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TO REGENERATION … WITH COMPLEMENT

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1. REGENERATIVE ABILITIES IN VERTEBRATES

Apart from wound healing (or wound repair), which is mostly closure of a wound by scar tissue, the degree of tissue renewal or regeneration in vertebrates varies in different tissues. In fact, what is different is the complexity involved in the mechanisms and magnitude of regeneration. The simplest form of regeneration is the axonal outgrowth seen in a severed nervous system. Regeneration by simple proliferation seen in organs, such as intestines, liver, or adrenal gland, is somewhat more complex. It involves proliferation of cells that compose the particular organ. Regeneration of other organs and tissues, on the other hand, can be channeled through proliferation and differentiation of stem cells. More complex types of regeneration involve dedifferentiation. In these cases (mostly seen in amphibia), a particular cell type at the damaged site dedifferentiates and then redifferentiates into the same type. Regeneration of CNS (brain, spinal cord), intestine and heart can be achieved by this mechanism of dedifferentiation. An even more complex type of regeneration involves transdifferentiation from one cell type to another and can be seen during pancreas regeneration. The most complex type of regeneration, however, is seen in amphibian limb and lens regeneration, in which cells at the damaged or amputated site dedifferentiate and transdifferentiate, but they are also able to build back an exact replica of the lost part. This is indeed the most spectacular type of regeneration, and is restricted to some urodeles. The other types of regeneration are rather widespread among vertebrates[1].

In our laboratories we have been committed to work with the amphibian models because they can provide information on all the mechanisms that are

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involved in the different types of regeneration. We strongly believe that knowledge from the regenerative abilities of amphibia could be applied to other animals as well. In the following sections, we will familiarize the reader with the process of limb and lens regeneration, the two systems we have selected in which to examine the role of the complement system.

2. LIMB REGENERATION

Among amphibia, both anura and urodeles are capable of limb regeneration; however, only some urodeles are capable of regeneration as adults. Most of the anura (frogs) lose their regenerative properties after metamorphosis. This makes some newts and salamanders the most gifted ones. These animals can regenerate their limbs throughout their lives and as many times as they are amputated[2,3].

Upon amputation the events that lead to regeneration of the missing part are initiated immediately. The first apparent histological event to be noticed is coverage of the wound by the so-called wound epithelium, which starts soon and ends within a few hours after amputation. The presence of this specialized wound epithelium is of great importance since its removal would not allow the subsequent events of dedifferentiation and regeneration to take place. Once the cover has been established, the remaining tissues of the intact limb (called the stump) undergo a dramatic cellular event and lose the characteristics of their origin. It is believed that the wound epithelium provides the critical signal for dedifferentiation[4].

The event of dedifferentiation is unique in these animals and is a necessary prerequisite for regeneration to occur. Terminally differentiated cells, such as muscle, virtually melt down and become mononucleated cells. The remodeling of the extracellular matrix is paramount for the process of dedifferentiation. Many proteins and enzymes — such as intergins, collagens, and collagenases — are expressed specifically during this period[1,2]. Dedifferentiation of the normal terminally differentiated tissues is the key to the formation of the blastema, which is a mass of dedifferentiated cells that proliferate for about 2 weeks and then redifferentiate to give rise to the missing part. In other words, the blastema cells are "embryonic-like," because they have the potential to form a normally patterned limb similar to the one formed during embryonic development[2,3].

The blastema is the product of dedifferentiation of many different tissues, such as muscle, cartilage, bone, and mesodermal cells. The blastema cells redifferentiate and produce the lost part of these tissues. A blastema cell needs not to differentiate to a cell it came from. In other words, blastema cells from muscle can transdifferentiate to form cartilage. This has been shown by clonal blastema cells derived from muscle and transplanted back onto a host-regenerating limb[2,5].

Several important factors have been found to be expressed during the above-mentioned phenomena. Expression of fibroblast growth factors and their receptors in the wound epithelium have been associated with the critical signal-

ing for dedifferentiation[6, 7]. It has been found that expression of FGF-8, FGFR-1, and FGFR-2 is correlated with the ability of limb regeneration in premetamorphic frogs[8,9]. On the other hand, retinoblastoma protein, Rb, has been implicated in reentry of the cell cycle during muscle dedifferentiation[10]. When multinucleated muscle cells are allowed to enter the cell cycle by serum stimulation (possible only in newt muscle), Rb becomes mostly hyperphosphorylated. This represents its inactive form. The active hypophosphorylated form inhibits entry into the S phase in myotubes. The serum factor that stimulates myotubes to dedifferentiate and enter the cell cycle seems to be thrombin[11]. The cascade of mechanisms involved in these events is still elusive.

