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RA regulation of keratin expression and myogenesis suggests different ways of regenerating muscle in adult amphibian limbs

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SUMMARY

Formation of a regeneration blastema following limb amputation is believed to occur through a process of dedifferentiation. It has been suggested, however, that the cells contributed to the blastema by the stump muscle are satellite-like cells, rather than cells originated by dedifferentiation. We have previously shown that simple epithelial keratins 8 and 18 are expressed in the mesenchymal progenitor cells of the regenerating amphibian limb and in cultured cells with myogenic potential, and that their expression appears to be causally related to changes in proliferation and differentiation. We show here that retinoic acid (RA) affects the expression of these keratins differently in myogenic cells originated from normal limb and limb blastema. Furthermore, we find that the effects of RA on proliferation, myogenic differentiation and adhesion of these cells also differ. In fact, whereas RA does not affect keratin expression, proliferation or myogenic differentiation in blastemal cells, it does decrease keratin levels and thymidine incorporation and increase myogenesis in cells from normal limb. Conversely, RA increases cell adhesion only in blastemal cells. Significantly, these effects of RA on cultured cells are consistent with those observed in vivo. Overall the results presented here suggest that in the urodele limb there are two distinct cell populations with myogenic potential, one originating from dedifferentiation and one equivalent to the satellite cells of the mammalian muscle, which are likely to be primarily involved in blastema formation and muscle repair, respectively.

Key words: Blastema, Dedifferentiation, Keratin, Limb, Muscle, Proliferation, Regeneration, Retinoic acid, Satellite cell, Urodele amphibian

INTRODUCTION

Adult urodele amphibians can regenerate their limbs because of their ability to form a blastema in response to amputation (Thornton, 1968; Wallace, 1981). The blastema is a mound of mesenchymal progenitors cells, the blastemal cells, which is covered by a specialized wound epidermis. After a nerve-dependent phase of fast proliferation the blastemal cells start to differentiate into the various limb tissues and undergo morphogenesis. The cellular and molecular basis of blastema formation, growth and differentiation have been tackled over the last few years, but many of the mechanisms underlying these events have yet to be elucidated (Brockes, 1997; Géraudie and Ferretti, 1998). One crucial issue concerns the origin of blastemal cells and the hypothesis of dedifferentiation of mature cells of the stump versus the existence in the newt limb of stem cells (Fig. 1A). The dedifferentiation hypothesis based on morphological evidence of cells pinching off from the injured muscle (Hay, 1959) has been recently corroborated, at least in the muscle, by elegant molecular studies (Lo et al., 1993; Tanaka et al., 1997). However, the existence of myogenic reserve cells in the urodele muscle, which would participate in blastema formation without invoking a process of dedifferentiation, has also been proposed (Cameron et al., 1986; Schrag and Cameron, 1983).

A few years ago we established newt cultures from normal limbs (TH4B) and regeneration blastemas (BlH1), which display very interesting properties in that they can differentiate into muscle but appear to be self-renewing and do not become senescent (Ferretti and Brockes, 1988; Powell et al., 1998). Furthermore, they express markers characteristic of blastemal cells in vivo and it has been suggested that expression of these markers in cultures from normal limb is induced in response to the injury caused by cutting the tissue and/or enzymatic dissociation (Ferretti and Brockes, 1988; Ferretti et al., 1989). Among these markers are the simple epithelial keratins 8 and 18 (named in the newt NvK8 and NvK18), which although normally considered markers of epithelial differentiation, are expressed in the mesenchymal progenitors of the regenerating limb. We have recently shown that downregulation of NvK8 and NvK18 in vitro by anti-sense oligonucleotides results in a decrease in cell proliferation and an increase in myogenic differentiation (Corcoran and Ferretti, 1997; Ferretti et al., 1989). Therefore, NvK8 and NvK18 expression appears to be causally related to changes in proliferation and differentiation.

It has been shown that the expression of many keratins,

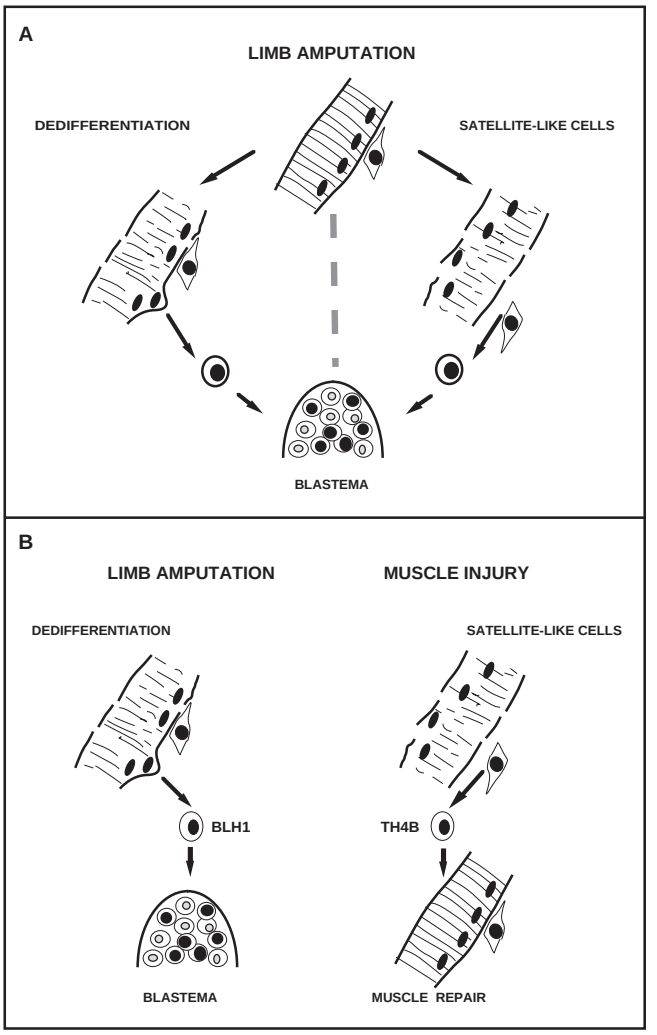

Fig. 1. (A) The two opposing views on the contribution of cells to the blastema from muscle through a process of dedifferentiaton (left hand side) or from putative satellite-like cells (right hand side). (B) A model consistent both with the occurrence of dedifferentiation and the existence of reserve cells, suggesting that different cell populations are recruited in response to different types of damage.

including K8 and K18, can be regulated by retinoic acid (RA) (Jing et al., 1996; Kim et al., 1987). RA can affect many fundamental biological processes such as growth and differentiation, and it has been shown that cultured myoblasts can be induced to differentiate by retinoid treatment (Alric et al., 1998). Furthermore, in the regenerating newt limb RA induces formation of extra segments in a dose-dependent manner, and is supposed to do so by changing the positional memory of blastemal cells. We therefore decided to examine how RA affected keratin expression, proliferation and differentiation of blastemal cells in vivo and in vitro in order to establish whether there is a causal relationship between changes in the expression of these molecules and the cellular and morphogenetic effects induced by RA.

