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Expression of transforming growth factor β-like molecules in normal and regenerating arms of the crinoid *Antedon mediterranea*: immunocytochemical and biochemical evidence

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The phylum Echinodermata is well known for its extensive regenerative capabilities. Although there are substantial data now available that describe the histological and cellular bases of this phenomenon, little is known about the regulatory molecules involved. Here, we use an immunochemical approach to explore the potential role played by putative members of the transforming growth factor-β (TGF-β) family of secreted proteins in the arm regeneration process of the crinoid *Antedon mediterranea*. We show that a TGF-β-like molecule is present in normal and regenerating arms both in a propeptide form and in a mature form. During regeneration, the expression of the mature form is increased and appears to be accompanied by the appearance of an additional isoform. Immunocytochemistry indicates that TGF-β-like molecules are normally present in the nervous tissue and are specifically localized in both neural elements and non-neural migratory cells, mainly at the level of the brachial nerve. This pattern increases during regeneration, when the blastemal cells show a particularly striking expression of this molecule. Our data indicate that a TGF-β-like molecule (or molecules) is normally present in the adult nervous tissues of *A. mediterranea* and is upregulated significantly during regeneration. We suggest that it can play an important part in the regenerative process.

Keywords: regeneration; echinoderms; transforming growth factor β

1. INTRODUCTION

Interest in echinodermic regeneration has grown among developmental biologists in recent years because of the remarkable potential of this phenomenon to replace adult tissues that are lost following trauma or experimentally induced amputation (Thorndyke & Candia Carnevali 2000). In the comatulid crinoid *Antedon mediterranea*, regeneration is an essentially epimorphic process and has been explored extensively from several perspectives (Candia Carnevali *et al.* 1993, 1998*a*,*b*). In crinoids, new structures develop from the recruitment of migratory, morphologically undifferentiated cells previously identified as amoebocytes (a non-neural population of satellite cells located around the brachial nerve) or coelomocytes (a population of wandering free cells in the coelomic canals) (Candia Carnevali *et al.* 1993, 1995, 1997, 1998*a*). The main sites of active cell proliferation in the regenerating arm are: (i) the apical regenerative blastema, which comprises a mass of proliferating and differentiating cells developing at the amputated arm tip; (ii) the brachial nerve and coelomic epithelium, which show evidence of intense proliferative activity throughout their length, in both the regenerate and stump region of the arm (Candia Carnevali *et al.* 1995, 1997). Other types of migratory cell, including phagocytes and granule cells (i.e. granule-containing cells that are exclusive to crinoids and described as 'wanderzellen' by Reichensperger (1912)) are widely and differentially employed during regeneration.

It has been proposed several times that specific factors are necessary for initiating and controlling the regenerative response. For instance, the importance of the nervous system and its production of neuropeptides and growth factors have been shown to be essential during regeneration in many animals (Brockes 1987; Ferretti & Gèraudie 1998). However, until now, only a few factors native to echinoderms have been identified (Thorndyke & Candia Carnevali 2000). In *A. mediterranea* it has been shown that monoamine neurotransmitters are widely present during regeneration (Candia Carnevali *et al.* 1996), while the presence of other molecules (neuropeptides and growth factors) has been proposed and demonstrated in recent studies by employing immunocytochemical methods (Candia Carnevali *et al.* 1998*a*,*b*). Secreted proteins of the transforming growth factor-β (TGF-β) superfamily are dimeric cytokines that modulate many biochemical signals between cells. These proteins are widely involved in a range of processes, including cell proliferation, differentiation, apoptosis, axial patterning, wound healing and tissue remodelling (Raftery & Sutherland 1999). The mature TGF-β dimer (about 25 kDa) is usually associated

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with an amino-terminal propeptide (LAP) of about 100 kDa (Saharinen *et al.* 1999). TGF-βs are only activated following breakdown of the non-covalent interactions between the LAP and TGF-β, enabling the latter molecule to bind to a family of cell-surface transmembrane receptors (RI, RII and RIII). Once the ligand has bound to the receptor, a class of recently identified proteins (Mad-related proteins) transduce the signal and act as transcriptional regulators that interact directly with DNA (Massagué 1998; Raftery & Sutherland 1999). TGF-β1 initially binds to RII and thus acts upstream of RI. According to Massagué (1996), the type II receptor should be regarded as the primary receptor because RI cannot bind to a free ligand, but only to a ligand already bound to a type II receptor. All these molecules are highly conserved throughout evolution. For example, recent studies show that all the vertebrate signalling pathways are also represented in *Drosophila* (Raftery & Sutherland 1999).

