Short Contribution

Correlation between RA-induced apoptosis and patterning defects in regenerating fins and limbs

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ABSTRACT We have compared the ability of RA to induce apoptosis in regenerating fins and limbs in order to establish whether there may be a possible causal relationship between RA-induced cell death and the different patterning abnormalities observed in these two systems following RA treatment. In regenerating fins RA affects the anteroposterior axis and induces narrowing of the fin and fusion of rays. In regenerating limbs however, it mainly affects the proximodistal axis, where dose-dependent duplications of segments are observed. We report here that RA increases cell death by apoptosis both in fin and limb regenerates independently from the route of administration, but in distinct cell populations. In regenerating fins, RA-induced apoptosis is observed only in the wound epidermis, whereas in regenerating limbs significant apoptosis is observed mainly in the blastema mesenchyme. Altogether, these findings support the view that RA-induced apoptosis is causally related to the patterning defects observed in fins and limbs.

KEY WORDS: *cell death, fin, limb, regeneration, zebrafish, newt, retinoids*

Retinoids, which include vitamin A and its metabolites, induce dysmorphogenesis in all vertebrate embryos and in regenerating appendages of adult urodele amphibians and zebrafish when administered at certain developmental stages (Stocum and Crawford, 1987; Stocum, 1991; Morriss-Kay, 1993; Géraudie *et al.*, 1994, 1995; Conlon, 1995; Means and Gudas, 1995). Retinoic acid (RA), one of the vitamin A metabolites present in the regenerating limb tissues (Scadding and Maden, 1994), affects patterning of both limbs and fins in a dose-dependent fashion, but the resulting defects in these two types of appendages are quite different. In zebrafish, RA induces narrowing of the fin and fusion of regenerating fin rays, but it does not affect the length of the regenerate, and the regenerated fin is never longer than the control one (Géraudie *et al.*, 1994, 1995). In contrast, in urodele amphibians such as the newt, the teratogenic effects induced by RA are rather different. The most striking and easily observed effect is the formation of segments proximal to the amputation plane (proximalization effect) resulting in serial duplication of skeletal elements and muscles at the level of amputation and regenerated limbs longer than controls (Thoms and Stocum, 1984; Stocum and Crawford, 1987). The effects of retinoids on the other two limb axes are only revealed when a half of the stump is surgically removed either along the anteroposterior or dorsoventral axis or a double half limb is created before retinoid treatment (review in Stocum, 1991). RA-treated anterior and dorsal half limbs not only regenerate the complementary half limb, but exhibit pattern duplication in the proximodistal axis. In contrast, RA-treated posterior and ventral half limbs do not regenerate (Stocum and Thoms, 1984; Kim and Stocum, 1986a,b). These results have been taken to indicate that retinoids can indeed affect all of the limb axes.

Analysis of RA-treated fins at the cellular level has shown that RA induces cell death by apoptosis in the epidermal compartment of the regenerating fin, but not in the epidermis of the stump (Ferretti and Géraudie, 1995). The wound epidermis covering regenerating appendages is believed to play a similar role to that of the apical epidermal ridge during limb development, and it has been shown that the width of the ridge affects the underlying mesenchyme and correlates with the number of digits formed (Lee and Tickle, 1985). These findings, together with our observation that RA increases cell death in the wound epidermis and induces narrowing of regenerating fins, led us to propose a causal relationship between RA-induced apoptosis and teratogenesis. If this were the case, the prediction is that RA will not induce an increase in apoptosis in the wound epidermis of regenerating limbs, since no significant patterning defect is observed along the anteroposterior axis of RA-treated limb regenerates. In order to test this hypothesis we compared the occurrence of apoptosis in fins and limbs treated with teratogenic doses of RA, and checked whether there was any correlation between these effects and the

Abbreviations used in this paper: RA, retinoic acid; TUNEL, Tdt-mediated dUTP-biotin Nick End Labeling; DMSO, dimethylsulfoxide.

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TABLE 1

EFFECT OF INTRAPERITONEAL INJECTION OF RA ON REGENERATING FINS

Dose µg/g body weight	Survival 5 days post injection	Effect ray fusion
100	lethal	n.a.
10	25% survival	none
5	75% survival	none

administration route used. In fact, in adult newts injection of RA, but not treatment by immersion, is effective in inducing duplications, whereas in all of the experiments examining the effects of RA on regenerating fins, the treatment was carried out by immersion.

