EFFECTS OF RETINOIC ACID AND DIMETHYLSULFOXIDE ON THE MORPHOGENESIS OF THE SEA URCHIN EMBRYO

Serafina Sciarrino and Valeria Matranga*

Istituto di Biologia dello Sviluppo, Consiglio Nazionale delle Ricerche
Via Archirafi 20, 90123 Palermo, Italy

*Corresponding author

ABSTRACT

Sea urchin embryos of the species Paracentrotus lividus were treated continuously with different concentrations of all-trans retinoic acid (RA) or dimethylsulfoxide (DMSO) at different developmental stages. A delay in embryonic development was observed when embryos were cultured in the presence of $2x10^{-5}$ M RA, between 1 and 12 hours of development. Hence, at 48 hours of development, while control embryos had reached the pluteus stage, RA-treated embryos were at the prism stage. At 72 hours of development RA-treated embryos recovered and continued normal development reaching the pluteus stage. No effect was observed when treatment was performed before 1 hour or after 12 hours of developmet. DMSO treatment had no effect on normal sea urchin embryo development, although we observed that pigment cells, clearly visible at the pluteus stage, become visible earlier with respect to control embryos. This report confirms the advantages that the sea urchin embryo offers for the study of problems in cellular and developmental biology.

INTRODUCTION

Retinoids, a group of natural or synthetic compounds that are structurally related to Vitamin A, function as signaling molecules for cell growth and differentiation during embryogenesis. The effects of exogenous treatment with retinoic acid (RA) on embryo development have been extensively studied in vertebrates, where RA has been shown to cause several fetal malformations. During gastrulation, RA excess modifies the development of the brain in *Xenopus* (Durston et al., 1989; Papalopulu et al., 1991;), zebrafish (Holder and Hill 1991), mouse (Marshall et al., 1992) and chicken (Sundin and Eichele 1992). RA is also a potent agent in controlling cellular differentiation during embryo development and it is currently used in the differentiation of cell lines as well as in the treatment of many tumors (for a review see Gudas 1994).Although less studied in terms of its basic molecular action mechanism dimethylsulfoxide (DMSO) is commonly used as a differentiation agent and in some cases it has been shown to enhance the expression of mesodermal territories. As an example, P19 embryonal carcinoma cells differentiate into mesoderm-like cells upon DMSO treatment (den Hertog et al., 1993).
The sea urchin embryo constitutes a very useful model system for the study of morphogenesis (Giudice 1986), as it is possible to obtain embryos that develop synchronously, in a short time, to the larval stage of pluteus. Furthermore,

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due to the transparency of the embryos, it is very easy to observe any morphological changes occurring during embryonic development, simply by microscopical inspection. In this work we investigated the effects of all-trans RA and DMSO on normal sea urchin embryo morphogenesis and development.

MATERIAL AND METHODS

Embryo cultures

Paracentrotus lividus eggs were fertilised with a freshly diluted sperm suspension and cultured at 16°C in Millipore-filtered natural sea water, containing penicillin (60 µg/ml) and streptomycin sulphate (50 µg/ml). Embryos were harvested at different developmental stages, their dilution being determined by counting an aliquot, and adjusted to $2x10^4$ embryos per ml. All reagents were from SIGMA Chemical Company unless otherwise specified.

Morphogenetic assay

96-well microtiter plates (PBI N° 11222) were prepared to contain, in a final volume of 100µl, about 200 embryos to be cultured in the presence of different concentrations of all-trans RA (Sigma Chemical Company R-2625, type XX) or DMSO (Merck cat. N° 2931). Retinoic acid dilutions in Millipore filtered sea water were prepared just before the experiment from a 10^{-2} M stock solution [2mg of retinoic acid in 66.6 µl DMSO, 600 µl 96% ethanol (Merck)]. Embryos were added last to microtiter wells, at the developmental stage needed, in a volume of 10 µl, and allowed to develop at 16°C in the dark in order to reduce RA degradation. Development was monitored by optic microscopy at various time intervals. When needed, control and treated embryos were fixed in 10% formalin (Merck) in Millipore filtered sea water and photographs were taken using an IM35 Zeiss inverted microscope.

