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Immunocalization of telomerase in cells of lizard tail after amputation suggests cell activation for tail regeneration

L. Alibardi

Comparative Histolab and Department of Bigea, University of Bologna, via Selmi 3, 40126 Bologna, Italy

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ABSTRACT

Tail amputation (autotomy) in most lizards elicits a remarkable regenerative response leading to a new although simplified tail. No information on the trigger mechanism following wounding is known but cells from the stump initiate to proliferate and form a regenerative blastema. The present study shows that telomerases are mainly activated in the nuclei of various connective and muscle satellite cells of the stump, and in other tissues, probably responding to the wound signals. Western blotting detection also indicates that telomerase positive bands increases in the regenerating blastema in comparison to the normal tail. Light and ultrastructural immunocytochemistry localization of telomerase shows that 4–14 days post-amputation in lizards immunopositive nuclei of sparse cells located among the wounded tissues are accumulating into the forming blastema. These cells mainly include fibroblasts and fat cells of the connective tissue and satellite cells of muscles. Also some immature basophilic and polychromatophilic erytroblasts, lymphoblasts and myelocytes present within the Bone Marrow of the vertebrae show telomerase localization in their nuclei, but their contribution to the formation of the regenerative blastema remains undetermined. The study proposes that one of the initial mechanisms triggering cell proliferation for the formation of the blastema in lizards involve gene activation for the production of telomerase that stimulates the following signaling pathways for cell division and migration.

1. Introduction

Regeneration of the tail in lizards is a remarkable case of amniote organ regeneration (Bellairs and Bryant, 1985; Alibardi, 2010a, 2014). Ultrastructural studies have shown that after amputation cells of different tissues in the tail stump accumulate on the free surface of the tail and give rise to a mass of mesenchymal-like cells called blastema, covered by a stratified (wound) epidermis, a cone-like structure resembling the developing tail bud formed during embryogenesis (Alibardi and Sala, 1988; Alibardi and Toni, 2005; Alibardi, 2010a,b). However the organogenetic potential of the tail blastema is much limited in comparison to the embryonic tail bud and simplified tissues are regenerated in comparison to the normal tail (Quattrini, 1954; Simpson, 1965; Werner, 1967; Cox, 1969; McLean and Vickaryous, 2011; Fisher et al., 2012; Gilbert et al., 2013; Lozito and Tuan, 2015).

The molecular signal that triggers the controlled proliferation, migration, and accumulation of blastema cells remains enigmatic, as well as the initial nuclear and cellular response of the injured tissues to the wound stimulation. Recent studies have indicated that the blastema derives from the activation of resident stem cells more than from tissue dedifferentiation (Alibardi, 2014). Other studies have shown that two cell regulator proteins, telomerase and p53/63, are activated during tail regeneration in lizards, addressing future studies on the mechanism allowing a controlled regulation of the process of tissue regeneration (Alibardi, 2015a,b). While p53/63 is mainly involved in the control of epidermal proliferation and differentiation, telomerase appears to be present in sparse connective cells. This enzyme consists in a protein component (TERT, Telomerase Retro Transcriptase) that associates to a special RNA-template (TERC, Telomerase RNA Component) for catalizing the addition of telomere sequences to chromosomal ends during DNA duplication. Since telomerase is believed to be constitutionally active in stem cells, or at least when they give rise to transient amplifying cells, the presence of few telomerase positive cells in the regenerating tail suggests that few stem cells are actually accumulated in regenerating tissues (Alibardi, 2015a,c), an observation also indicated in a recent molecular study on other stem cell markers (Hutchins et al., 2014). This initial observation has suggested searching for the presence of telomerase at earlier stages of wounding and blastema formation in comparison to normal tail tissues using immunological methods.

In the present study we have examined, using light and ultrastructural immunocytochemistry, and western blotting, for the

E-mail address: lorenzo.alibardi@unibo.it

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presence of telomerase in the early events of regeneration, the first 4–14 days post-wounding, when an extensive tissue degeneration, blood cell infiltration, and tissue recovering is taking place, preparing the surface of the tail stump to accumulate mesenchymal-like cells (Alibardi and Sala, 1988; Alibardi, 2010b; McLean and Vickaryous, 2011).

2. Materials and methods

2.1. Western blotting

Two normal tails from *Podarcis muralis* were utilized for protein extraction. Three other samples from *P. muralis* and two from *Anolis carolinensis*, consisting of the stump (1–2 mm) and their relative conical blastema (2–3 mm in length) formed at 14 days after amputation were also utilized. Before analysis, the tail stump was separated from the blastema, and the two pieces were analyzed independently. Tissues were homogenized in 8 M urea/50 mM Tris–HCl at pH 7.6 containing 0.1 M 2-mercaptoethanol/1 mM dithiothreithol/ and 1% protease inhibitor (SIGMA, St. Louis, USA). The particulate matter was removed by centrifugation at 10,000 × *g* for 5 min, and protein concentration was assayed by the Bradford method before electrophoresis.

For the electrophoresis analysis, 70 μg of proteins were loaded in each lane and separated in 12% SDS-polyacrylamide gels (SDS-PAGE) using the MiniProtean III electrophoresis apparatus (Bio-Rad). For western blotting, the proteins separated in SDS-PAGE were transferred to nitrocellulose paper. After Western blot, membranes were stained with Ponceau red to verify the protein transfer and incubated with primary anti-rabbit antibody. In controls, the primary antibody was omitted. Detection was performed using the enhanced chemiluminescence procedure developed by Amersham (ECL, Plex Western Blotting System, GE Healthcare, UK).