The present investigation focuses on TGF-β1 because of its well-established role in the wound healing processes of both embryos and adults (Nodder & Martin 1997; O'Kane & Ferguson 1997). Furthermore, this molecule has been implicated in neuronal differentiation, the stimulation or inhibition of cell proliferation (Tomoda *et al.* 1996), as well as the regulation of epithelial–mesenchymal interactions that are fundamental for pattern determination in regenerating systems (Ferretti & Gèraudie 1998). Previous morphological studies of *Antedon* demonstrated rapid regeneration in response to injury. The rate of crinoid blastemal regeneration is impressive and at 24 h post amputation (pa) the repair process is more or less complete and the regenerative phase proper begins. Typically for epimorphic regeneration, the early regenerative bud essentially comprises a blastema of undifferentiated cells, which at this stage are represented by accumulating migratory amoebocytes. The brachial nerve of the stump already shows extensive axonal re-growth and by 72 h the regenerative process is well underway with growth, morphogenesis and early differentiation evident in the blastemal region of the regenerating arm tip. At one week post ablation, the regenerating arm tip can already be identified morphologically as a new arm and shows clear signs of tissue differentiation. In particular, a new endoskeleton is growing rapidly at this time and new muscle, ligaments and nerve cells are evident in the proximal region of the regenerate (Candia Carnevali *et al.* 1999). These findings, together with preliminary molecular data obtained in our laboratories (Candia Carnevali *et al.* 1998*a*; Patruno 2001) suggest members of the TGF-β superfamily are prime candidates for putative roles in both the early repair (reparative–proliferative) and advanced regenerative (proliferative–differentiative) phases of regeneration in echinoderms. In this paper, we have employed a biochemical and immunocytochemical approach to explore (i) the presence of TGF-β1 and its primary type II receptor in arm tissues of *A. mediterranea*, and (ii) the potential changes in its distribution during the regenerative process.

2. MATERIAL AND METHODS

Specimens of *A. mediterranea* were collected by scuba divers in the Gulf of Taranto (Italy) and maintained in the laboratory in a circulating artificial seawater system. The experimental regeneration of arms was induced by mimicking the conditions of natural autotomy (see for details Candia Carnevali *et al.* 1993). A scalpel was used to stimulate gently the arm at the level of the autotomy plane and this resulted almost immediately in the autotomy reaction. By this method, the distal portions of three different arms were removed from each intact animal at approximately one- to two-thirds of the distance between the arm tip and disc. Tissue samples were prepared from both normal intact arms and regenerating arms. Regeneration was monitored at fixed times, early in the regenerative period (24, 48 and 72 h PA) and at an advanced stage (one week PA) when a well-developed regenerate was detectable at the amputated arm tip.

(a) *Gel electrophoresis and Western blotting*

Two-dimensional (2D) gel analysis was carried out according to Hussey *et al.* (1988) and one-dimensional (1D) gel electrophoresis as described by Patruno *et al.* (2001). In brief, at least three regenerating arms of *A. mediterranea* were crushed in liquid nitrogen and solublized in boiling sample buffer (Laemmli 1970). Insoluble materials were removed by centrifugation at 10 000 *g* for 3 min. Proteins in the aqueous phase were acetone precipitated and pelleted by centrifugation at 3000 *g* for 3 min. The air-dried pellet was resuspended in 100 µl of lysis buffer (5 mM $MgCl_2$, 20 mM Tris–HCl pH 7.6, 1% w/v Nonidet P-40) to form a slurry. Prior to electrophoresis, protein concentration was determined using a protein assay (BioRad Laboratories) with bovine serum albumin (BSA) as a standard. For each gel, 20–50 µg of total protein was loaded. First dimension (IEF) gels were run at 150 V for 1–2 h, then at 400 V for 16 h and then at 800 V for a further 3 h. Gels were equilibrated in Laemmli (1970) sample buffer for 20 min, placed on top of the SDS–PAGE slab gels (15% acrylamide) for the second dimension. These gels were run for 4 h at 200 V. For 1D electrophoresis, crinoid proteins were separated on a 10% SDS gel and stained with Coomassie blue for protein visualization.

Western blotting was as described by Towbin *et al.* (1979). The immunodevelopment of Western blots was carried out with MAS 729 (Harlan, Sera-Lab) mouse anti-human TGF-β1 (1:500) and TGF-β RII (Santa Cruz) rabbit anti-type II receptor for the TGF-β proteins (1:500). As a negative control, an anti-Hsp72 antibody (Stressgen SPA-810) was used. A further control used an overnight pre-incubation step of the MAS 729 mAb in a homogenate prepared from normal crinoid arms. This pre-absorped antibody was used subsequently in the blotting procedure. Finally, human lymphocytes (H9 cell line) were heat shocked (1 h at 43 °C + 3 h recovery) and immunoblotted as a positive control for Hsp72 induction as described in Patruno *et al.* (2001).