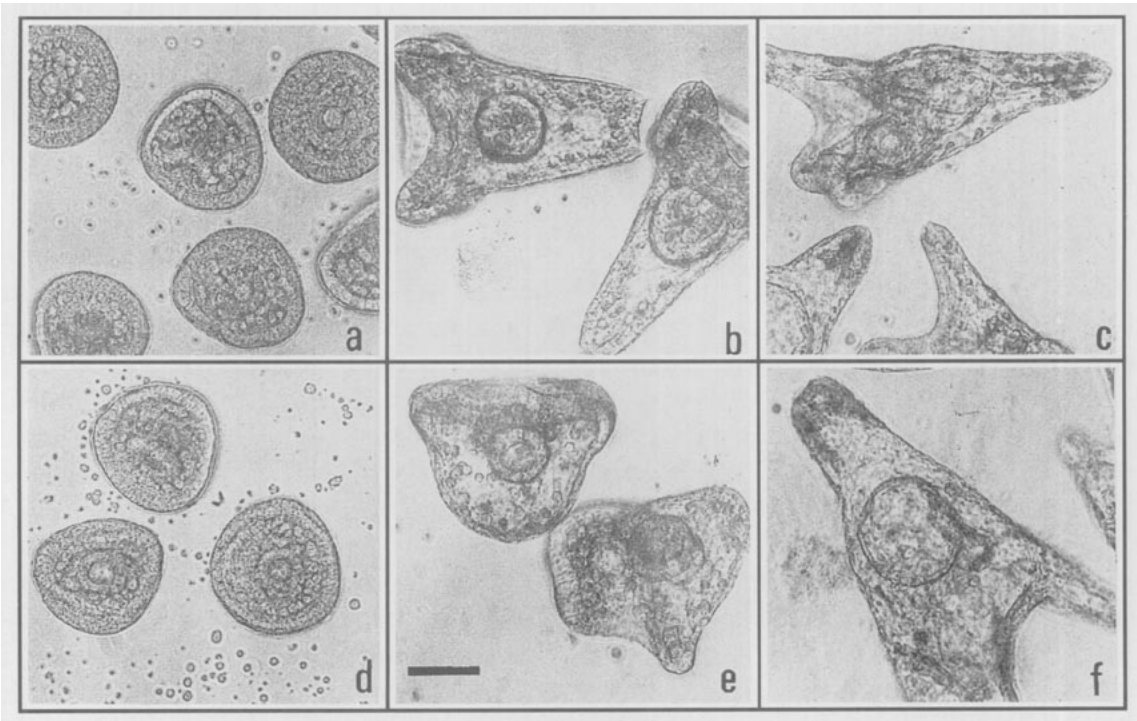
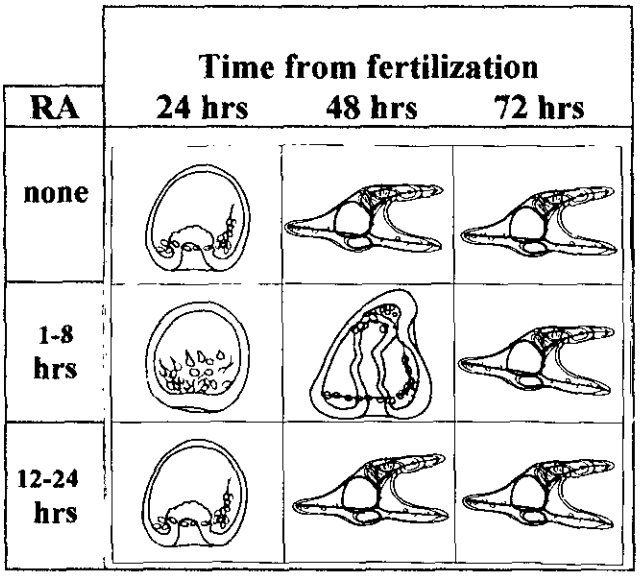