In order to establish whether injection of RA induced apoptosis in the wound epidermis of the regenerating fin as described in animals treated with RA by immersion (Ferretti and Géraudie, 1995), we injected zebrafish intraperitoneally with different doses of RA, and assessed their effects both on cell death and skeletal patterning. The injected RA precipitates in the peritoneal cavity and is then slowly released into the bloodstream (Stocum and Maden, 1990). We started from a 100 µg/g body weight dose of RA because doses between 100 and 150 µg/g produce maximal proximodistal duplications in regenerating axolotl limbs whereas a 20 µg/g dose has minimal effects on axis duplication of regenerating axolotl double half limbs.

We found that injection of 100 µg/g body weight dose of RA into zebrafish was lethal within 24 h after injection, well before any effect on patterning could be assessed. After injecting 10 and 5 µg/g RA, 25% and 75% of the treated animals, respectively, were still alive 5 days after injection (Table 1), but no obvious fin abnormality was observed. It should be pointed out that at the early stages of regeneration (5-7 days) small patterning defects may be difficult to detect. Nonetheless, of the animals which survived till completion of the regenerative process (40%) none displayed any significant skeletal abnormality. Therefore, it appears that sublethal doses of RA are not teratogenic. This indicates that such doses do not allow accumulation of RA in the regenerating fins of injected animals at sufficient levels and/or for a sufficient length of time to produce patterning defects. This is supported by previous time-course and dose response studies on the effects of RA treatment by immersion, which showed that both concentration and length of exposure to RA are proportional to the teratogenic effects induced (Géraudie *et al.*, 1994, 1995). Therefore, under certain experimental conditions not only the effect of RA on growth retardation, but also that on patterning, is reversible.

When analysis of cell death was performed 24 h after RA injection, a significant increase in cell death in the wound epidermis similar to that induced after treatment by immersion for 24 h was observed (Fig. 1A-C). This is consistent with previous work showing that immersion for only 24 h is sufficient to induce cell death, although a longer treatment, which results in sustained cell death, is required to induce patterning defects (Géraudie *et al.*, 1994, 1995; Ferretti and Géraudie, 1995). Therefore, these experiments clearly demonstrate that RA induces cell death in the wound epidermis, but not in the mesenchyme, independently from the administration route.

In contrast to the pattern of cell death observed in the zebrafish fin, numerous scattered apoptotic figures are observed both in the blastema mesenchyme, and in the dedifferentiating distal part of the stump in newts injected with a dose of RA which induces proximodistal duplications (Fig. 1C-D). Only a few apoptotic cells are observed in the epidermis, and are mainly located in the outer layers where apoptosis normally occurs as a physiological process controlling the epidermis homeostasis. Although increased cell death in the stump of RA-treated animals has been previously reported by Thoms and Stocum (1984), this is the first demonstration that RA-induced cell death in regenerating limbs occurs through an apoptotic mechanism.

As summarized in Table 2, the effects of RA on different cellular compartments appear to correlate with the different patterning abnormalities in regenerating fins and limbs. In fact, not only in the regenerating fin, but also in developing mammalian limb buds where RA affects the pattern along the anteroposterior axis, increased cell death is observed in the epidermal compartment (Sulik and Dehart, 1988). In contrast, in the regenerating limb where RA's most striking effect is on the proximodistal axis, the significant change in cell death is not observed in the wound epidermis, but in the mesenchyme. The significance of this massive apoptosis in the mesenchyme of RA-treated limb blastemas and its possible relevance to the duplication effects induced is presently not clear. It would be tempting to speculate that the proximalization effect induced by RA is due to the fact that increased cell death in the stump mesenchyme leads to recruitment of more proximal cells than in control animals. An experiment by Thoms and Stocum (1984) where RA treatment appeared to induce proximalization of distal limb blastemas inserted in the orbit, suggested the occurrence of re-specification of blastemal cells rather than recruitment of more proximal limb cells. Indeed, the regenerated limb forms from dedifferentiated and cycling blastemal cells whose exposure to RA can result in various effects going from growth arrest, to respecification of the three limb axes, to death by apoptosis. Stocum and his colleagues (Ludolph *et al.*, 1990) proposed that RA not only induces proximalization but also posteriorization and ventralization of cells located respectively in the anterior and dorsal quadrant, suggesting the existence of subpopulations of blastemal cells with different sensitivity to retinoids in respect to axis specification. Therefore, it is conceivable that the RA-induced apoptosis we have observed in regenerating limbs specifically affects a subpopulation of blastemal cells, and that its disappearance can trigger environmental changes resulting in re-sorting and re-specification of the remaining blastemal cell populations.