Anti-mouse and anti-rabbit horseradish peroxidase conjugates were used for secondary immunodevelopment. Peroxidase was detected using the enhanced chemiluminescence (ECL) method for 1 min (Amersham). After exposure to X-rays, total protein on the nitrocellulose membrane was stained using a colloidal silver method (Kovarik *et al.* 1987).

Densitometric analysis of blots was performed using UVP gel documentation and analysis systems (UVP, Inc., Upland, CA, USA). At least three replicates were carried out for each sample. For protein extracts, all values quoted are expressed relative to normal arms (p0; 100%). A paired *t*-test was used for the statistical analysis. The differences between the protein extracts from normal and regenerating crinoid arms were considered significant if $p \leqslant 0.05$.

(b) *Immunocytochemistry*

The samples were fixed in either Bouin's fluid or in a mixture of paraformaldehyde (4% w/v)-glutaraldehyde (0.5% w/v) in 0.1 M phosphate buffer for 2 h. Following an overnight wash in the same buffer, the samples were dehydrated and embedded in paraffin wax or Epon-Araldite resin, respectively, according to standard protocols (Candia Carnevali *et al.* 1995). Sagittal semithin resin sections were cut with an LKB Ultratome V and Reichert Ultracut E and processed using either the immunoperoxidase ABC system (Vector) or immunofluorescence (IF). In the latter case, either fluorescein- or Texas Red-conjugated secondary antibodies were used. Immunocytochemistry was carried out with MAS 729 (Harlan, Sera-Lab) and TGF-β RII (Santa Cruz) antibodies at a dilution of 1 : 50. The ultrathin sections were treated as described in Candia Carnevali *et al.* (1998*a*,*b*).

Microscopy was with a Jenaval optical microscope or Zeiss Axioplan with fluorescence attachments or a Leica TCS-4D confocal laser scanning microscope (CLSM). Immunocytochemistry controls were prepared as described in Candia Carnevali *et al.* (1998*a*,*b*) using primary antisera pre-adsorbed with an appropriate antigen.

3. RESULTS

(a) *Western blot analysis*

The monoclonal MAS 729 antibody used in the immunoblot analysis of arms of *A. mediterranea* recognized a band of *ca*. 25 kDa. The intensity of the band showed a noticeable increase in regenerating arms when compared with non-regenerating arms (figure 1*a*). In particular, in both early (24 h pa, figure 1*a*, lane 3) and advanced (one week pa, figure 1*a*, lane 4) phases of regeneration, at least a fivefold increase ($p < 0.01$) in densitometric intensity of the 25 kDa band was detected compared with that from non-regenerating arms (figure 1*a*, lane 1).

A negative control using a monoclonal antibody (SPA-810) against the heat-shock protein hsp72 was used in order to detect any non-specific binding to crinoid proteins. The SPA-810 antibody recognized a single specific band of *ca*. 72 kDa (figure 1*a*, lane 5 and figure 1*b*, lane 1). Some non-specific bands were observed at very high molecular weights (close to the top of the gel) and in cases of long exposure (more than 4 min) of the nitrocellulose to the X-rays (data not shown). Figure 1*a* (lane 2) shows an absorption control using the mAb MAS 729. This antibody was pre-incubated overnight in a homogenate prepared from normal non-regenerating crinoid arms and subsequently used in the blotting procedure (figure 1*a*, lane 2).

The analysis of protein samples prepared from regenerating and non-regenerating arms using 2D SDS–PAGE showed that more proteins were expressed in regenerating arms only at 72 h pa (figure 2*a*,*b*). When transferred to nitro-cellulose membranes and probed with the MAS 729 antibody, two closely related TGF-β isoforms of *ca*. 25 kDa were identified in protein samples prepared from normal arms (figure 2*c*). By contrast, samples from regenerating arms at 72 h pa, appeared to include an extra acidic isoform with a molecular weight similar to the two main isoforms (figure 2*d*).

The polyclonal anti-TGF-β type II receptor antibody recognized a distinct band of *ca*. 70 kDa (figure 1*b*, lanes 2–4). In samples prepared from normal and regenerating arms the intensity of the band recognized with this antibody did not change significantly. Again, the mAb against the heat-shock protein Hsp72 was used as a negative control and showed a specific band of *ca*. 72 kDa (figure 2*b*, lane 1).

