Adenosine enhances progenitor cell recruitment and nerve growth via its A2B receptor during adult fin regeneration

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Abstract A major issue in regenerative medicine is the control of progenitor cell mobilisation. Apoptosis has been reported as playing a role in cell plasticity, and it has been recently shown that apoptosis is necessary for organ and appendage regeneration. In this context, we explore its possible mode of action in progenitor cell recruitment during adult regeneration in zebrafish. Here, we show that apoptosis inhibition impairs blastema formation and nerve growth, both of which can be restored by exogenous adenosine acting through its A2B receptor. Moreover, adenosine increases the number of progenitor cells. Purinergic signalling is therefore an early and essential event in the pathway from lesion to blastema formation and provides new targets for manipulating cell plasticity in the adult.

Keywords Apoptosis · Stem cells · Adenosine · A2B receptor · Nerve growth · Proliferation · Zebrafish · Regeneration

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Introduction

The ability to regenerate amputated or injured body parts varies greatly from one species to another [1, 2]. In adult amphibians and fish, the regeneration of complex structures such as appendages or organs involves the coordinated renewal of many cell types from a small group of cells [3, 1, 2]. Following amputation, cells of different lineages from the stump respond to injury by dedifferentiating and acquiring a progenitor identity [1]. The progenitor cells then accumulate at the damaged surface underneath the wound epithelium and form a mass of proliferating cells—the blastema [4, 5, 1, 6]. The missing structure will be replaced through blastema growth differentiation and morphogenesis. In regenerative models, the replacement of damaged organs or appendices requires the coordination of three modules: an early module that corresponds to the early injury response [7]; a second module that is interlocked with the first module and one that still needs to be clarified but includes apoptosis and reactive oxygen species (ROS) signalling [8–11] and is nerve-dependant [12–14]; and the last module, which corresponds to blastema growth and developmental programmes to be activated again to drive the differentiation and morphogenesis of a correct structure [1, 15]. Crucial questions that remain unanswered include how amputation drives the dedifferentiation of cells into a progenitor state and the nature of the signalling system that induces their recruitment and migration to form the blastema [16].

Immediate injury-induced apoptosis is part of the early wound healing response [7], and it has been shown that programmed cell death—and, more directly, caspase activity—is essential to the regenerative capacities of different

regenerative models [17, 8, 10, 11]. Apoptotic cells release different signalling molecules involved in proliferation and cell plasticity. In invertebrates, it has been shown that apoptotic cells release Wnt and BMP signals that are directly responsible for compensatory proliferation in injured *Drosophila* imaginal discs [18] and head regeneration in hydra [8]. In mammals, it has been shown that during the process of apoptosis, activated caspases stimulate Ca²⁺-independent phospholipase A2 (iPLA2) to trigger the production of prostaglandin (PGE2), which in turn induces compensatory proliferation [19, 20]. This induction of cell proliferation, via apoptosis/PGE2, near the dying cells is essential for skin wound healing and liver regeneration in mice. In addition, this induction of proliferation by apoptotic cells is also engaged as a compensatory proliferation mechanism during radiotherapy [21, 22]. One of the known mechanisms of apoptotic cells during wound healing is the release of ATP through pannexin-1 channel activation by caspases [23]. Extracellular ATP signalling appeared very early in evolution and relies on receptors and translocators [24]. Once secreted, ATP may signal via direct interaction with receptors [25] or after hydrolysis by ectonucleotidase [26]. ATP hydrolysis gives rise to different metabolites, the last one being adenosine which can signal via adenosine receptors (AdoR) or translocators (Fredholm, 2001). ATP and adenosine signalling pathways are not mutually exclusive and are subject to considerable crosstalk [27]. ATP release participates in the “find me signal,” which attracts phagocytes to the lesion site to resolve the wound [28, 23]. The infiltration of macrophages is required for blastema formation in newts regenerating their limbs [29], as well as in adult zebrafish regenerating their caudal fin and their heart [30, 31]. Within the first 24 hpa, these macrophages release proteases, growth factors, and cytokines that are important for tissue remodelling and the stimulation of proliferation. Recent studies have shown that purinergic signalling is a key element in cell proliferation [32–35] and in nerve physiology [36–38]. Finally, ATP is an essential molecule in the communication between neuron and glia [39], an essential function in systems in which regeneration is nerve-dependent [40, 14].