Whereas we did not find any relationship between RA effects on keratin expression in the blastema and morphogenetic effects, some interesting correlation between RA, keratin and myogenesis in different populations of limb myogenic cells emerged from this study. In fact, RA downregulates NvK8 and NvK18 in cells derived from normal limb but not in blastemal cells either in vivo or in vitro. Furthermore, it affects the behaviour of the myogenic cells TH4B and BlH1, originating from normal limb and blastema, respectively, in a different fashion. This demonstrates that these myogenic cells, unlike what was previously believed, are significantly different, and this is consistent with their different origin. The relevance of these findings to our understanding of muscle regeneration will be discussed.

MATERIALS AND METHODS

Animals and surgery

Adult *Notophthalmus viridescens* supplied by Nasco (Wisconsin, USA), were maintained at 20°C and fed shredded bovine heart on alternate days. All surgical procedures were performed on newts anaesthetized in 0.1% tricaine (3-aminobenzoic acid ethylester methanesulphate salt; Sigma, Poole, UK). Following bilateral forelimb amputation at mid-humerus level and recovery in 0.5% sulfamerazine (Sigma, Poole, UK), the regenerating newts were maintained at 25°C.

Retinoic acid (RA) treatment in vivo

Retinoic acid (RA; all-trans retinoic acid, Sigma, Poole, UK) treatment was performed by injecting intraperitoneally 10 μl of an RA-DMSO (dimethyl sulfoxide, Sigma, Poole, UK) solution (30 mg/ml); this dose reproducibly proximilizes the regenerating limb. Control animals were injected with 10 μl DMSO. Newts were injected either with RA or DMSO 7 days after proximal limb amputation. RA- and DMSO-treated blastemas were collected either 7 days or 12 days later. Livers were collected 4 days after injection to assess whether changes in keratin expression occurred at an earlier time after injection.

RNA preparation and analysis

RNA was extracted from newt tissues by the guanidine isothiocyanate procedure as previously described (Brown and Brockes, 1991). All the RNA samples were standardized by OD measurements at 260 nm and RNAse protection with the satellite 2 probe pSP6D6 (Epstein and Gall, 1987). For the analysis of NvK8 and NvK18 expression, ^{35}S-labelled riboprobes were prepared from a 300-bp *Pst*I-*Pst*I fragment, which encodes part of helix 2 of NvK8, and from a 290-bp *Bgl*II-*Eco*RI fragment, which encodes part of the α-helix 1A of NvK18 (Corcoran and Ferretti, 1997). RNAse protection was performed as previously described (Casimir et al., 1988; Ferretti et al., 1991).

Cell cultures

Long-term cultures of limb cells that express the blastemal phenotype were used in this study (Ferretti and Brockes, 1988; Ferretti et al., 1989). These long-term myogenic cultures originated from cells that had migrated from small explants of either normal limbs (TH4B) or limb blastemas (BlH1). Although these cells have not been cloned because of their inability to grow efficiently at clonal density, they appear to be a homogeneous population, as indicated not only by their behaviour and immunoreactivities, but also by analysis of muscle and connective tissue lineage marker hypometilations (Casimir et al., 1988). Cells were grown and passaged essentially as previously described (Ferretti and Brockes, 1988), apart from the fact that 0.1% bovine skin gelatine (Sigma, Poole, UK), instead of collagen, was used to coat the dishes. Usually 750 cells/well were plated in 96-well plates for proliferation assays and 5,000 cells/well were plated in 24-well plates or on 13 mm diameter glass coverslips for immunocytochemistry. In certain experiments the percentage of foetal