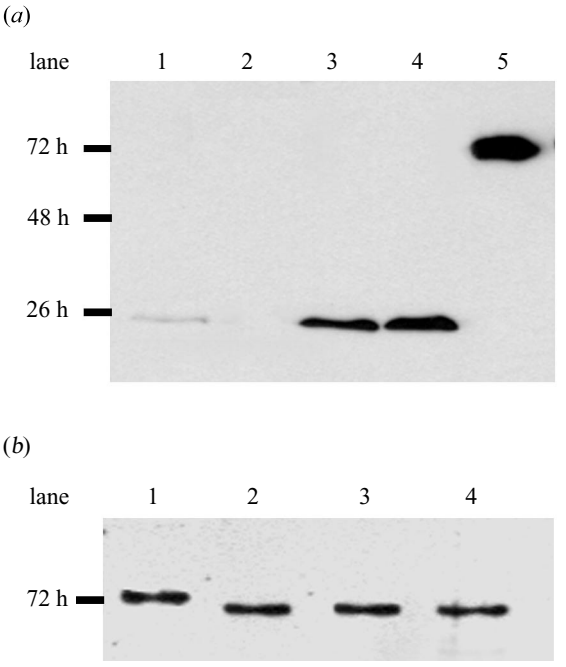

Figure 1. Levels of TGF-β1 and type II receptor expression during regeneration in arms of *A. mediterranea*. (*a*) Western blots of protein extracts prepared from normal adult and regenerating arms (distal tip of the arm) immunodeveloped with the MAS 729 anti-TGF-β1 antibody (lanes 1–4) and the anti-Hsp72 antibody (SPA-810) (lane 5). The Western blot shown in this figure is representative of three experiments. Lane 1, normal adult arms; lane 2, absorption control with the MAS 729 antibody pre-absorped with the 'normal' arm protein extract (refer to § 2); lane 3, regenerating arms at 24 h pa; lane 4, regenerate at one week pa; lane 5, negative control with the anti-Hsp72 antibody on the regenerating protein extract (24 h pa). (*b*) Western blot of protein extracts from normal adult and regenerating arms immunodeveloped with the anti-receptor RII antibody (lanes 2–4). The Western blot shown in this figure is representative of three experiments. Lane 1, negative control with the anti-Hsp72 antibody on the regenerating protein extract (24 h pa); lane 2, normal adult arm; lane 3, regenerating arm at 24 h pa; lane 4, regeneration at one week pa. For both (*a*) and (*b*) the protein loading was 60 μg per track and the samples were separated on a 10% SDS–PAGE.

(b) *Immunocytochemical studies*

Figure 3 shows a sagittal section of a regenerating arm at 72 h pa. The main anatomical features of the arm are illustrated.

Immunoreactivity using the anti-TGF-β1 antibody MAS 729 on semithin sections of non-regenerating arms was restricted to neural cells and processes, mainly at the level of the brachial nerve (figure 4*d*, arrow).

By contrast, in regenerating arms at different stages (from 24 h to one week pa) a much wider distribution was evident. A markedly enhanced reaction was found as follows:

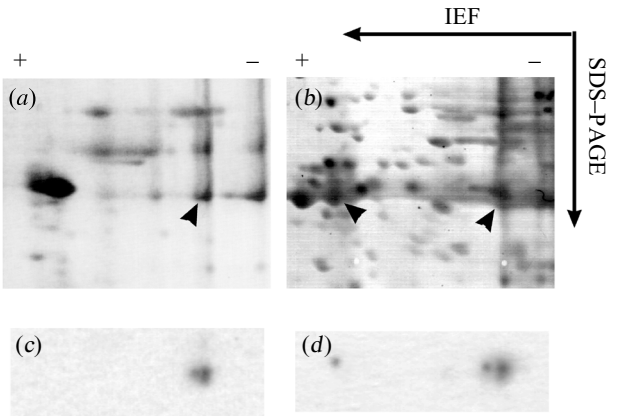

Figure 2. Two-dimensional SDS–PAGE of a protein extract from *A. mediterranea* prepared from (*a*) normal adult arms; (*b*) regenerating arms at 72 h pa; (*c*,*d*) Western blots of (*a*) and (*b*), respectively. The arrowheads on the 2D SDS gels indicate the spots detected by the MAS 729 anti-TGF-β1 antibody.