Purinergic signalling components are very conserved amongst vertebrates and have been identified and studied in zebrafish (for review, see [41]). Recent studies showed that acute restraint stress enhanced ROS production in the brain [42] and modified purinergic signalling in adult fish [43]. Considering that caudal fin amputation triggers ROS production which is essential for regeneration [10], we decided to test directly whether the purinergic signalling engaged by apoptotic cells during adult caudal fin regeneration is of importance for cell plasticity, i.e. progenitor recruitment and nerve regrowth.

Materials and methods

Fish care, surgery and quantification of regeneration

Zebrafish colonies (AB-Tu and gold fish) were maintained using standard methods [44]. In order to maintain a healthy colony, a cycle of 14 h light–10 h dark was used and a water temperature of 28 °C was maintained with five fish per litre maximal density. Water filtration depends on aquatic habitats stand-alone fish housing and operates automatically (Aquatic Habitat Inc, FL, USA). Fish were feed twice a day with live 2-day-old paramecia. For manipulation and amputation, the adult zebrafish (5–10 months of age) were anaesthetised in 0.1 % tricaine (ethyl-*m*-aminobenzoate), the caudal fins were amputated at the level of the first ray bifurcation and the fins were allowed to regenerate for various lengths of time. The fish were then anaesthetised, and the regenerating fins were collected for further analysis. The efficiency of regeneration was quantified at 3 dpa (day post-amputation). The surface of the blastema was measured and subsequently divided by the square length of the amputation plane for each fish. The efficiency of regeneration is expressed as a percentage of the control.

Pharmacological treatments

Four to six fish (most of the time five) were incubated in 200 ml of water for all pharmaceutical treatments. NS3694 (Apo-i) was purchased from Calbiochem (# 178494) and used at a final concentration of 5 µM. The fish were kept in the dark and put back into the light for 1 h per day for feeding and water change. Fish incubated in water or DMSO (1/10,000) comprised the control group. AMP (Sigma # 01930), ATP (Sigma # A1852) and adenosine (Sigma # A9251) were prepared extemporaneously in water. If not specified, adenosine was used at a final concentration of 2.6 nM. 2-Chloro-*N*6-cyclopentyl-adenosine (CCPA) (Sigma #C7938), 2-*p*-(2-carboxyethyl)-phenethylamino-5'-*N*-ethylcarboxamido-adenosine (CGS21680) (Sigma #C141), *N*-ethylcarboxamido-adenosine (NECA) (Sigma #E2387), MRS1754 (Sigma #M6316), AMPCP (sigma #M8386) and M50054 (Merck #178488) were prepared in DMSO.

Labelling of dividing cells with BrdU

The labelling of proliferating cells with bromodeoxyuridine (BrdU) was performed according to [45].

Statistical analysis

Continuous variables were expressed as the means±SEM. Comparisons between multiple groups were performed by

one-way ANOVA followed by Tukey's post tests. Comparisons between the two groups were subsequently performed by a Student's *t* test. *p* values <0.05 were considered to indicate statistical significance.

Results

Purinergic pathway downstream of apoptotic cells is essential for blastema formation

We have previously shown that apoptosome inhibition with NS3694 inhibitor (Apo-i) leads to a reduction in progenitor cell expression and blastema formation [10]. In order to test whether purinergic signalling is downstream of apoptotic cell signalling, we tested the effects of purines, ATP and adenosine (Ado) on rescuing the inhibition of blastema formation caused by Apo-i. The caudal fins were amputated, and

the fish were incubated in Apo-i and purines from the time of amputation to 3 dpa. The size of the regenerate was then measured (Fig. 1a–g). As previously shown [10], Apo-i strongly reduced the size of the regenerate at 3 dpa (Fig. 1b, g). This effect was rescued with ATP and adenosine treatments from the time of amputation to 3 dpa (Fig. 1c–e, g), demonstrating that purinergic signalling acts downstream of apoptosis. Furthermore, adenosine alone increased the size of the blastema (Fig. 1f–h), identifying it as a molecule that stimulates regeneration, whereas ATP molecules do not (Fig. 1d, g and Fig. S1). To better characterise the mode of adenosine production, we used an ADP analogue (AMPCP, 5 μ M) to block ectonucleotidase activity. Fish were treated with AMPCP or AMPCP and Ado from the time of amputation to 3 dpa. The size of the regenerate was then measured (Fig. 1i–k). AMPCP reduced the size of the regenerate, and this effect was rescued with Ado. Together, these results suggest that adenosine, produced by ectonucleotidases, is the purine engaged by apoptotic cells for blastema formation.