TABLE 2

SUMMARY OF EFFECTS OF DIFFERENT ROUTES OF ADMINISTRATION OF RA ON PATTERNING AND CELL DEATH IN REGENERATING ZEBRAFISH FINS AND NEWT LIMBS

	Immersion		Injection	
	pattern defect	*cell death*	*pattern defect*	*cell death*
zebrafish fin	ray fusion	WE	none?	WE
newt limb	none	nd	proximodistal duplication	blastema mesenchyme

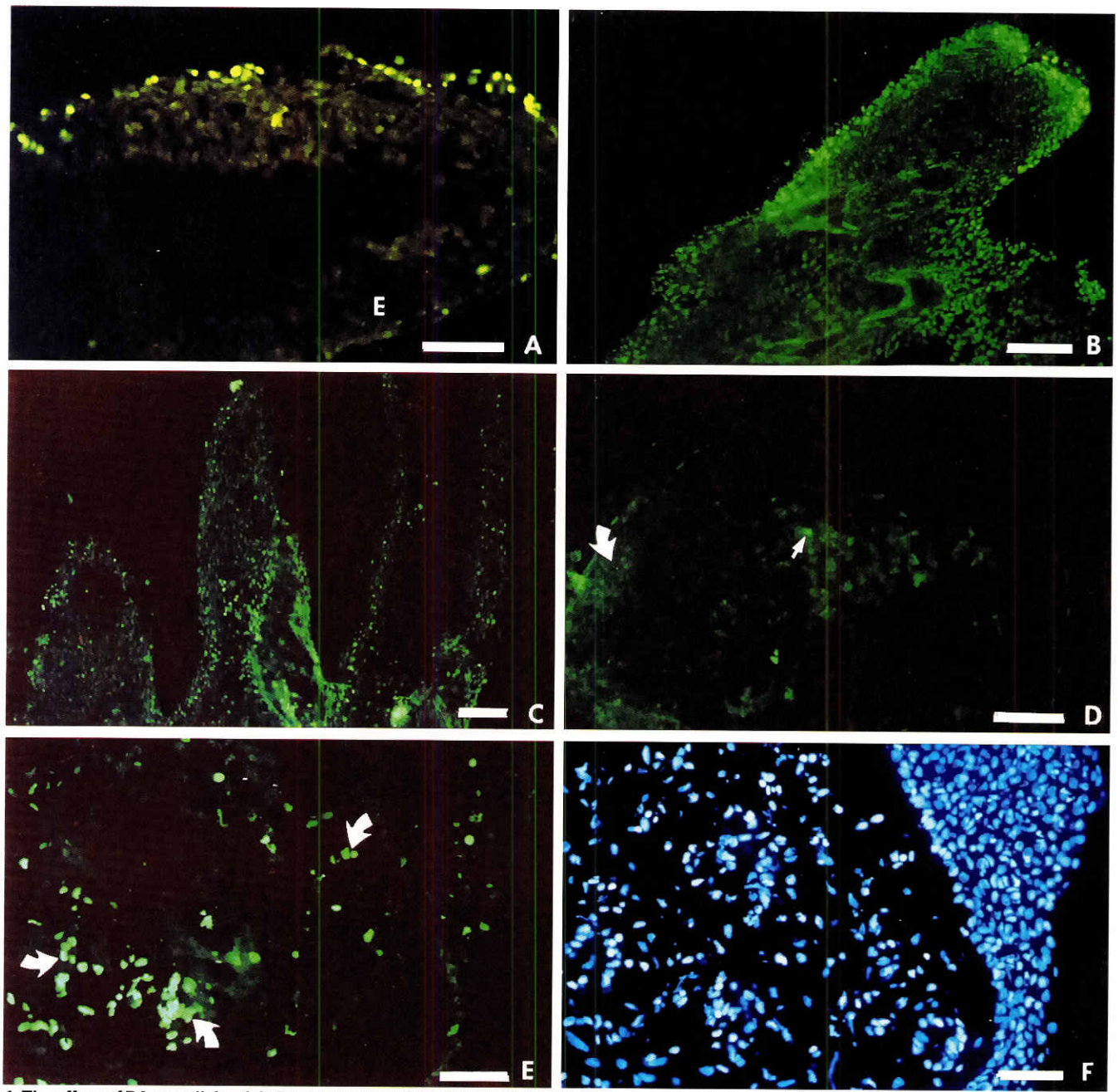

Fig. 1. The effect of RA on cell death in RA-treated zebrafish fins and newt limbs was evaluated by the TUNEL technique on longitudinal sections of regenerating fins (A-C) and limbs (D-F). (A) *5-day control fin regenerate. Note that apoptotic cells, identified by their green fluorescent nuclei, are present only in the outer epidermal layer and are part of the normal shedding of the epidermis (E, epidermis). Bar, 75 μm.* **(B)** *5-day fin regenerate treated with 10^{-6} M RA by immersion for 24 h starting 4 days after amputation. Note that apoptotic cells are not confined to the outer epidermal layer, but are scattered throughout the thickness of the epidermis. Bar, 300 μm.* **(C)** *5-day fin regenerate treated by injection of 4 μl of 10^{-6}M RA 4 days after amputation. Same result and same magnification as in* **B**. **(D)** *8-day control newt limb regenerate injected intraperitoneally with 10 μl of DMSO 4 days after amputation. Note that some apoptotic cells are present in the epidermis, particularly at the stump/blastema junction (curved arrow), but apoptotic figures in the mesenchyme are very rare (arrow). Bar, 200 μm.* **(E)** *Newt limb regenerate injected intraperitoneally with a dose of RA which induces proximodistal duplications (10 μl of 30 mg/ml RA) 6 days after amputation and harvested 48 h later. Note the high number of apoptotic cells present in the mesenchyme (curved arrows). The same effect was observed when blastemas were analyzed 24 h after RA injection (not shown). Bar, 100 μm.* **(F)** *Corresponding nuclear staining with DAPI. Same magnification as in* **E**.

In order to clarify whether the limb defects induced by RA could be due to recruitment of blastemal cells at more proximal levels, to re-sorting of surviving blastemal cells, or to a combination of the two, it will be necessary to carefully investigate the contribution to the blastema of cells from different stump levels and their behavior in normal and RA-treated animals. Surprisingly, a thorough analysis of the origin of blastema cells in RA-treated animals which could help to clarify this important issue has yet to be carried out.