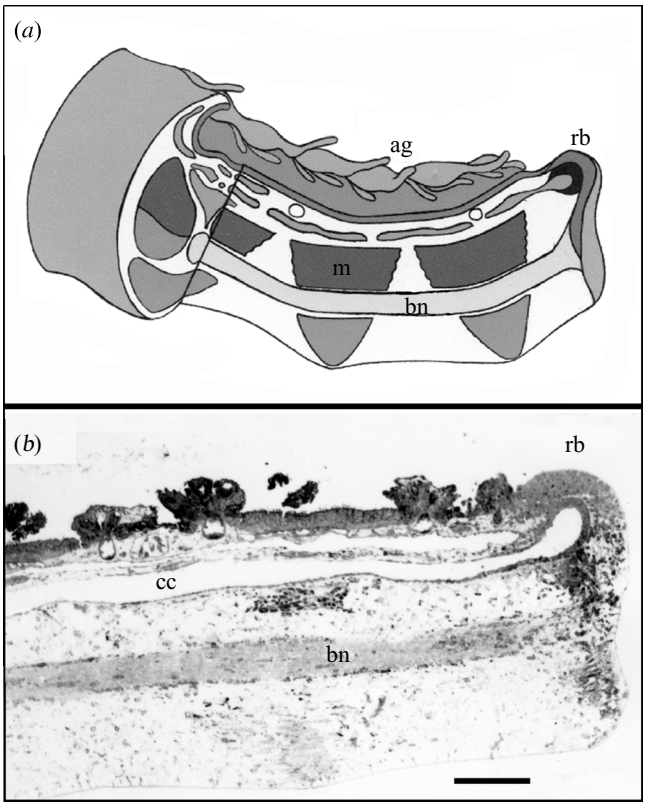

Figure 3. (*a*) A schematic reconstruction showing the main anatomical features of the regenerating arm (early regenerative stage of *ca*. 48–72 h pa) of *A. mediterranea*. (*b*) Histological section of a regenerating arm at 48 h pa. Abbreviations: rb, regenerating blastema; bn, brachial nerve; cc, coelomic canals; ag, ambulacral groove; m, muscle. Bar, 100 μm.

(i) at the level of the brachial nerve (figure 4*a*, arrow and figure 4*c*);
(ii) in the apical blastema (from 48 h pa and throughout the regenerative phase; figure 4*a*, arrowhead); and
(iii) in the distal portion of the regenerating new arm (apical regenerate) beginning at one week pa (figure 4*b*).

Positive elements immunoreactive to the anti-TGF-β1 antibody were detected in both neural and non-neural tissues: in particular, at the level of the apical blastema (figure 4*a*). The blastemal cells were strongly labelled and, at the level of the brachial nerve, the granule cells (figure 4*c*, arrowheads) and migratory undifferentiated cells (amoebocytes) were also immunoreactive (figure 4*c*, arrow).

Furthermore, in the early stages of regeneration, immunolabelling with the anti-TGF-β1 antibody showed a localization in both the cytoplasm (where staining appeared to be stronger; figure 4*e* arrow) and in the nucleus (figure 4*e*, arrowhead) of several migratory amoebocytes close to the amputation surface. The anti-type II receptor antibody showed a marked cytoplasmic staining especially in migrating amoebocytes in the brachial nerve (figure 4*f*, arrow).

Immunogold staining using the MAS 729 antibody on ultra-thin sections of the regenerating blastema (72 h pa) showed particularly strong labelling in blastemal cells, especially in association with the nuclei and nucleoli (figure 4*g*, arrowheads). At this early stage, the blastemal cells are still morphologically undifferentiated and are structurally similar to migratory amoebocytes.

4. DISCUSSION

The evidence presented here offers clear support for the presence of a TGF-β-like molecule (or molecules) in crinoid echinoderms together with the implication that it might have a role during regeneration. The presence of such molecules in echinoderms is not surprising, since this group of growth regulators and their receptors are clearly very ancient, with representatives recently described from sponges (Suga *et al.* 1999). Moreover, members of this superfamily of growth factors have been described also in other echinoderm classes, albeit restricted to their larval stages. For example, TGF-β family members have been reported in the embryonic stages of two echinoid species where it was suggested that they have a part in early patterning events (Stenzel *et al.* 1994; Hwang *et al.* 1999). Our current results indicate the presence of TGF-β-like molecules in normal and regenerating arms of *A. mediterranea*. Western blotting analysis revealed an immunoreactive band in non-regenerating arms with a molecular weight close to that observed for other members of the TGF-β superfamily of growth regulators (Mozes *et al.* 1999). On this basis, we speculate that this band might correspond to the mature form of a TGF-β-like molecule. The Western blot (from the 2D gel) performed on material from non-regenerating arms further suggested that this TGF-β-like molecule might exist in at least two isoforms. The close proximity of the isoforms to each other implies that they could result from a single post-translational modification such as phosphorylation (Cohen 1983; Massagué 1998).

During regeneration, significant changes in this profile are evident. First, there is a significant increase in intensity of the 25 kDa band in the blotted 1D gel in all the regenerating phases studied. Second, the 2D gel indicates an overall increase in protein expression that is especially