Fig. 1 Purinergic signalling downstream of apoptosis. **a–g** ATP (10 nM) and adenosine (2.6 nM) supplementation rescued the inhibition of blastema formation caused by the apoptosis inhibitor Apo-i (5 μ M). Representative fins are shown. **g** The efficiency of regeneration was quantified at 3 dpa and is expressed as a percentage of the control. **h** Adenosine enhances the regenerate size at 3 dpa. **i** AMPCP (5 μ M) inhibits regeneration that can be rescued by adenosine. **j, k** Representative fins are shown. Error bars represent the SEM (**p*<0.05; ***p*<0.01; ****p*<0.001). Arrowheads indicate the amputation plane. *n* values were indicated at the bottom of each bar of the graph

Fig. 2 Adenosine signals through its A2B receptor. **a** The effect of different adenosine receptor agonists and an antagonist on fin regeneration. Only 5-*N*-ethylcarboxamido-adenosine (NECA 10 nM), a preferential A2 receptor agonist, stimulated fin regeneration, and the A2B antagonist MRS17541 (10 nM) inhibited blastema formation. **a** In combination with adenosine, MRS17541 (1 nM) abolished the effect of adenosine on

blastema size. **b–e** NECA can rescue inhibition by Apo-i. Fish were incubated in different compounds, and the efficiency of regeneration was quantified at 3 dpa and is expressed as a percentage of the control. Representative fins are shown. Error bars represent the SEM (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). *n* values were indicated at the bottom of each bar of the graph

Adenosine acts via its A2B receptor

We tested whether adenosine signals via a receptor or nucleoside transporter using a pharmacological approach (Fig. 2 and Fig. S1). Four adenosine receptor (AdoR) subtypes (A1, A2A, A2B and A3) and two transporters

(ENT1 and 2) have been identified in vertebrates [46] and the subtypes A1 [43] as well as A2A and B [47] studied in zebrafish [41]. After caudal fin amputation, the fish were incubated in agonists or an antagonist of AdoR, and the size of the regenerate was analysed at 3 dpa (Fig. 2a). The nucleoside transporter agonist (NBTI+dipy, 10 μ M

Fig. 3 Adenosine is involved in nerve regrowth after amputation. **a–d** Growing nerves were detected in the regenerating fin (24 hpa) by immunodetection of acetylated β -tubulin in fish incubated in Apo-i, adenosine or vehicle. The quantification corresponds to the number of rays with growing nerves per half fin (maximum=9). **e** Apo-i inhibits nerve regrowth, which can be rescued with adenosine. Error bars represent the SEM (*** $p < 0.001$). DMSO: $n=11$, Apo-i: $n=11$, Ado: $n=10$, Apo-i+Ado: $n=10$

each) did not have an effect on fin regeneration and neither did the agonists for AdoR A1 (CCPA) and A2A (cgs 21680) (Fig. 2a–c and Fig. S1). Either these molecules play no role in blastema formation or the drugs used are not efficient on zebrafish receptors as they are on mammalian ones. However, NECA, an AdoR A2 agonist, stimulated blastema formation to the same degree as adenosine, whereas MRS17541, an AdoR A2B antagonist, inhibited blastema formation (Fig. 2a and Fig. S1). Furthermore, the AdoR A2 agonist (NECA) was able to rescue the phenotype observed after apoptosis inhibition (Fig. 2b–e). Furthermore, the positive effect of adenosine on regeneration was suppressed by the AdoR A2B antagonist MRS17541 (Fig. 2a), which suggested that adenosine stimulation of regeneration occurred via the A2B receptor. Collectively, these data demonstrate that adenosine signals through its A2B receptor and identify adenosine as a relay between apoptotic cells and blastema formation or growth.