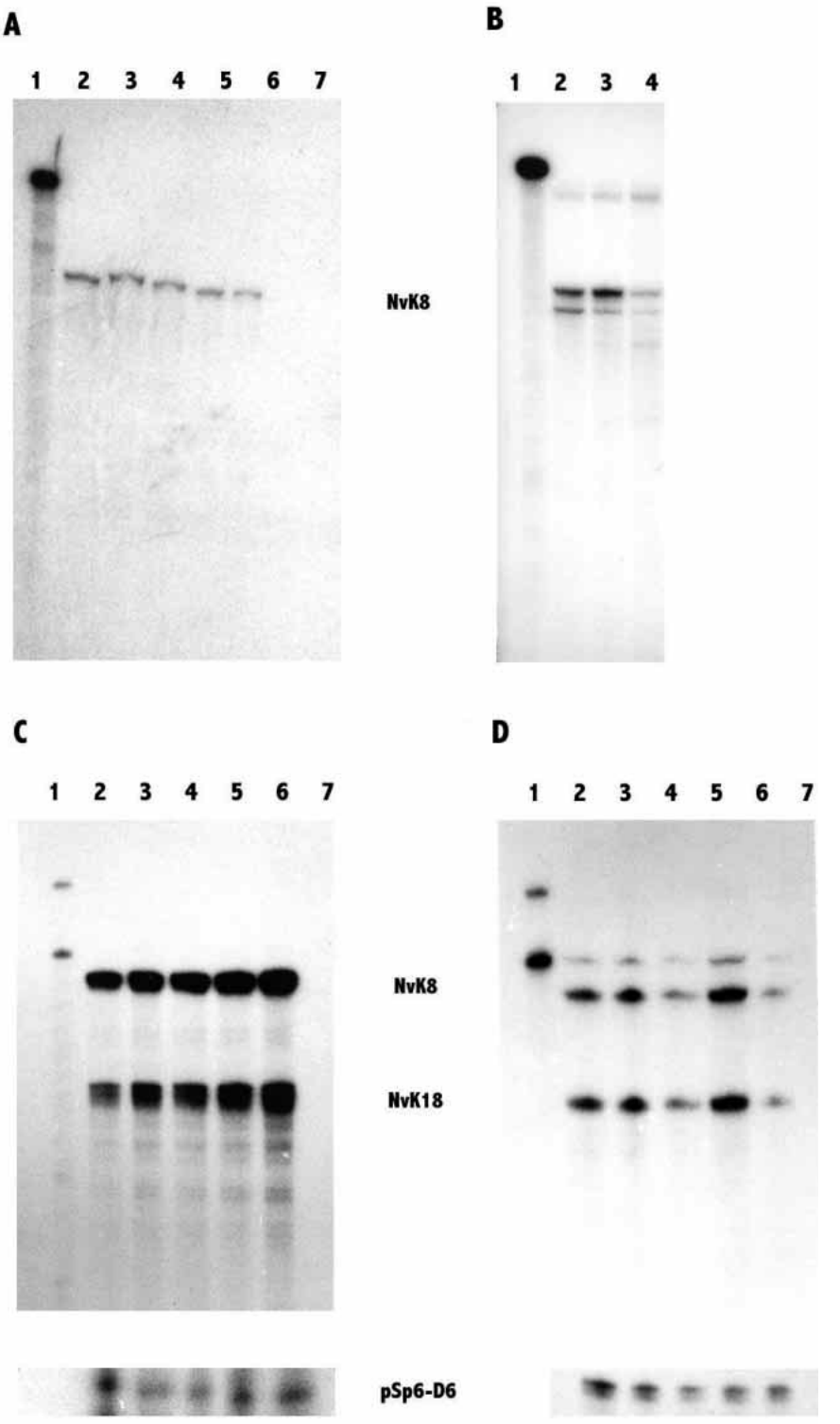

Fig. 2. RNAse protection analysis of the effects of RA on the expression of NvK8 and NvK18 in different tissues in vivo and in vitro. Regenerating animals were injected 7 days after amputation either with RA dissolved in DMSO, or with DMSO alone. 5 μg of total RNA per lane were used. (A) Effects of a proximalizing dose of RA (see Materials and methods) on the expression of NvK8 in the limb blastema. Lanes: 1, NvK8 probe; 2, untreated blastema; 3, blastema 7 days after DMSO injection; 4, blastema 7 days after RA injection; 5, blastema 12 days after DMSO injection; 6, blastema 12 days after RA injection; 7, tRNA. Note that RA does not affect the expression of NvK8 in the regenerating blastema. (B) Effects of the dose of RA used in A on the expression of NvK8 in the liver. Lanes: 1, NvK8 probe; 2, liver from untreated animals; 3, liver 4 days after DMSO injection; 4, liver 4 days after RA injection. Note that RA downregulates NvK8 in the liver. (C) Effects of 10^{-7} M RA and its vehicle DMSO on the expression of NvK8 and NvK18 in cultured cells derived from limb blastema (BlH1). Lanes: 1, NvK8 and NvK18 probes; 2, untreated cultures; 3, 6 hours DMSO treatment; 4, 6 hours RA treatment; 5, 3 days DMSO treatment; 6, 3 days RA treatment; 7, tRNA. (D) Effects of 10^{-7} M RA on the expression of NvK8 and NvK18 in cultured cells derived from normal limb (TH4B). Lanes are as in C. Normalization of the RNA samples with the satellite probe (pSp6-D6) is shown in C and D. Note that NvK8 and NvK18 transcripts are downregulated by RA in the TH4B cells whereas RA does not affect their expression in the BlH1 cells.

calf serum was lowered from 10% to 1% to speed myogenic differentiation and in others serum which had been delipidated using charcoal and dextran to remove retinoids was used (Fuchs and Green, 1981). In certain experiments RA at different concentrations (1×10^{-8}, 1×10^{-7} or 1×10^{-6} M) was added to the medium and its effects studied for different lengths of time as specified in Results. The RA-containing medium was always changed every 3 days. RA treatment was started the day after plating, when the cells had attached and spread onto the substrate. FGF-2 (Gibco, UK) was dissolved in A-PBS (amphibian phosphate buffer saline: 0.1 M phosphate buffer, pH 7.4, 0.12 M NaCl) and used at a final concentration of 10 nM; TPA (12-0-tetradecanoyl phorbol-13-acetate; Sigma, Poole, UK) was dissolved in ethanol and used at a final concentration of 1 μM. FGF-2 and TPA treatment was started after maintaining the cells in serum-free medium for 2 days.

Morphological analysis

The morphology of the cells was monitored throughout the duration

of each experiment and micrographs of live cells in 96-well plates taken using an IM45 inverted microscope (Zeiss, Germany).

Western blotting and immunohistochemistry

The anti-keratin monoclonal antibodies (mAbs) LP1K (Lane et al., 1985) and the muscle marker 12/101 (Kintner and Brockes, 1984) were used to assess the extent of differentiation of the cultured cells under different experimental conditions. For western blotting, detergent-insoluble and detergent-soluble protein fractions were separated on a 10% polyacrylamide gel, blotted onto nitrocellulose using a semi-dry gel transfer apparatus and stained either with India Ink for total proteins or with monoclonal antibodies as previously described (Ferretti et al., 1989).

Immunohistochemistry was essentially performed as previously described (Ferretti et al., 1989). Bound antibodies were detected by a rhodamine-conjugated rabbit anti-mouse-immunoglobulin antibody (Dako, Denmark), and the nuclei stained with 1.25 μg/ml of Hoechst dye 33258 (Sigma, Poole, UK). The results were recorded using an Axiophot (Zeiss, Germany) fluorescence photomicroscope.

Proliferation assay

The effects of RA, FGF-2 and TPA on DNA synthesis in TH4B and BlH1 cells were evaluated by using a thymidine incorporation assay; [^{3}H]thymidine (methyl-1′,2′-[^{3}H]thymidine, 124 Ci/mmol, Amersham, UK) was added to culture medium at 1 mCi/ml 24 hours before collecting the cells onto filters using an automatic cell harvester. The filters were washed, dried and the incorporated radioactivity measured by liquid scintillation counting. Experiments were repeated at least twice, and more usually three times; each experimental group usually consisted of at least five samples. The effect of treatment was evaluated by one-way variance analysis using the statistical software SigmaStat.