Experimental Procedures

Animals

Amputations of limbs of adult newts *Notophthalmus viridescens*, and either pectoral or caudal fins of adult zebrafish *Danio rerio* were performed in animals anesthetized in buffered 0.1% MS 222 (Sigma). Zebrafish fins were amputated proximal to the first branching fork of the rays and the fish maintained at 26°-28°C (Géraudie *et al.*, 1994, 1995). Newt forelimbs were amputated at the mid-humerus level, and after recovery in 0.5% sulfamerazine (Sigma), the regenerating newts were maintained at 25°C.

RA treatment

RA treatment was performed either by immersion in 10^{-6} M RA for 24 h 4 days after fin amputation as previously described (Géraudie *et al.*, 1994, 1995), or by intraperitoneal injection, and at least 5 animals per group were used. Four microliters of 100, 10 or 5 μg/g body weight of all-*trans* RA (Sigma) dissolved in DMSO (Sigma) were injected 4 days after fin amputation, and the regenerates either analyzed 24 h later for their content of apoptotic cells or left to regenerate fully in order to examine the patterning of the regenerated skeleton by Alcian blue and Alizarin red S staining (Géraudie *et al.*, 1994, 1995). Control animals were injected with DMSO. Newts were injected intraperitoneally with 8 μl of RA (30 mg/ml which is approximately equivalent to 125 μg/g body weight dissolved in DMSO) 6 days after amputation and the blastemas analyzed either 24 or 48 h later. Control animals were injected with DMSO. RA treatment of regenerating newts by immersion was not performed, since this administration route is never effective in inducing limb abnormalities in adult newts, probably because of the low levels of RA which can accumulate in the blastema under these conditions.

Identification of apoptotic cells

TUNEL technique (Tdt-mediated dUTP-biotin Nick End Labeling) was used to identify apoptotic cells in cryostat sections fixed with 1% paraformaldehyde as previously described (Gavrieli *et al.*, 1992). In brief, sections were incubated with a nucleotide mix containing biotinylated-dUTP and TdT (Boehringer Mannheim). The incorporated biotinylated-dUTP was detected by FITC-conjugated extravidine (Sigma). Nuclei were counterstained with DAPI.

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