Figure 1. Retardation of embryo development upon RA treatment.
2 x 10^{-5} M RA was added to 8-hour embryos and photographs were taken 24 hours (d), 48 hours (e) and 72 (f) after fertilization. Control embryos (a, b, c) were photographed at the same developmental stages. Bar = 50 µm

RESULTS

To define the concentration range to be used for the treatment we decided to perform a first series of experiments using early blastula embryos and different RA concentrations, comprised between 10^{-4}M and 10^{-6}M. Similar concentrations are commonly used in several other systems. Since the number of embryos subjected to the treatment was also crucial, as already reported (Berg et al., 1990), we always used 200 embryos per well. We observed that high RA concentrations (10^{-4}M) were lethal to the embryos: in fact they reached the morula stage and eventually died. By contrast the micromolar range (10^{-6}M) did not affect sea urchin morphogenesis. Embryonic development was affected for RA concentrations comprised between $0.4x10^{-5}$ M and $4x10^{-5}$M. Figure 1 shows the effects of $2x10^{-5}$M RA on embryos treated continuously from the early blastula stage and observed 24, 48 and 72 hours after fertilization. The development proceeded quite normally for the first hours after treatment and no effect was observed at 24 hours of development (compare Fig. 1a and 1d). At 48 hours after fertilization the effect observed was a delay in embryonic development: while control embryos had reached the pluteus stage (Fig. 1b), RA-treated embryos were at the prism stage (Fig. 1e). At 72 hours after fertilization RA-treated embryos recovered and continued embryonic development, reaching the pluteus stage (compare Fig. 1c and Fig. 1f).

Table 1. Schematic drawing of embryo development upon RA continuous treatment.



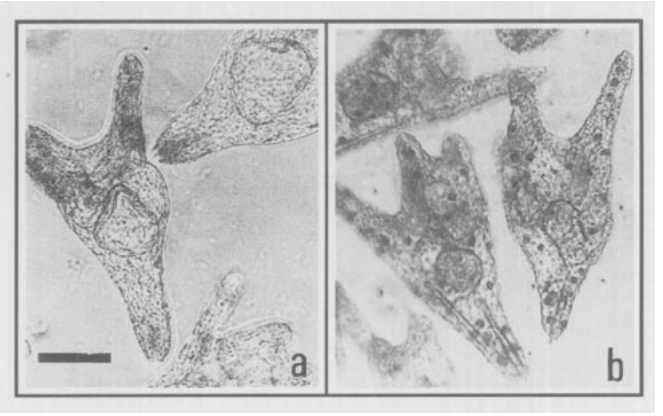


Figure 2. Effects of DMSO treatment on the differentiation of pigmented cells.
a) control, b) .002% DMSO treated embryos. Pictures were taken 48 hours after fertilization. Bar = 50 μm.

It has been reported that not only the dosage but also the time at which RA is administered during embryogenesis is important in order to observe any malformation (Shenefelt 1972). Therefore we tested the effects of $2 x10^{-5}$M RA on embryos at 1, 2, 4, 6, 8, 12, 16 and 24 hours of development. In Table I are schematically summarised the results we obtained. When RA was added between 1 and 8 hours from fertilization a delay in embryonic development was observed at 48 hours of development in 99% of the embryos. At 72 hours of development 100% of RA-treated embryos recovered and reached the pluteus stage. On the contrary, no effect was observed when RA was added before 1 hour or after 12 hours of development.

To analyse the correct expression of the three germ layers on control and RA-treated embryos, we performed indirect immunofluorescence experiments on whole mounts, using McAbs specific for ectoderm, mesoderm and endoderm.

We found a topic expression of the three germ layer markers in RA-treated embryos too (not shown).