RESULTS

Effect of RA on K8 and K18 expression

In order to assess whether RA regulates expression of NvK8 in the regenerating limb, newts were injected 7 days after amputation either with RA or with its vehicle, DMSO. The effects of RA were examined by RNase protection analysis 7 and 12 days after injection, that is at the end of the RA-induced delay in regeneration (Maden, 1983; Thoms and Stocum, 1984) and at the mid-bud stage, respectively. Because of the initial effect of RA on blastema growth, it is difficult to collect clean blastemal tissue at earlier time points. As shown in Fig. 2A, RA does not change the level of expression of NvK8 in the blastema at either of the times analysed.

To rule out the possibility that RA had affected keratin expression at an earlier stage, we examined its effect on the liver, another tissue which expresses high levels of NvK8, 4 days after injection. We found that RA strongly reduces NvK8 mRNA in the liver (Fig. 2B), suggesting that RA might affect keratin expression in the blastema at an earlier time post-

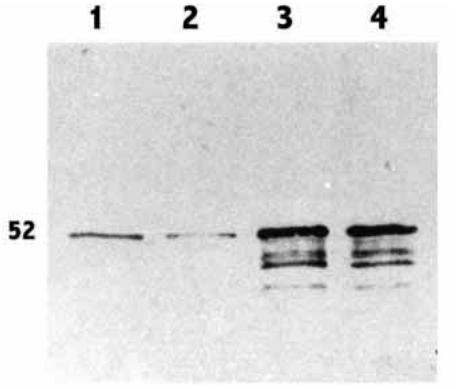

Fig. 3. Analysis of NvK8 protein expression by western blot analysis of cytoskeletal fractions (5 μg protein/lane) from cultured TH4B and BlH1 cells treated with 10^{-7} M RA for 2 weeks. NvK8 was detected using the mAb LP1K. Lanes: 1, TH4B controls; 2, TH4B cultured in 10^{-7} M RA; 3, BlH1 controls; 4, BlH1 cultured in 10^{-7} M RA. Note that NvK8 (52×10^3 M_r) is downregulated by RA in the TH4B but not in the BlH1 cultures.

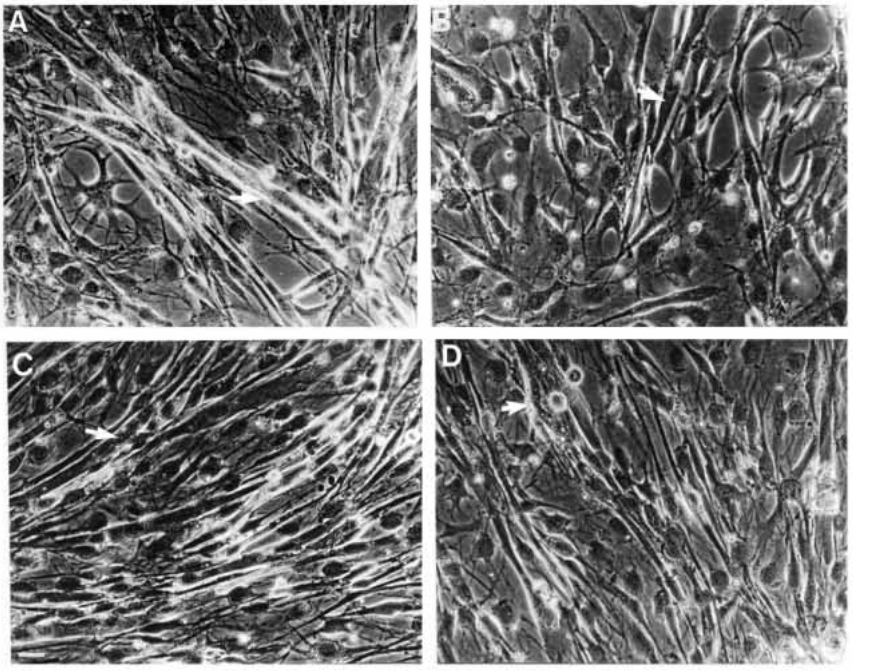

Fig. 4. Effect of cell density on myogenesis of TH4B and BlH1 cells. Phase contrast micrographs of representative cultures of TH4B (A,C) and BlH1 (B,D) at different cell densities. In low density cultures, myotube formation is evident in TH4B (A) but rare in BlH1 (B). In dense cultures a comparable degree of myogenesis is observed in TH4B (C) and BlH1 (D), but the myotubes formed in the BlH1 cultures are less well aligned and tend to be broader than those present in TH4B. Some myotubes are indicated by white arrows. Bar, 50 μm.