2.2. Fixation and microscopic methods

Eight wall lizards (*P. muralis*) and three anole lizards (*A. carolinensis*) were utilized in the present microscopic study. The adopted procedures for care and handling the animals followed the Italian guidelines (Art. 5, DL 116/92). After inducing autotomy in *P. muralis*, a natural process of tail amputation that does not harm the animal, the tail was allowed to repair for 4 ($n = 4$), 8 ($n = 2$) and 14 ($n = 2$) days at room temperature (25–30 °C) when the blastema was formed over the tail stump. Also for *A. carolinensis* the tail was obtained by induced autotomy (grabbing and twisting the tail to exploit the release of the tail by the animal), and the tail was left to regenerate for 8 days (1 animal) and 14 days (2 animals) until the blastema was formed on the tail stump. The initial, more proximal part of the amputated tail was also fixed or collected for biochemical analysis (see later), and served later as control of the adjacent stump. The stumps or the regenerating tails were collected, halved and the two pieces were fixed for 5 h in cold (0–4 °C) 4% paraformaldehyde in 0.1 M Phosphate buffer at pH 7.4, rinsed in buffer for about 30 min, dehydrated in ethanol (70°, 80°, 95°, 100°), and embedded in Bioacryl Resin (Scala et al., 1992).

The tissues were sectioned using an ultramicrotome, and 2–3 μm thick sections were collected on glass slides, and stained with 1% Toluidine blue for histology, or were attached to precoated slides for the following immunostaining. Thin sections of 40–90 nm in thickness were collected on Nickel grids for immunogold detection at the transmission electron microscope. The primary antibody, an anti-telomerase-1 component antibody selected on a specific epitope of the lizard *A. carolinensis*, was produced and affinity purified from immunized rabbits and previously characterized (Alibardi, 2015a), using a Biotechnology company (Davids Biotechnology, Germany).

For light microscopy immunocytochemistry the sections were incubated overnight for 4 h at room temperature with the primary antibody diluted 1:100 in buffer (Tris 0.05 M at pH 7.6 containing 5% BSA). After rinsing in the buffer, the sections were incubated for 60 min at room temperature with a fluorescein-conjugated anti-rabbit antibody (FITC, Sigma, diluted 1: 200), rinsed in buffer, mounted in anti-fading medium (Fluoroshield, Sigma, USA), and observed under a fluorescence microscope using a fluorescein filter. Pictures were taken by a digital camera (Euromex, The Netherlands) and digitalized into a computer using the Adobe Photoshop Program.

For electron microscopy immunocytochemistry, sections on Nickel grids were incubated for 4 h at room temperature in the primary antibody diluted in 0.05 M Tris–HCl buffer at pH 7.6 containing 1% Cold Water Fish Gelatin. In negative controls, the primary antibody was omitted. After the incubation period the sections were rinsed in buffer and incubated for 1 h at room temperature with goat anti-rabbit Gold-conjugated secondary antibody (Sigma, 5 or 10 nm gold particles). Some grids went through a silver enhancing treatment, to increase the size of gold particles and make the labeling more easily detectable under the electron microscope. The suggested intensification method followed the manufacturer instructions (British Biocell International, SEKB250). Grids were rinsed in the buffer, dried and stained for 4 min with 2% uranyl acetate, and then observed under an electron microscope (Zeiss C10).

3. Results

3.1. Western blotting

The tail extracts from *A. carolinensis* showed weak bands at 150–160 and weaker at 60 kDa in the tail stump (Fig. 1, first lane). The blastema instead showed more intense bands, mainly at 150–160 kDa, and around 200, 120, and 60–70 kDa (Fig. 1, lane 2). In the control of the regenerating tissues (blastema), no bands were seen (Fig. 1, lane 3). The normal tails and stumps of *P. muralis* showed similar faint labeling in the expected range, at 100–150 kDa and only a main band around 40–45 kDa was seen (Fig. 1, lane 4).

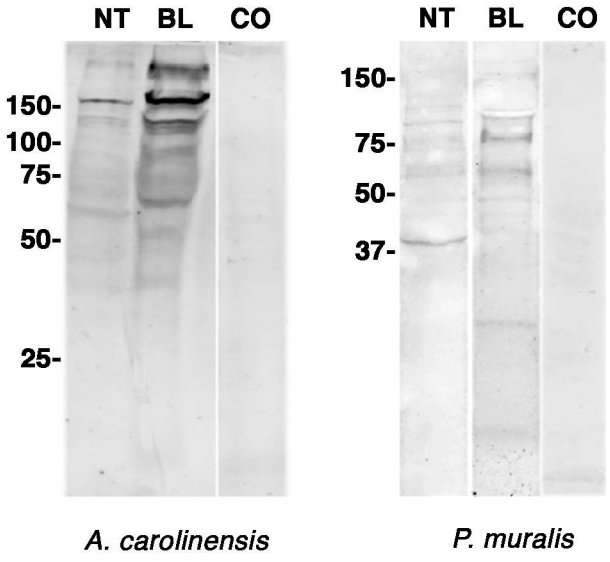

Fig. 1. Western blot of protein extracts and immunoreacted for telomerase from the normal tail (NT) in comparison to the regenerative blastema (BL, 14 days regeneration) in *A. carolinensis* and *P. muralis*. CO, controls (blastema). Numbers on the left indicate the molecular weight in kilo-daltons (see text for description).