3. LENS REGENERATION

When the lens of a newt is removed, the process of regeneration is always initiated from the dorsal iris. The pigment epithelial cells (PECs) from the dorsal iris proliferate, dedifferentiate, and then transdifferentiate into lens cells. The process of proliferation and dedifferentiation takes a few days. During this period PECs initiate DNA synthesis and eventually lose their characteristics of origin, such as pigmentation. At about 7–10 days post-lentectomy a small vesicle has been formed at the tip of the dorsal iris. Cells in this vesicle then transdifferentiate into lens cells and form the lens vesicle (10–15 days). Cells from the posterior part of the lens vesicle differentiate to form the lens fibers (15–20 days). Lens regeneration is complete by 25 days post-lentectomy[1,12].

FGF signaling seems paramount in lens regeneration as well. FGFR-1 protein has been found to be specifically expressed during dedifferentiation in the dorsal iris, and its inhibition by specific FGFR-1 inhibitors results in no lens regeneration[13,14]. Other factors that seem to be involved in lens regeneration are the homeo-box-containing Pax-6 and Prox-1 genes[15-17] as well as retinoid receptors[18]. These regulatory factors are also expressed in regeneration-competent tissues of the dorsal iris and might regulate the normal ability of eye tissues to transdifferentiate to lens.

The reader should not fail to see that some of the mechanisms involved in limb and lens regeneration are similar. Especially FGF signaling could be common in both regenerative systems[1]. Indeed, the eye, for example, is not a necessary physical environment for transdifferentiation and lens formation. If dorsal PECs are transplanted in the regenerating limb blastema they transdifferentiate and form a perfect lens! The ventral PECs are not able to do this[19]. This means that the factor(s) responsible for signaling must be common during limb and lens regeneration.

However, when PECs from the dorsal or ventral iris from any animal, including old humans, are placed in vitro they all have the capacity for transdifferentiation[20]. It seems that dissociation and culturing of cells, which alters their extracellular environment considerably, can be an inductive mechanism. The

study, therefore, of limb and lens regeneration in parallel could be very informative when it relates to mechanisms of regeneration. That is why in this proposal both systems will be studied in order to pinpoint common themes in regeneration. This could be more useful when it comes to more broad applications in the different fields of regeneration.

4. THE COMPLEMENT SYSTEM

The complement system is comprised of several serum proteins, membrane-bound receptors, and regulatory proteins that constitute a phylogenetically ancient mechanism of innate immunity[21]. The functions of the complement system in host defense and inflammation are mediated mainly through sequential activation and proteolytic cleavage of serum proteins. Complement activation occurs through three distinct pathways (classical, alternative, and lectin) — all of which converge at activation of C3, the third component of complement. C3 can interact with a wide spectrum of factors, and because of that it is able to mediate a wide variety of functions[22,23]. C3 interacts with several proteins that are involved in differentiation, such as fibronectin and integrins[24,25]. Other complement factors share homologies with domains of extracellular matrix proteins, such as collagen binding, which might indicate that complement factors could be involved in such interactions in the extracellular matrix[26]. Therefore, some complement functions might not be immunologic. For example, C3 is expressed in myoblasts and is also associated with proliferation and growth of B-cells in vitro[27].

   C5, the fifth component of complement, has also been found to have novel noninflammatory functions in various tissues. Studies in a human neuroblastoma cell line have suggested that C5a (a fragment of C5) participates in apoptotic signal transduction pathways through its binding to the neuronal C5a receptor[28,29]. It has also been shown that the terminal complex system C5b-9 (MAC) in sublytic doses can induce DNA synthesis and cell proliferation in cultured mouse fibroblasts[30], human aortic smooth muscle cells[31], oligodendrocytes[32], and glomerular epithelial cells, in the absence of other growth factors[33]. Also, sublytic concentrations can activate monocytes and induce cytokine release through activation of NF-kappaB signaling pathways, which are critical for cell cycle transition into DNA synthesis[34].