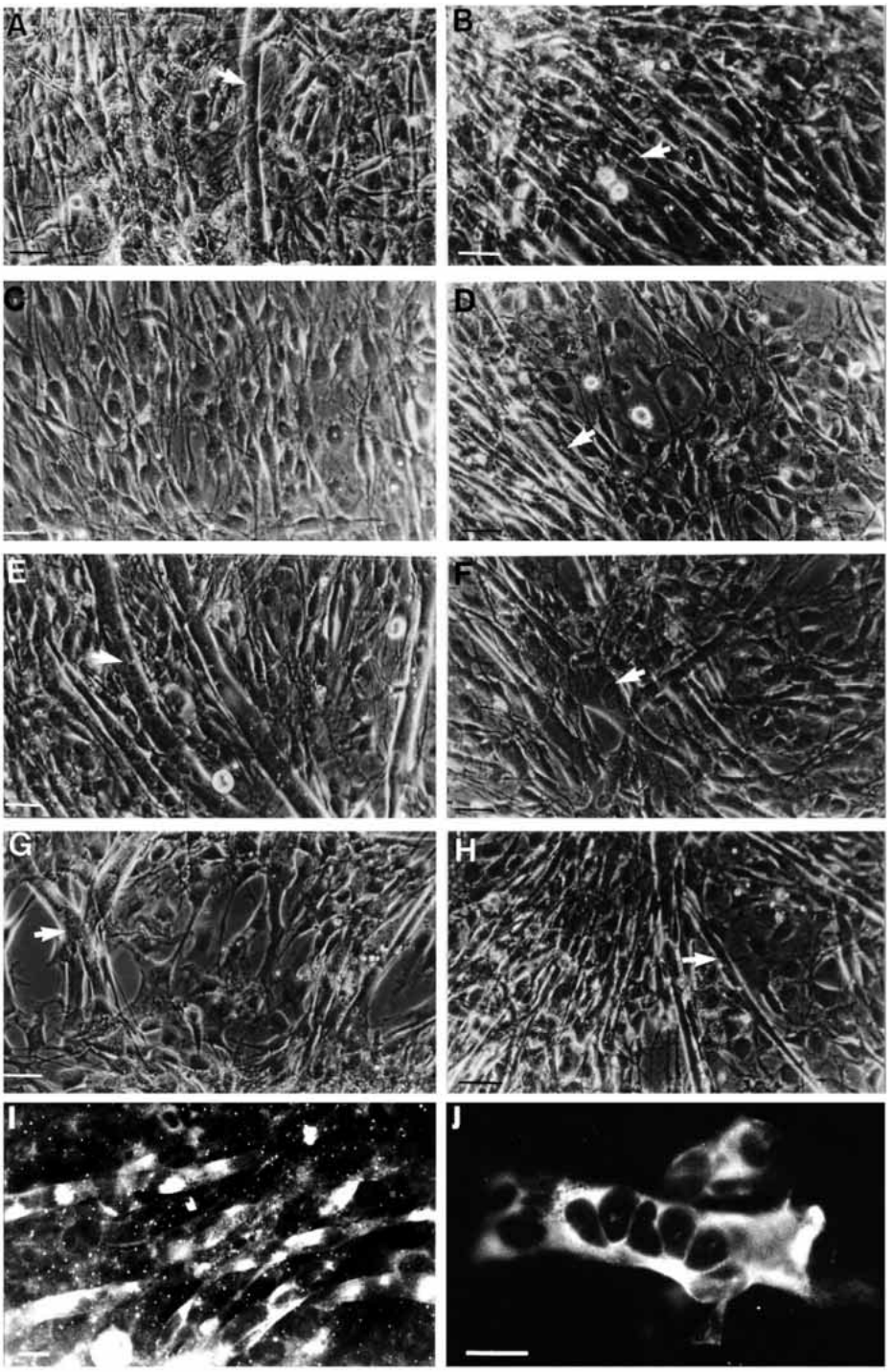

Fig. 5. Effect of RA on myogenesis of TH4B and BlH1 cells. Phase contrast micrographs (A-H) and reactivity of the muscle marker 12/101 (I,J) in representative cultures of TH4B (A,C,E,G,I) and BlH1 cells (B,D,F,H,J) grown for 12 and 8 days, respectively, in medium containing normal serum (A,B), delipidated serum (C,D) and delipidated serum plus 1×10^{-8} M RA (E,F) and 1×10^{-7} M RA (G-J). BlH1 cells were examined at an earlier time point then TH4B cells because of the occurrence of clamping in older dense cultures (see Fig. 7). Note that BlH1 cells form myotubes in all of the media used (B,D,F,H,J). In contrast, in TH4B cultures grown in medium containing delipidated serum (B), the cells align but little or no myogenesis occurs (C). Addition of RA to the medium re-establishes their ability to form myotubes (E,G). Note the difference in myotube morphology in TH4B and BlH1 cells, highlighted by 12/101 staining. Some myotubes are indicated by a white arrow. Bars, 50 μm.

treatment. We therefore used the BlH1 and TH4B long-term cell cultures derived from limb blastema and normal limb, respectively, to address this issue. When NvK8 and NvK18 transcripts were examined in BlH1 cultures 6 hours and 3 days after adding RA to the medium, no change in their level of expression was observed (Fig. 2C), confirming that keratin levels in blastemal cells are not regulated by RA. This indicates the lack of any causal relationship between the morphogenetic effects of RA on the blastema and keratin expression. In contrast, when the same analysis was carried out on TH4B cultures a clear downregulation of these keratins was already apparent at 6 hours and this decrease was maintained after 3 days (Fig. 2D). Consistent with the changes in mRNA expression, a decrease in the NvK8 protein was detected by western blotting in TH4B, but not in BlH1 cells after 2 weeks of RA treatment (Fig. 3). The finding that simple epithelia keratins are differently regulated by RA in TH4B and BlH1 cells is surprising, since these cells appear to have very similar morphology, and both express the regeneration markers NvK8, NvK18, 22/18 and vimentin and can undergo myogenic differentiation (Corcoran and Ferretti, 1997; Ferretti and Brockes, 1988, 1990).

The different regulation of keratin expression by RA in cultured myogenic limb cells of different origins mirrors the response to RA observed in vivo. Since this difference in keratin regulation by RA could be just one of the fundamental differences between these two cell populations and may reflect the existence of different myogenic precursors for regeneration in vivo, we further analyzed proliferation and myogenic differentiation of these cells under different culture conditions.

Myogenesis and RA

The initial analysis of myogenesis in BlH1 and TH4B cells was carried out in cultures grown in medium containing 1% instead of 10% serum, since cells differentiate more rapidly under these conditions. Alignment and fusion of cells to form myotubes was observed in TH4B cultures when cells were still at a relatively low density, whereas myogenesis was very rarely observed in BlH1 cells at a similar density (Fig. 4A-B). At a higher cell density myogenic differentiation occurred both in BlH1 and TH4B cultures, but myotubes tended to be better aligned and more elongated in TH4B than in BlH1 cultures, where broader myotubes were observed (Fig. 4C-D). Therefore it appears that although both BlH1 and TH4B cultures are myogenic their differentiation is regulated in a different fashion.

In order to study the effect of RA on myogenesis of BlH1 and TH4B, cells were grown in the presence of serum from which RA and its metabolites had been removed (delipidated serum). Myogenesis in BlH1 cultures grown in delipidated serum was comparable to that observed in cells grown in normal serum. In contrast, in TH4B cultures grown in delipidated serum myogenesis was inhibited, although cell alignment was still observed (Fig. 5A-D). To confirm that the effect observed was due to the RA that had been removed from the serum, we added to the delipidated serum different concentrations of RA. Addition of 10^{-8} M and 10^{-7} M RA did indeed restore the capability of forming myotubes in TH4B cultures, whereas no significant difference in myogenesis was observed in BlH1 cultures, as observed both morphologically and by immunocytochemistry (Fig. 5E-H). The same response was initially observed also with 10^{-6} M RA, but after 12 days treatment a toxic effect of this concentration of RA was apparent and the cell number significantly decreased (not shown).