To confirm that the effects obtained were not due to the organic solvent used to dissolve RA, namely DMSO, parallel control experiments were performed using the same DMSO dilutions as the ones used to dilute RA, namely .004%, .002% and .0004% DMSO in natural sea water on embryos at 8 hours of development. We found that none of the DMSO concentrations used caused a delay in sea urchin embryo development. In Figure 2 are shown the results of .002% DMSO continuous treatment of embryos, observed at 48 hours of development. What appears evident is that pigment cells, which in control embryos usually become visible by optical microscopy between 68 and 72 hours of development (not shown), become visible earlier in DMSO-treated embryos. This effect was not observed in RA-treated embryos albeit the drug was dissolved in DMSO, possibly because RA, causing an overall delay in development, masked DMSO differentiating action.

DISCUSSION

We examined the effects of continuous treatment with exogenous RA and DMSO on the development of sea urchin embryos. We found that continuous RA treatment of embryos caused a delay in embryonic development, although morphogenesis was recovered after long periods of time. The effect observed was not due to cytoxicity of the drug, since we observed a spontaneous recovery, which indicates the reversibility of the phenomenon. The recovery of embryo development can be explained by the fact that RA is readily metabolised, as already described (Westarp et al., 1993), and therefore its action cannot be exerted any more. Since RA treatment caused a delay in development, its action, possibly exerted through a putative receptor, could involve a block in cell proliferation. This possibility remains to be investigated. It is well known that RA signals through RA specific receptors, whose presence in the sea urchin embryo, to our knowledge, has not been demonstrated so far. However, recent interesting experiments, coming from zebrafish embryogenesis, showed that the expression of different RA receptors can be affected by exogenous retinoic acid treatment of the embryos (Joore et al., 1994).

Another possible effect of RA-induced reversible delay in development could be a delay in production of needed zygotic mRNA at the blastula stage, since it is well known that sea urchin embryos can make it to the blastula stage even if mRNA synthesis is inhibited (Giudice 1986). Still another possibility to explain the delay in development could be the induction of a heat-shock like response that would allow repair and/or adaptation to RA induced damage. On the other hand the synthesis of heat-shock proteins in developing sea urchins in response to stress has been fully documented (Roccheri et al., 1981, Sconzo et al., 1986).

The effects we observed on embryo retardation are the result of exogenous addition of RA during embryonic development. Whether endogenous RA plays a role in normal sea urchin embryo development remains to be investigated. However, it is important to note that the vast majority of experiments that have been reported involve the application of exogenous RA to developing embryos, which causes great changes in the expression of a variety of different genes.

In this report we have also shown that DMSO treatment caused pigment cells to be visible at earlier developmental stages than in control embryos. Pigment cells, or echinophores, are elongated and branched cells, usually dispersed through the ectoderm, although they have been shown to be of mesodermal origin (Gibson and Burke 1985).

In the late fifties Monroy and coworkers showed that in the sea urchin species *Paracentrotus lividus* the total content of the carotenoid pigment increases from the gastrula stage on, being twice as much at the pluteus stage (Monroy et al., 1951). More recently,

Nemer and coworkers (1985) have shown that inhibition of mesodermal derivatives induced by zinc treatment affects the echinochrome pigment content of the embryos. Consequently, our results would suggest that an increase in metabolism of the carotenoid pigments is produced as a consequence of a precocious mesodermal differentiation of pigment cells induced by DMSO treatment. Although we observed an increase in the content of pigment per single pigment cell, we never observed an increase in the number of pigment cells in DMSO-treated embryos, which appeared to be as many as those described for the sea urchin species *Strongylocentrotus purpuratus*, roughly 30 at the pluteus stage (Gibson and Burke 1985).

In conclusion, our results are in agreement with cell lineage studies which have demonstrated the mesodermal nature of pigment cells (Cameron and Davidson 1991) and also confirm the ability of DMSO to induce mesodermic structures to differentiate, as it does in the case of embryonal carcinoma cells (den Hertog et al., 1993).

Taken all together the experiments described here show once again that the sea urchin embryo model system is very helpful in the understanding of developmental biology problems. The finding that the sea urchin embryo is responsive to RA opens the way to the search for putative RA receptors in this system and to studies on the regulation of those genes that are under RA control.