Adenosine is responsible for nerve regrowth after amputation

In parallel to wound healing, injured nerves regrow. It is well known that innervation plays a crucial role in blastema formation and growth [12, 13] (for review, see [48, 1, 14]). As adenosine is a key regulator of neuron physiology [49, 50], we analysed the impact of apoptosis inhibition and adenosine treatment on sensory neuron regrowth after amputation (Fig. 3). After amputation of the caudal fin, fish were incubated in Apo-i and/or adenosine until 24 hpa, and sensory axons were visualised with an anti-acetylated β -tubulin antibody. The number of rays with growing nerves that overpass the amputation plane was counted (Fig. 3e). We observed that apoptosis inhibition significantly reduced the number of growing nerves (Fig. 3a–b, e) and that this inhibition could be rescued by adenosine treatment (Fig. 3d–e). It therefore appears that after amputation, nerves require apoptosis and purinergic signalling to regrow.

Fig. 4 Adenosine increases epidermal cell proliferation and the number of recruited progenitor cells. **a** BrdU was injected in the peritoneal cavity at 15 or 24 hpa, and the fin was fixed at 24 or 35 hpa. BrdU-positive cells were immunostained. **b, c** Staining on whole fin and transversal sections showed an increase in the number of positive cells in samples treated with adenosine (*right panels* represent a higher magnification of the inlet in the corresponding left panels). It is noticeable that in the control, BrdU-positive cells are mainly found in the inter-rays, while adenosine is able to enhance proliferation in both rays and inter-rays. **d, e** Progenitor cells were detected on longitudinal sections by bromodeoxyuridine (BrdU)

incorporation in fish allowed to regenerate in presence of adenosine. **d** At 24 hpa, adenosine enhances the number of mesenchymal mitotic cells. Fin longitudinal sections are shown. **e** When the BrdU pulse was performed at a later time point (between 24 and 35 hpa) and the fins were analysed at 35 hpa, numerous BrdU-positive cells were detected under the wound epidermis (*arrow*) in samples incubated with adenosine compared to the controls. Representative longitudinal sections are shown. The *dashed lines* indicate the amputation plane. Error bars represent the SEM (* $p<0.05$; ** $p<0.01$). Scale bars=100 μ m. Four fish per condition were used in all the experiments

Adenosine stimulates stump epidermal cell proliferation and increases the number of progenitor cells

It has been previously shown that apoptosis is responsible for compensatory proliferation observed in the stump epidermis at the level of inter-rays shortly after amputation [10]. To analyse whether adenosine is involved in this process, we performed a BrdU experiment to label proliferating cells from 15 to 24 hpa. After the amputation of the caudal fin, adenosine was added to the water of the tanks at the time of amputation, and BrdU was injected in the abdominal cavity at 15 hpa. BrdU incorporation was analysed at 24 hpa (Fig. 4a–b). In the absence of adenosine, BrdU-positive cells were mainly detected in the interray epidermis, where apoptosis occurred (Fig. 4b). In adenosine-treated fish, BrdU-positive cells were uniformly distributed in the epidermis and were much more abundant than in the control (Fig. 4b–c). These results indicate that adenosine stimulates epidermal cell proliferation after lesion formation and that epidermal cells localised in both rays and inter-rays can respond to adenosine.

To further characterise the role of adenosine in progenitor cell recruitment, we then analysed adenosine impact on the number of recruited progenitor cells. Although the expression of progenitor cell markers has been reported in fish [51, 10] and amphibians [52], unbiased *in situ* visualisation of the recruited cells is still required. Recruited mesenchymal cells in the stump mesenchyme re-enter into the cell cycle and migrate to the wound while still dividing. This cell cycle entry is the current criterion used to identify progenitor cells that have dedifferentiated and migrated to the wound epidermis. The caudal fins were amputated, and the fish were incubated with or without adenosine from the time of amputation; BrdU was injected in the abdominal cavity either at 15 or 24 hpa (Fig. 4a, d–e). BrdU-positive cells were analysed at 24 hpa to detect the progenitor cells in the mesenchyme while they are migrating to the distal part of the stump (Fig. 4d) or at 35 hpa when they have reached the wound (Fig. 4e). Adenosine treatment between 15 and 24 hpa enhanced the number of BrdU-positive cells (twofold) in the mesenchyme (Fig. 4d), and the elongated shape of the positive nuclei suggested that they correspond to migrating cells. BrdU incorporation between 24 and 35 hpa showed that adenosine increases the number of positive cells that have overpassed the amputation plane to form the blastema (threefold) (Fig. 4e). Adenosine therefore appears to increase the number of progenitor cells. However, this experiment does not discriminate between an increased number of recruited cells forming the blastema or a higher rate of proliferation of these cells. To specify the effect of adenosine, we addressed its impact on the rate of blastema growth. After amputation, the size of the regenerate was measured everyday until 7 dpa. Figure 5 shows that the adenosine effect is restricted to the period of blastemal cell

