative morphogen in the developing inner ear.

The c-fos proto-oncogene belongs to a group of rapidly induced genes encoding proteins that form complexes regulating transcription (see Herschman, 1989 and Curran, 1988). Several growth factors are known to induce c-fos gene and protein (Müller et al. 1984; Lau and Nathans, 1987; Almendrals et al. 1988). Yet, the relationship between c-fos expression and the processes of cell proliferation and differentiation, as judged from studies on cultured cell lines, is still unclear. Expression of c-fos has been measured in mouse embryonic extracts during days 1 to 10 of prenatal development (Müller et al. 1982) and, using in situ hybridization techniques, c-fos mRNA has been detected in certain areas of the nervous system in late stages of mouse development (Caubet, 1989). However, no functional relation to defined events during embryogenesis has been demonstrated. The results reported here show (1) a transient expression of c-fos throughout the development of the otic vesicle, (2) the rapid induction of c-fos by bombesin and (3) its inhibition by retinoic acid. The first point is of interest because it shows the constitutive expression of c-fos in
the otic vesicle and that it is stage dependent. Moreover, high levels of c-fos expression coincide with the most active period of the proliferative phase in the otic vesicle suggesting that the physiological regulation of c-fos is associated with the control of cell proliferation. This is also supported by the fact that a mitogen like bombesin is able to induce the expression of c-fos and, additionally, by the association between the inhibitory effects of RA and the suppression of c-fos induction. This indicates that c-fos expression may be a critical element for regulation of cell proliferation in the otic vesicle. Moreover, c-fos appears to be a target for retinoic acid. The developmental effects of RA, therefore, might be in part exerted via the regulation of the expression of c-fos.

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References