   The possible role of complement in differentiation of muscle and its interaction with molecules that are involved in cell-to-cell communication and signaling prompted us to examine expression of complement factor C3 in limb regeneration. We have found expression of C3 during blastema formation and limb regeneration[35]. The fact that C3 is not expressed in the intact or developing limb indicated to us specificity for the regeneration process. Furthermore, we were able to show expression of C3 in dedifferentiated muscle cells in vitro.
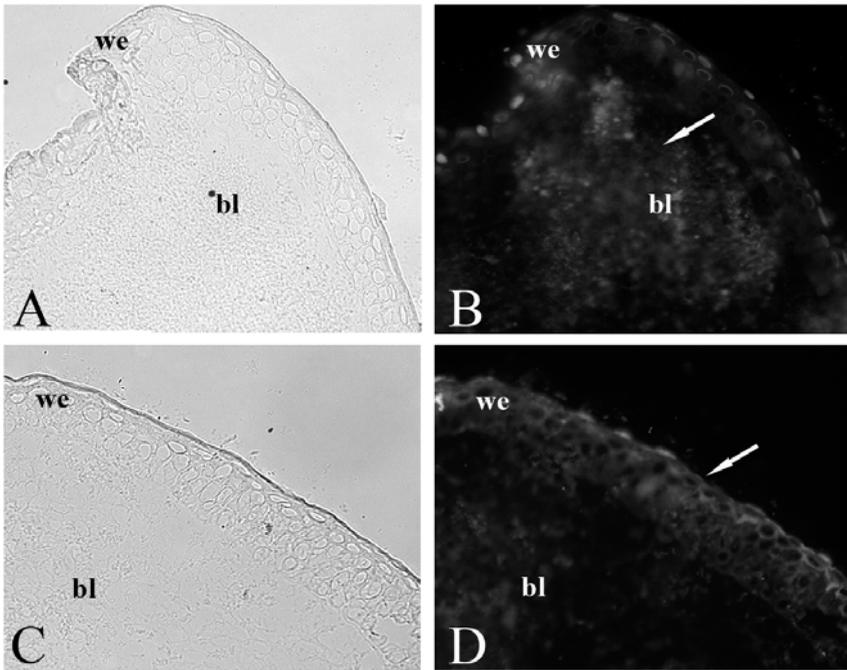
Figure 1 (see color insert, Fig. 5.1). Expression of C3 (A,B) and C5 (C,D) during limb regeneration, 3 weeks after amputation. Note expression of C3 in the blastema cells (bl; arrow) and of C5 in the wound epithelium (we; arrow). B and D have been counter-stained with DAPI.

Encouraged by these interesting results, we asked the question of whether complement factors are expressed in other regenerative tissues. C3 was expressed during lens regeneration as well, strengthening our conviction for a role in the control of regeneration processes in amphibia[36]. Experiments from our team dealing with liver regeneration in mice, which can be regenerated by proliferation of the remaining hepatic cells, showed that C5 is imperative for the process. Mice lacking C5 were not able to repair the liver, and they were able to do so only when they were reconstituted with C5[37].

All this information gives credit to our idea that the complement system has a new role — namely, control of regenerative processes. The long-term goal of this research is to establish the relationship between complement and regeneration and identify the mechanisms by which complement controls regeneration. Biochemistry of the complement factors is well understood, and this will enable us to dissect the mechanisms whereby the complement factors control regenerative processes. This knowledge can be applied in other systems and eventually might allow us to devise strategies that can be used to induce regeneration.
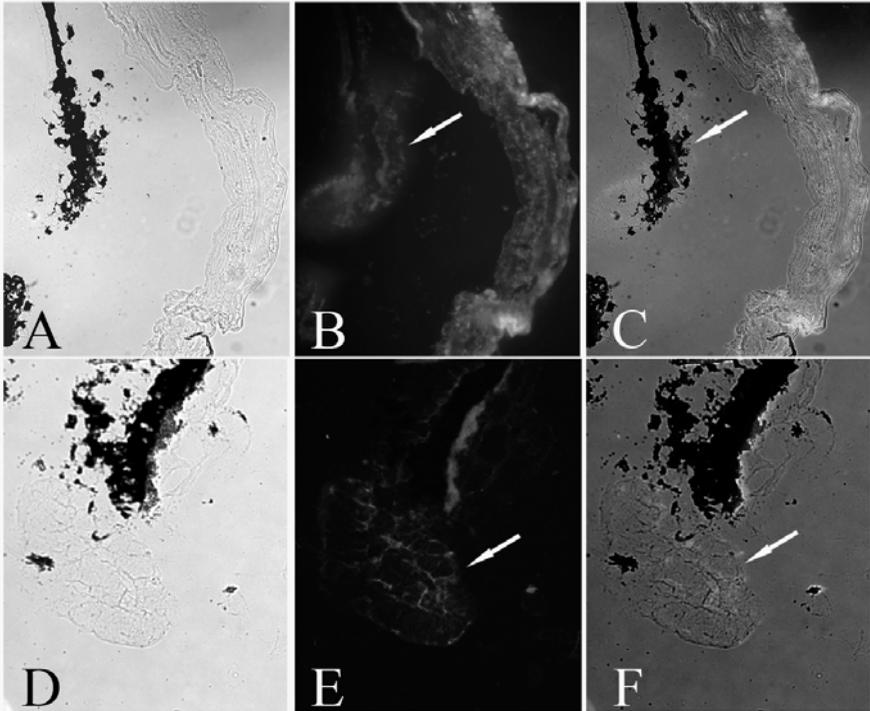
Figure 2 (see color insert, Fig. 5.2). Expression of C3 (A–C) and C5 (D–F) during lens regeneration 20 days post-lentectomy. Note expression of C3 on the iris (B,C; arrow) and of C5 in the regenerating lens (E,F; arrow). B and E have been counter-stained with DAPI.

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