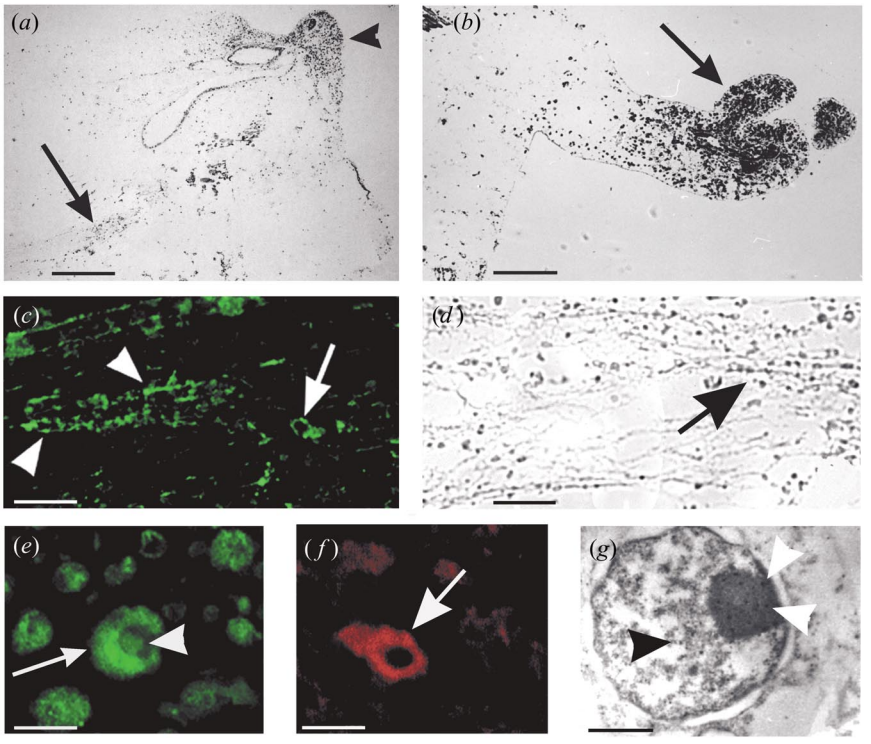

Figure 4. Immunocytochemical localization of a TGF-β-like molecule and its type II receptor using the MAS 729 anti-human TGF-β1 antibody (*a–e,g*) in regenerating arms of *A. mediterranea*. (*a*) From 48 h up to one week pa, a strong immunoreactivity is observed in the regenerating blastema (arrowhead) and in the brachial nerve (arrow). Bar, 200 μm. (*b*) At advanced phases of regeneration the immunoreactivity is particularly strong in the regenerate at the level of its apical portion and new developing lateral branches (pinnules, arrow). Bar, 100 μm. (*c*) In the regenerating nerve tissue of *A. mediterranea* several granule cells are positive (arrowheads); a migratory amoebocyte shows distinct localization at the cytoplasmic level (arrow). Bar, 50 μm. (*d*) In the normal non-regenerating arm of *A. mediterranea* the brachial nerve presents a diffuse but not particularly intense immunoreactivity at the level of neural cells and processes (arrow). Bar, 50 μm. (*e*) Staining for TGF-β1 using a fluorescein isothiocyanate indirect method. A migratory cell of the brachial nerve (amoebocyte) close to the amputation site shows marked cytoplasmic (arrow) and weaker nuclear staining (arrowhead) for the anti-TGF-β1 antibody. Bar, 20 μm. (*f*) Staining for the type II TGF-β receptor using a Texas Red indirect method. A migratory amoebocyte of the brachial nerve shows cytoplasmic (arrow) staining for the type II TGF-β receptor. Bar, 20 μm. (*g*) In thin sections, an undifferentiated blastemal cell of the apical blastema presents clear immunogold labelling (arrowheads) for the anti-TGF-β1 antibody. Bar, 2 μm.

marked at 72 h pa. At this stage, the repair phase is terminated and substantial cell migration (towards the distal amputated tip of the arm) and proliferation (in the regenerative blastema and in the brachial nerve) are observed. It could be argued that, at this phase of regeneration, the increase in protein content might come from an 'inflammatory-like' response to injury in the stump. However, in *Antedon*, the arm regeneration process seems to have acquired a dynamic stress-response programme that quickly enables the re-growth of the lost part without an apparent cell injury-response mechanism (Patruno *et al.* 2001).

Western blots of protein extracts from non-regenerating arms separated using 2D SDS–PAGE indicate that there are two TGF-β isoforms. However, in protein extracts from regenerating tissues at 72 h pa we also detected an additional acidic TGF-β isoform. The distance between the latter and the main isoforms suggests that either a strong acidic group has been added to a single site or that a number of acidic residues have been added to several different sites on the protein. In both cases, the size of the isoform would not be altered. Alternatively, there is also the possibility that expression of another member of the TGF-β gene family is switched on at this stage of regeneration. Molecular characterization of the crinoid TGF-β gene family will be required to support this suggestion (see Patruno 2001). The immunocytochemical data indicate that TGF-β expression in 'normal' non-regenerating arms is localized to neural elements in the brachial nerve. During regeneration, the cellular localization shows an overall increase in terms of numbers of cells involved and appears to be a reflection of the massive proliferative activity that accompanies the regenerative process. In particular, blastemal cells expressed strikingly strong TGF-β-like immunoreactivity. Our previous studies have shown that the blastema is a site of intense cell proliferation with the proliferating cells derived mainly from stem elements that migrate from both the brachial nerve and the coelomic canals (Candia Carnevali *et al.* 1995). In extreme conditions, such as those represented by isolated arm explants, these migrating–proliferating cells can also originate from dedifferentiation of other tissues, particularly muscle (Candia Carnevali *et al.* 1998*a*).