The effect of RA on myogenesis was also examined by western blotting using 12/101 as an indicator of muscle differentiation (Fig. 6). Analysis of the 12/101 protein confirmed the morphological observations, as an increase in its levels was observed in RA-treated TH4B but not in RA-treated BlH1. Immunostaining of myotubes with 12/101 made clearer the difference in the morphology of myotubes that was first noticed looking at the live cultures. In fact, whereas myotubes forming in TH4B cultures are always long, stringy and aligned, those forming in BlH1 cultures are usually much broader and shorter and either more randomly organized or, in older cultures where cells tend to clump, radiating from the clumps or sitting on top of them. The 'clumping effect' observed in BlH1 cultures grown in normal serum for 10 days (Fig. 7A) does not seem to occur in BlH1 grown in delipidated serum (Fig. 7B), but could be reinduced by adding RA to such medium (Fig. 7C). Therefore, although RA does not affect myogenesis in BlH1 cultures, it seems to increase cell-cell adhesion.

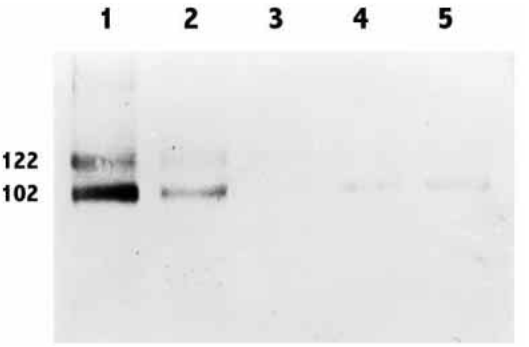

Fig. 6. Analysis of the expression of the muscle marker identified by mAb 12/101 by western blot of soluble protein fractions from cultured TH4B and BlH1 cells treated with 10^{-7} M RA for 2 weeks. Lanes: 1, limb muscle; 2, RA-treated TH4B; 3, control TH4B; 4, RA-treated BlH1; 5, control BlH1. 5 μg of protein were loaded in lanes 1-3, and 10 μg were loaded in lanes 4 and 5. 12/101 reacts with two proteins of 102×10^3 and 122×10^3 M_r. Note an increase in 12/101 levels in RA-treated TH4B cultures (lane 2), but not in RA-treated BlH1 cultures (B, lane 4) as compared to controls (lanes 3 and 5).

These data (summarized in Table 1) indicate that RA, in addition to displaying a difference in its ability to regulate keratin expression in TH4B and BlH1 cells, differentially affects myotube formation and cell adhesion in these cultures.

Effect of RA on cell proliferation

Since RA can also affect cell proliferation, we examined its effect on DNA synthesis in TH4B and BlH1 cultures grown in delipidated serum. The delipidated serum appeared to increase DNA synthesis in TH4B, as compared to control cells grown in normal serum, but it did not significantly affect BlH1 cells. This suggested that the factor extracted from the serum through

Table 1. Summary of the effects of different culture conditions on keratin expression, myogenic differentiation, adhesion and proliferation of BlH1 and TH4B cells

	Keratin expression		Myogenesis			Adhesion	Proliferation		
Cells	RA	anti-sense*	RA	low density	anti-sense*	RA	RA	TPA / FGF	anti-sense*
BlH1	=	⇓	=	±	⇑	⇑	=	⇑	⇓
TH4B	⇓	⇓	⇑	+	⇑	=	⇓	⇑	⇓

Note that in BlH1 cells keratin expression, myogenic differentiation and proliferation are not regulated by RA, whereas in TH4B cells proliferation and myogenic differentiation are regulated by RA, which also decreases K8. Note also that whenever keratin expression is downregulated, either by RA or anti-sense oligonucleotide treatment, myogenesis decreases and proliferation decreases.

*Data on the effects of K8 and K18 anti-sense oligonucleotides are from Corcoran and Ferretti (1997).

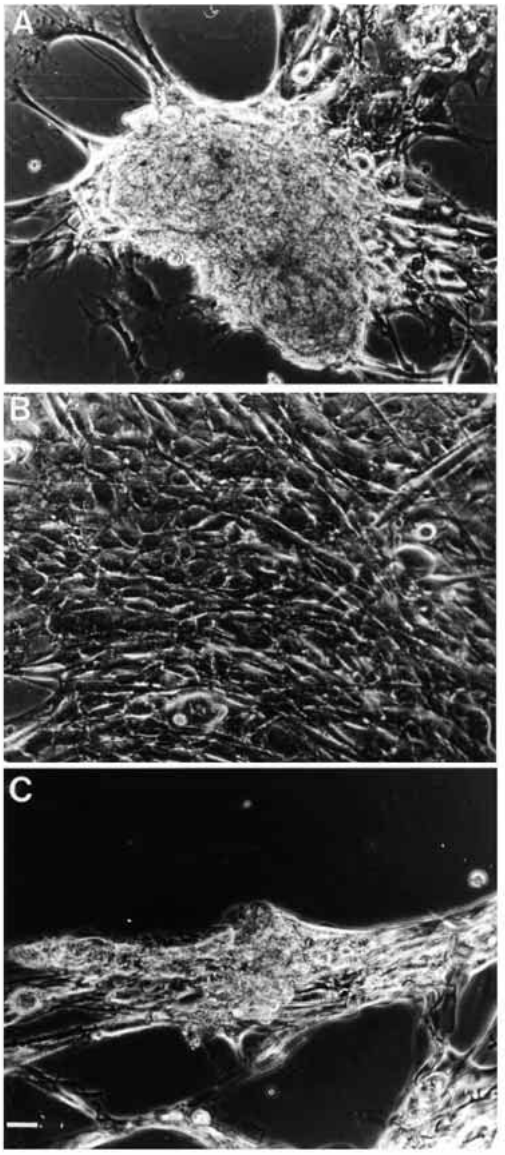

Fig. 7. Effect of RA on adhesive properties of BlH1 cells. Phase contrast micrographs of representative cultures grown for 10 days in medium containing normal serum (A), delipidated serum (B) and delipidated serum plus 1×10^{-8} M RA (C). Note the occurrence of cell clamping in normal serum (A) and delipidated serum supplemented with RA (C), but not in delipidated serum (B). Bar, 50 μm. A, B and C are at the same magnification.

delipidation, which causes inhibition of proliferation in TH4B, is RA. In order to confirm that this is the case, we treated these cultures with different concentrations of RA (10^{-8}, 10^{-7} and 10^{-6} M) and evaluated its effect on [^{3}H]thymidine incorporation. Addition of 10^{-7} M and 10^{-6} M RA did indeed decrease [^{3}H]thymidine incorporation in TH4B cells within 3 days, bringing it down to the level found in cultures grown in the presence of serum, but it did not have any significant effect on BlH1 cells (Fig. 8A,B). RA effect on TH4B cell proliferation was more marked after the 7-day treatment (not shown) when a significant decrease in [^{3}H]thymidine incorporation, as compared to the delipidated serum, was also observed after addition of 10^{-8} M RA; at this time, however, some toxic effects were observed in cultures treated with 10^{-6} M RA. Whereas RA has a negative effect on proliferation of TH4B, but not of BlH1 cells, we found that proliferative agents such as FGF-2 and TPA increase [^{3}H]thymidine incorporation in both cell populations (Table 1).