recruitment from 1 to 3 dpa. Adenosine had no effect on the rate of blastema growth once the cells were established. This suggests that adenosine enhances the number of progenitor cells recruited, but even if the number of blastemal cells is increased during blastema formation, tight proliferation regulation takes place in the growing blastema and limits the rate of division.

Discussion

In recent years, the definition of progenitor cells regarding their origin and maintenance has evolved, and the re-programming of differentiated cells has become a hot topic of research interest. A major issue that remains is how to control the cellular neighbourhoods in order to promote the differentiated/progenitor transition *in vivo*, and in this context, epimorphic regeneration in amphibians and fish provides relevant models of integrated regeneration [15, 16]. Although it seems that any type of differentiated cell is able to participate in blastema formation [6, 53–57], only a few types will respond to injury by progressing towards progenitor identity. The lesion must induce a response that is differentially sensed by stump cells. In turn, mobilised cells must tell the surrounding cells to keep their genetic expression programme intact to assume tissue integrity. There is still a long way to go to fully understand this process and be able to control it; however,

Fig. 5 Rate of blastema growth for adenosine-treated fish. Fish were incubated in adenosine (2.6 nM) from the time of amputation to 7 dpa. The regenerate size was quantified everyday. Error bars represent the SEM (* $p < 0.05$; ** $p < 0.01$). Thirty fish were used for each condition

here, we show that adenosine signalling is a piece of the puzzle.

The promotion of cell division by apoptotic cells has been well documented in the last few years and can now be considered a key element in tissue homeostasis [58, 11]. We provide evidence that the A2B receptor is involved in the regulation of compensatory proliferation shortly after amputation, and this correlates with findings in A2B receptor physiology [59]. Indeed, it has been shown that A2B receptor expression is induced by hypoxia and that A2B signalling plays a tissue protective role in many models [60].

The second impact of the inhibition of apoptosis/adenosine signalling is the reduction of progenitor (blastemal) cell recruitment. At this stage, we have no basis on which to correlate the two phenomena, and no direct link has been drawn between proliferation in the stump epidermis and blastemal cell recruitment to date. A direct effect of epidermal cell proliferation on blastemal cell recruitment presumes a long-range signalling mechanism. This could be achieved directly, as it has been shown that adenosine can diffuse up to 120 μm in a tissue [41] or indirectly by mechanical forces induced by epithelium deformation. The concept of “mechano-niche” has recently been proposed [61], and studies on the response of stem or progenitor cells to mechanical forces have recently been published (see [62] for review). However, the design of conclusive experiments in adults is still difficult.

Finally, nerves and macrophages are ideally suited to fulfil the relay role between apoptotic cells: adenosine in the stump epidermis and blastemal cell recruitment in the mesenchyme. They are present during this process, and they respond to adenosine. In parallel to epidermal cell proliferation, injured nerves regrow, and it is well known that innervation plays a crucial role in blastema formation and growth (see [12, 13] and [48, 1] for review). As it has been shown that adenosine signalling controls the guidance of sensory neuron growth cones [36], one can hypothesise that nerve regeneration stimulated by adenosine could be a relay between epidermal apoptosis and progenitor recruitment. Finally, adenosine signalling also regulates the immune response [28, 27], and it has been recently shown that macrophages are required for adult limb regeneration in newts [29].

Our results provide new insight into the signalling mechanisms for body part regeneration. Cell death is necessary for blastema formation and sensory nerve regeneration. We identified adenosine as the relay between apoptotic cells, leading to epidermal cell proliferation, nerve regrowth and blastema formation. Adenosine is sufficient to stimulate progenitor cell recruitment during fin regeneration and acts via the A2B receptor. We note that purinergic signalling is an early and essential event in the pathway from lesion to blastema formation, and these results provide new targets for manipulating cell plasticity in the adult.

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