Our data suggest that, although a TGF-β-like molecule is present in 'normal' non-regenerating arm tissues, where it presumably has a fundamental role in normal tissue maintenance, it can be significantly upregulated during regeneration, in particular within migratory stem cells as

well as committed blastemal cells, which form the basis of cellular and tissue renewal. This is supported by evidence showing that in the early phase (reparative–proliferative) of arm regeneration, as well as in the advanced phase (proliferative–differentiative), migratory stem cells and blastemal cells show a strong staining for both the anti-TGF-β1 and the anti-type II receptor antibodies.

In blotting analyses, the polyclonal antibody against the TGF-β type II receptor recognized a clear band that did not increase in optical density in regenerating samples compared with normal arms. It is important to note that during the early phases of the signalling cascade established for these molecules, it is the type II receptor that is thought to be the first to recognize the ligand and that can also be associated with other TGF-β-binding proteins in the initial steps of signal transduction (Massagué 1998).

Thus, in other animal models, TGF-β has been shown to have a wide range of functions, many of which are in keeping with the roles suggested here for crinoid regeneration. For example: (i) stimulating the synthesis of extracellular matrix components during the formation of the early fibrous cicatricial layer and acting as a specific neurotrotrophic–neuroprotective factor important for both repair and developmental phenomena at the level of the nervous tissue (Fawcett 1997); (ii) exerting an indirect neurotrophic action on neuron survival by regulating the expression of other factors such as nerve growth factor from both neural and non-neural cells (Lindholm *et al.* 1992); (iii) regulating cell proliferation (Husmann *et al.* 1996).

Events and processes similar to those in the previous paragraph must clearly take place during regenerative developmental growth in crinoid arms. Here, it is relevant that our previous results (Candia Carnevali *et al.* 1995, 1997, 1998*a*) indicate massive cell proliferation, migration, differentiation and dedifferentiation in tissues where the expression of the TGF-β-like molecule is particularly strong. It is, therefore, a plausible hypothesis that TGF-β-like factors are putatively responsible, at least in part, for the regulation of these phenomena. Understanding the precise nature of this role (or roles) will depend on the characterization of the native crinoid molecule(s) and we are now exploring the use of molecular techniques in order to identify potential crinoid homologues of these multifunctional growth regulators.

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REFERENCES

Brockes, J. P. 1987 The nerve dependence of amphibian limb regeneration. *J. Exp. Biol.* **132**, 79–91.

Candia Carnevali, M. D., Lucca, E. & Bonasoro, F. 1993 Mechanism of arm regeneration in the feather star *Antedon mediterranea*: healing of wound and early stages of development. *J. Exp. Zool.* **267**, 299–317.

Candia Carnevali, M. D., Bonasoro, F., Lucca, E. & Thorndyke, M. C. 1995 Pattern of cell proliferation in the early stages of arm regeneration in the feather star *Antedon mediterranea*. *J. Exp. Zool.* **272**, 464–474.

Candia Carnevali, M. D., Bonasoro, F., Invernizzi, R., Lucca, E., Welsch, U. & Thorndyke, M. C. 1996 Tissue distribution of monoamine neurotransmitters in normal and regenerating arms of the feather star *Antedon mediterranea*. *Cell Tissue Res.* **285**, 341–352.

Candia Carnevali, M. D., Bonasoro, F. & Biale, A. 1997 Pattern of bromodeoxyuridine incorporation in the advanced stages of arm regeneration in the feather star *Antedon mediterranea*. *Cell Tissue Res.* **289**, 363–374.

Candia Carnevali, M. D., Bonasoro, F., Patruno, M. & Thorndyke, M. C. 1998*a* Cellular and molecular mechanisms of arm regeneration in crinoid echinoderms: the potential of arm explants. *Dev. Genes Evol.* **208**, 421–430.

Candia Carnevali, M. D., Bonasoro, F., Welsch, U. & Thorndyke, M. C. 1998*b* Arm regeneration and growth factors in crinoids. In *Echinoderms: San Francisco* (ed. R. Mooi & M. Telford), pp. 145–150. Rotterdam: Balkema.

Candia Carnevali, M. D., Bonasoro, F., Trezzi, M. & Giardina, A. 1999 Nerve-dependent myogenesis in arm regeneration of *Antedon mediterranea*. In *Echinoderm research* (ed. M. D. Candia Carnevali & F. Bonasoro), pp. 139–143. Rotterdam: Balkema.