These data show that RA differentially affects proliferation of TH4B and BlH1 cells, and this is consistent with its effects on keratin regulation and myogenic differentiation, as summarized in Table 1.

DISCUSSION

It has been shown (Carlson, 1970) that there are important differences between limb muscle that has regenerated following mincing in the absence of amputation (tissue regeneration or repair), and the muscle that regenerates following limb amputation (epimorphic regeneration). In fact, whereas the regenerated minced muscle displays clear morphological defects, the muscle of the regenerated limb is virtually indistinguishable from that of a normal limb. This might be expected if different mechanisms were employed to regenerate the muscle under these different circumstances. The differences in the two populations of myogenic cells obtained from normal limb (TH4B cells) and blastema (BlH1 cells) reported here support this view and suggest that two distinct cell populations are involved in muscle repair and regeneration (see Fig. 1B).

TH4B and BlH1 differ in their regulation of NvK8 and NvK18

K8 and K18 are normally expressed in simple epithelia and glands such as the liver. In the newt they are expressed also in the blastemal cells of the regenerating limb both in vivo and in vitro (Corcoran and Ferretti, 1997; Ferretti et al., 1989). We have shown here that these keratins, like their mammalian homologues, can be regulated by RA at the mRNA level. This regulation is relatively fast, as an increase in these mRNAs can be detected within 6 hours from the addition of RA to TH4B cultures. Nonetheless, the fact that this regulation is observed in the TH4B cells and in the liver, but not in blastemal cells either in vivo or in vitro, suggests that the effect of RA on NvK8 and NvK18 is indirect. This is also supported by the fact that, although retinoic acid response elements (RAREs) have been found in the promoter of some keratins (Navarro et al., 1995; Ohtsuki et al., 1992), they do not appear to be present in the promoters of the mouse K8 and K18 genes (Oshima et al., 1990, 1996; Sémat et al., 1988). The lack of an effect of RA on keratin expression in blastemal cells in vivo does not seem to be due to the late time points we were able to analyze, since blastemal cells in vitro, unlike TH4B cells, do not show any regulation after either 6-hour or 3-day treatments. Therefore, although the effect of downregulation of NvK8 and NvK18 by anti-sense oligonucleotides is inhibition of proliferation and induction of differentiation both in TH4B and BlH1 (Corcoran and Ferretti, 1997), the mechanisms by which their expression is regulated in these cells appear to differ.

Furthermore, both RNAse protection and western blot analysis indicate that keratin content is higher in BlH1 than in TH4B cells, and this is consistent with their higher proliferative activity. This correlation between higher proliferative activity

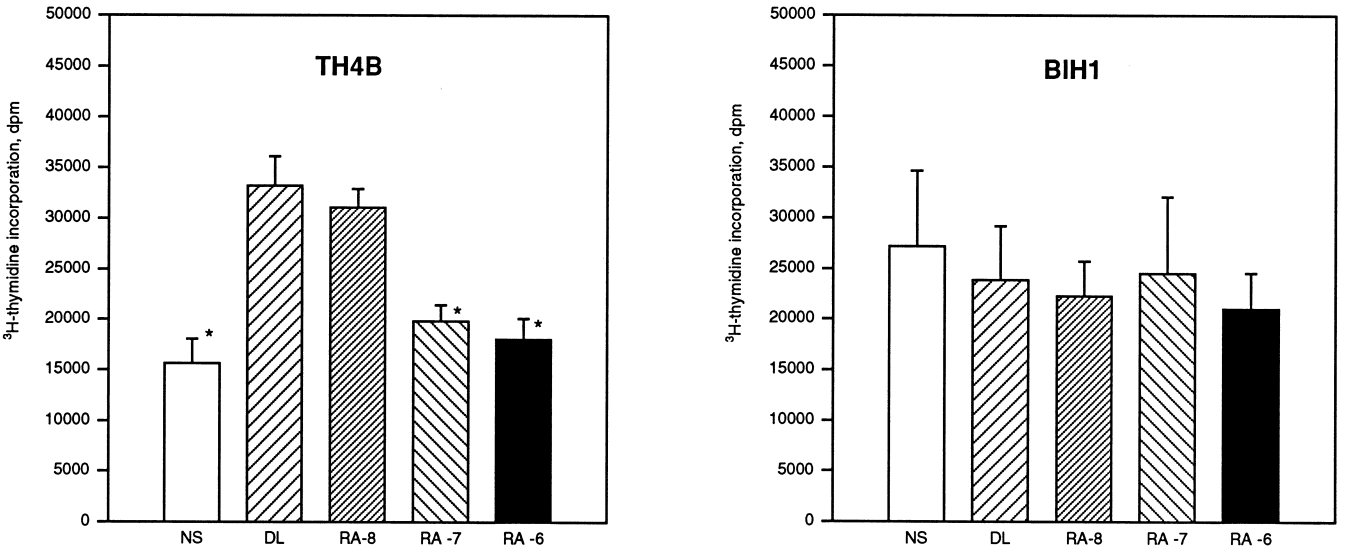

Fig. 8. Effect of RA on DNA synthesis in cultured newt limb cells. Cultures were incubated with 10^{-8} , 10^{-7} or 10^{-6} M RA for 3 days, and [^{3}H]thymidine was added 24 hours before harvesting the cells. (A) TH4B cells; (B) BlH1 cells. NS, normal serum control; DL, delipidated serum; RA-8, delipidated serum+10^{-8} M RA; RA-7, delipidated serum+10^{-7} M RA; RA-6, delipidated serum+10^{-6} M RA. An increase in DNA synthesis is observed in TH4B cells but not in BlH1 cells grown in delipidated serum, and is reversed by RA treatment (*P<0.001 by one-way analysis of variance as compared to [^{3}H]thymidine incorporation in delipidated serum; n=4 in TH4B-NS; n=5 in BlH1-NS and 6 in other groups; error bars represent the s.e.m.).

and keratin expression is apparent also in vivo, since no keratin expression is detectable either in blastemal cells before the onset of proliferative activity or in the putative satellite-like cells of normal muscle, which are presumably quiescent or very slowly cycling (Corcoran and Ferretti, 1997; Ferretti et al., 1989). This is in contrast to the cytoskeletal antigen 22/18, which is expressed not only during regeneration of minced muscle and early after limb amputation, where it labels cells whose division depends on the presence of the nerve, but also in putative myogenic mononucleate cells (Cameron et al., 1986; Ferretti and Brockes, 1990; Gordon and Brockes, 1988; Griffin et al., 1987). Therefore, whereas in the newt limb 22/18 may be a general marker of nerve-dependent regeneration, simple epithelial keratins appear to be associated with the onset and maintenance of proliferation and of the undifferentiated state during the regenerative process.