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References

Berg, R. W. and McBurney, M. W. (1990) Cell density and cell cycle effects on retinoic acid-induced embryonal carcinoma cell differentiation. *Develop. Biol.* 138, 123-135.

Cameron, R.A. and Davidson, E. H. (1991) Cell type specification during sea urchin development. *TIG*, 7, 212-218.

den Hertog, J., Pals, C.E.G.M., Peppelenbosch, M.P., Tertoolen, L.G.J., de Laat, S.W. and Kruijer, W. (1993) Receptor protein tyrosine phosphatase α activates $pp60^{c\text{-}src}$ and is involved in neuronal differentiation. *EMBO J.* 12, 3789-3798.

Durston, A. J., Timmermans, J., Hage, W. J., Hendriks, H. F., de Vries, N., Heideveld, M. and Nieuwkoop, P. (1989) Retinoic acid causes an anteroposterior transformation in the developing central nervous system. *Nature* 340, 140-144.

Holder, N. and Hill, J. (1991) Retinoic acid modifies development of the midbrain-hindbrain border and affects cranial ganglion formation in zebrafish embryos. *Development*113: 1159-1170.

Gibson, A. W. and Burke, R. D. (1985) The origin of pigment cells in embryos of the sea urchin *Strongylocentrotus purpuratus*. *Develop. Biol.* 107, 414-419.

Giudice, G. (1986) The sea urchin embryo. Springer-Verlag Ed., Heidelberg.

Gudas, L. J. (1994) Retinoids and vertebrate development. *J. Biol. Chem.* 269, 15399-15402.

Joore, J., van der Lans, G.B.L.J., Lanser, P. H., Vervaart, J.M.A., Zivkovic, D., Speksnijder, J. E. and Kruijer, W. (1994) Effects of retinoic acid on the expression of retinoic acid receptors during zebrafish embryogenesis. *Mech. Develop.* 46, 137-150.

Marshall, H., Nonchev, S., Sham, M. H., Muchamore, I., Lumsden, A. and Krumlauf, R. (1992) Retinoic acid alters hindbrain hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. *Nature* 360, 737-741.

Monroy, A., Oddo, A. M., and De Nicola, M. (1951) The carotenoid pigments during early development of the egg of the sea urchin

Paracentrotus lividus Exp. Cell Res. 2, 700-702.

Nemer, M., Wilkinson D. G. and Travaglini E. C. (1985) Primary differentiation and Ectoderm-specific gene expression in the animalized sea urchin embryo. *Develop. Biol.* 109, 418-427.

Papalopulu, N., Clarke, J. D., Bradley L., Wilkinson D. and Krumlauf R., Holder N. (1991) Retinoic acid causes abnormal development and segmental patterning of the anterior hindbrain in Xenopus embryos. *Development* 113, 1145-1148.

Roccheri, M. C., Di Bernardo, M. G. and Giudice, G. (1981) Synthesis of heat-shock proteins in developing sea urchins. *Develop. Biol.* 83, 173-177.

Sconzo, G., Roccheri, M. C., La Rosa, M., Oliva, D., Abrignani, A. and Giudice (1986) Acquisition of thermotollerance in sea urchin embryos correlates with the synthesis and age of heat shock proteins. *Cell Differ.* 19, 173-177.

Shenefelt, R. (1972) Morphogenesis of. malformations in hamsters caused by retinoic acid: relation to dose and stage treatment. *Teratology* 5, 103-118.

Sundin, O. and Eichele, G. (1992) An early marker of axial pattern in the chick embryo and its respecification by retinoic acid. *Development* 114: 841-852.

Westarp, M.E., Westarp, M. P., Bruynseels, J., Bollag, W. and Kornhuber, H. H. (1993) Oral liarozole as a catabolic inhibitor potently increases retinoic acid in vivo: first experience from an ongoing therapeutic trial in highly malignant primary brain tumors. *Onkologie* 16: 22-25.

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