Cohen, P. 1983 Protein phosphorylation and the control of glycogen metabolism in skeletal muscle. *Phil. Trans. R. Soc. Lond.* B **302**, 13–25.

Fawcett, J. W. 1997 Astrocytic and neuronal factors affecting axon regeneration in the damaged central nervous system. *Cell. Tissue Res.* **290**, 371–377.

Ferretti, P. & Gèraudie, J. 1998 *Cellular and molecular basis of regeneration: from invertebrates to humans*. Chichester, UK: Wiley.

Husmann, L., Souiet, L., Gautron, J., Martelly, L. & Barritault, D. 1996 Growth factors in skeletal muscle regeneration. *Cytokine Growth Factor Rev.* **7**, 249–258.

Hussey, P. J., Lloyd, C. W. & Gull, K. 1988 Differential and developmental expression of β-tubulins in a higher plant. *J. Biol. Chem.* **263**, 5474–5479.

Hwang, S. P. L., Chen, C. A. & Chen, C. P. 1999 Sea urchin TgBMP2/4 gene encoding a bone morphogenetic protein closely related to vertebrate BMP2 and BMP4 with maximal expression at the later stages of embryonic development. *Biochem. Biophys. Res. Commun.* **258**, 457–463.

Kovarik, A., Hlubinova, K., Vrbenska, A. & Prachar, J. 1987 An improved colloidal silver staining method of protein blots on nitro-cellulose membranes. *Folia Biol.* **33**, 254–257.

Laemmli, E. K. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lindholm, D., Castren, E., Kiefer, R., Zafra, F. & Thoenen, H. 1992 Transforming growth factor-β in the rat brain: increase after injury and inhibition of astrocyte proliferation. *J. Cell Biol.* **117**, 395–400.

Massagué, J. 1996 TGF-β signalling: receptors, transducers and Mad proteins. *Cell* **85**, 947–950.

Massagué, J. 1998 TGF-β signal transduction. *A. Rev. Biochem.* **67**, 753–791.

Mozes, M. M., Hodics, T. & Kopp, J. B. 1999 Isoform specificity of commercially available anti-TGF-β antibodies. *J. Immun. Meth.* **225**, 87–93.

Nodder, S. & Martin, P. 1997 Wound healing in embryos: a review. *Anat. Embryol.* **195**, 215–228.

O'Kane, S. & Ferguson, M. W. J. 1997 Transforming growth factor βs and wound healing. *Int. J. Biochem. Cell. Biol.* **29**, 63–78.

Patruno, M. 2001 The role of stress proteins and the trans-

forming growth factor-β family (TGF-βs) in echinoderm regeneration. PhD thesis, University of London.

Patruno, M., Thorndyke, M. C., Candia Carnevali, M. D., Bonasoro, F. & Beesley, P. 2001 Changes in ubiquitin conjugates and Hsp72 levels during arm regeneration in echinoderms. *Mar. Biotechnol.* **3**, 4–15.

Raftery, L. A. & Sutherland, D. J. 1999 TFGβ family signal transduction in *Drosophila* development: from MA to Smads. *Dev. Biol.* **210**, 251–268.

Reichensperger, A. 1912 Beitrage zur Histologie und zum Verlauf der Regeneration bei Crinoiden. *Ztschr. Wiss. Zool.* **101**, 1–69.

Saharinen, J., Hyytianen, M., Taipale, J. & Keski-Oja, J. 1999 Latent transforming factor-β binding proteins (LTBPs) structural extracellular matrix proteins for targeting TGF-β action. *Cytokine Growth Factor Rev.* **10**, 99–117.

Stenzel, P., Angerer, L. M., Smith, B. J., Angerer, R. C. & Vale, W. W. 1994 The univin gene encodes a member of the transforming growth factor-β superfamily with restricted expression in the sea urchin embryo. *Dev. Biol.* **166**, 149–158.

Suga, H., Kanako, O. & Miyata, T. 1999 Multiple TGF-β receptor related genes in sponge and ancient gene duplications before the parazoan–eumetazoan split. *FEBS Lett.* **453**, 346–350.

Thorndyke, M. C. & Candia Carnevali, M. D. 2000 Regeneration neurohormones and growth factors in echinoderms. *Can. J. Zool.* **79**, 1171–1208.

Tomoda, T., Shirasawa, T., Yahagi, Y., Ishii, K., Takagi, H., Furiya, Y., Arai, K., Mori, H. & Muramatsu, M. 1996 Transforming growth factor-β is a survival factor for neonate cortical neurones: coincident expression of type I receptors in developing cerebral cortices. *Dev. Biol.* **179**, 79–90.

Towbin, H., Staehelin, T. & Goedon, J. 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA* **76**, 4350–4354.

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