These results further support a causal relationship between keratin expression, proliferation and myogenic differentiation, as RA downregulation of keratins in TH4B is associated with a decrease in proliferation and myogenesis, whereas lack of keratin regulation by RA in BlH1 is associated with the maintenance of the undifferentiated state. Furthermore, when keratin expression is downregulated in BlH1 cells by anti-sense oligonucleotides, these cells also decrease their proliferative activity and start to differentiate (Corcoran and Ferretti, 1997). It is not clear whether the primary effect of RA on TH4B cells is downregulation of keratins and activation of the myogenic differentiation programme, which would then result in mitotic arrest, or inhibition of proliferation, and as a consequence myogenic differentiation. Given the fact that proliferative agents such as FGF-2 and TPA appear to affect TH4B and the BlH1 in a similar fashion, and that RA rapidly downregulates keratin expression in TH4B cultures, it is tempting to speculate that in these cells the primary effect of RA is on differentiation rather than proliferation.

Differences between TH4B and the BlH1 cells reflect their tissue origin

The differences observed between the TH4B and the BlH1 cells appear to reflect their origin from normal muscle and blastema, respectively. It has been previously suggested that mononucleate cells located outside the external lamina of the newt muscle may be equivalent to the myogenic reserve cells, satellite cells, present beneath the external lamina of the muscle fibers in higher vertebrates (Cameron et al., 1986; Mauro, 1961) Analysis of newt muscle explants has supported the hypothesis that these cells are myogenic, and led to the suggestion that they play a key role in blastema formation (Cameron et al., 1986; Schrag and Cameron, 1983). The TH4B cells, which like the cells studied by Cameron et al. (1986) originated from muscle explants, do display characteristics typical of muscle satellite cells in other species. Both satellite cells and the newt TH4B cells are determined to the muscle lineage, and RA, which causes a decrease in proliferation and induces differentiation of muscle satellite cells (Halevy and Lerman, 1993), has the same effect on TH4B cells. In conclusion, the behaviour of TH4B cells is consistent with the behaviour of muscle satellite cells in other species.

The behaviour of TH4B differs from that of BlH1 in ways other than in relation to their keratin content and regulation by RA of proliferation and differentiation. In fact, although both TH4B and BlH1 cells are myogenic, the myotubes formed in these cultures are different in morphology. This morphological difference does not seem to reflect a difference in fiber type, since myotubes from both cultures express 12/101. In the newt limb this protein is expressed in those muscle fibers which do not express cardioskeletal myosin, and in the rabbit in fast muscle fibers (Casimir et al., 1988; D. M. Fekete and P. M. Bennet, unpublished results). An intrinsic difference between the TH4B and the BlH1 cells is that TH4B but not BlH1 cells can differentiate at low cell density and their differentiation can

be directly regulated by RA. In vivo, blastemal cells begin to differentiate only after reaching a critical mass. The BlH1 cells seem to behave in a similar fashion, and might require numerous cell-cell interactions before fusing to form muscle; this might imply that BlH1 cells are less committed than TH4B cells, consistent with their origin through a process of dedifferentiation and the undifferentiated state of the blastema. Although we have never noticed any differentiated cell type other than muscle in BlH1 cultures, even under conditions permissive for chondrogenic differentiation, it might be that BlH1 cells are pluripotent, and under certain culture conditions could differentiate into other cell types.

Finally we have found that BlH1, but not TH4B cells, change their adhesive properties when RA is present in the culture medium and become more densely packed. An increase in adhesion of blastemal cells following RA treatment is also observed in vivo in both regenerating limbs and fins (Ferretti and Géraudie, 1995; Scadding, 1989). The behaviour of BlH1 cells in response to RA is consistent with the behaviour of blastemal cells in vivo: they are able to differentiate after reaching a critical mass, their keratin content is not affected by RA, but their adhesive properties are.

Whereas the behaviour of BlH1 cells mirrors that of blastemal cells in vivo, as discussed above TH4B cells appear to have properties similar to those of satellite cells in higher vertebrates, and to be significantly different from blastemal cell. It therefore appears that TH4B cells do indeed reflect the existence of a population of reserve cells in the newt limb, but their behaviour seems to indicate that, unlike previously suggested, they are not involved in epimorphic regeneration. We propose that, as in other vertebrates, they are activated in response to muscle injury (Fig. 1B). This interpretation is consistent with the differences observed in muscle regeneration following injury or limb amputation, with recent compelling evidence of the occurrence of muscle dedifferentiation following amputation, and with the view that this process is fundamental to blastema formation (Brockes, 1997; Carlson, 1970; Lo et al., 1993; Tanaka et al., 1997). Although this study does not allow us to completely rule out that myogenic reserve cells may contribute to the blastema, the fact that blastemal cells in vivo and in vitro display the same behaviour with respect to their keratin regulation and changes in adhesion upon RA treatment suggests that the contribution of reserve cells to epimorphic regeneration, if any, is rather small.

In conclusion, our analysis of the effects of RA on keratin expression and cell behaviour in vivo and in vitro points to the existence of two different sources of myogenic cells in the newt limb: a reserve cell population used for muscle repair following injury, and a progenitor cell population originating through muscle dedifferentiation following limb amputation. This reconciles opposing views concerning the occurrence of dedifferentiation and the existence and role of muscle progenitor cells in limb regeneration. Furthermore, this indicates the co-existence of two different repair mechanisms in lower vertebrates, one based on the existence of reserve cells, which has been maintained throughout evolution, and one based on the ability of mature tissues to dedifferentiate, which appears to have been largely lost in higher vertebrates.

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