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REVIEW

Organ Frame Elements or Free Intercellular Gel-Like Matrix as Necessary Conditions for Building Organ Structures during Regeneration

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Abstract—Over the past decades, an unimaginably large number of attempts have been made to restore the structure of mammalian organs after injury by introducing stem cells into them. However, this procedure does not lead to full recovery. At the same time, it is known that complete regeneration (restitution without fibrosis) is possible in organs with proliferating parenchymal cells. An analysis of such models allows to conclude that the most important condition for the repair of histological structures of an organ (in the presence of stem cells) is preservation of the collagen frame structures in it, which serve as "guide rails" for proliferating and differentiating cells. An alternative condition for complete reconstruction of organ structures is the presence of a free "morphogenetic space" containing a gel-like matrix of the embryonic-type connective tissue, which exists during embryonal development of organs in mammals or during complete regeneration in amphibians. Approaches aimed at preserving frame structures or creating a "morphogenetic space" could radically improve the results of organ regeneration using both local and exogenous stem cells.

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INTRODUCTION

The notion that regeneration of organs after injury is one of the most important and still unresolved problems in biology and medicine hardly needs justification. Some aspects of this problem, such as roles of various cytokines, stromal and stem cells, have been investigated in great detail, while others have not practically been discussed in the literature. This work is devoted to consideration and justification of the approach to full regeneration (restitution) of organ structures, which has never been considered as a general key requirement, although several examples of successful realization of this approach in experimental models have been reported. It seems that non-compliance with this requirement is the reason for regular failures of the attempts to regenerate organs both in experiments and in clinic through introduction of various growth factors and stem cells.

UNSUCCESSFUL ATTEMPTS TO REGENERATE ORGAN STRUCTURES AFTER INJURY WITH THE HELP OF STEM CELLS

First attempts to achieve complete regeneration of organs in mammals and humans by introduction of stem cells (initially embryonic stem cells) after organ injury have been made very long ago [1-16]. However, the results of these attempts were discouragingly insignificant. Although it was assumed at the beginning that the cause of failures was incompatibility of donor embryonic stem cells with the host organism, use of induced autologous pluripotent cells did not improve the situation [8]. At the same time, there is no doubts that in many cases formation of new differentiated cells (even formation of areas of the uniformly structured tissues such as, for example, myocardium) from the transplanted pool of stem cell has been observed [1-16]. However, the complex organ structures (such

as nephrons) as well as whole organs was not possible to regenerate. Obviously, in this case the suggested initial concept implying that the pool of stem cells is the main component required for organ regeneration contradicts the available experimental data. Contradictions of this concept to two other groups of the facts also exist: (i) pool of stem cells is always present in many organs such as skin, gastrointestinal tract, liver, kidneys; (ii) proliferation of the relatively differentiated elements plays the main role in reparative regeneration under natural conditions because stem cells in normal tissues are present in very small numbers (less than 0.1% of population) and they divide quire rarely, but regeneration occurs within a relatively short time. Hence, stem cells are more important for self-maintenance of cell population, than for reparative regeneration itself [17].

Unsuccessful attempts of using stem cells resulted in the shift of research direction towards investigation of the role of 'stem-cell niches' and cytokines in regeneration [16, 18-22]. But not much attention has been paid to the group of facts indicating that the organs with their own pool of proliferating parenchyma cells are capable in a number of cases completely regenerate their structure after damage.

STEM AND OTHER PROLIFERATING CELLS ARE PRESENT IN MANY ORGANS AND ARE CAPABLE OF REGENERATING THE ORGAN STRUCTURE UNDER CERTAIN CONDITIONS

Such parenchymatous organs in mammals as liver and kidney are capable of complete regeneration of their structure in the case of certain types of damages. In particular, complete regeneration of liver structure is possible under the action of thioacetamide causing apoptosis (but not necrosis) with active proliferation and hypertrophy of the remaining hepatocytes [23, 24] (Fig. 1, a and b). It is well-known from clinical observations that outcome of the acute (contrary to chronic) kidney failure in the vast majority of cases (upon recovery) consists of complete restoration of the nephron structure without the development of chronic kidney failure [25-27]. Obviously, this is associated with the fact that these organs have their own pool of low-differentiated cells (including stem cells) without which regeneration is impossible, similar to impossibility of regeneration after myocardium infarction [3, 14]. However, presence of the pool of stem cells in these organs does not necessary leads to regeneration of the damaged organ. Moreover, failure of regeneration is observed in most cases of damages especially under chronic infectious hepatitis and toxic liver damage, which results in liver cirrhosis or under pyelonephritis and infarctions leading to development of rough scars in kidneys [25]. Comparison of the conditions leading to these two outcomes could be very useful for understanding the mechanisms of complete regeneration. In this respect the case of acute toxic damages of kidneys that results in mass death of epithelial cells in renal tubules and their removal with urine without destruction of basement membranes is very interesting. As has been mentioned above, acute kidney failure developing during intoxication, as a rule, ends with complete restoration of the nephron structure. However, in the rare occasions, when the acute kidney failure leads to the development of chronic kidney failure with fibrosis (such as in the cases of mercury compaunds poisoning), it is necessarily accompanied by not only death of epithelia in renal tubules, but also by tubulorrhexis with basement membrane damage, appearance of urine components and residues of necrotised cells in interstitium, and development of inflammation [25]. Similarly, if not the small doses of thioacetamide are used to induce experimental liver damage, but other damage-inducing agents causing necrosis (but not apoptosis) of hepatocytes with development of inflammation, liver fibrosis is observed in these experiments [23, 24, 28] (Fig. 1, c and d).

Another remarkable example is regeneration of testicle. Following exposure to moderate radiation spermatogenic cells are almost completely killed, but basic three-dimensional structure of the testis tubules is maintained, therefore, organ structure is completely restored after certain time; in the case of mechanical damage or chemical necrosis the affected part is subjected to resorption with time, and its space is filled via elongation of the preserved testis tubules growing in the direction away from testicular network [29].

These examples imply that not only the organs with own pool of stem cells are capable of complete regeneration (restitution), but also provide indications of the particular conditions that make this regeneration either possible or impossible.

STEM CELLS ARE NOT CAPABLE OF RECREATING ORGAN STRUCTURES IN AN ADULT ORGANISM

It is important to note that although the elements of stem system (primarily induced pluripotent stem cells) have very wide potential for differentiation, this does not mean that the possibility for building of complex and ordered organ structures from these cells are equally as wide in a post-fetal organism. Architectural potential of stem cells is clearly visible in the case of tumor growth especially of highly differentiated neoplasia. In this case mass of tissue structures could be formed such as plates, globules, alveoli, tubules, rosettes, and in

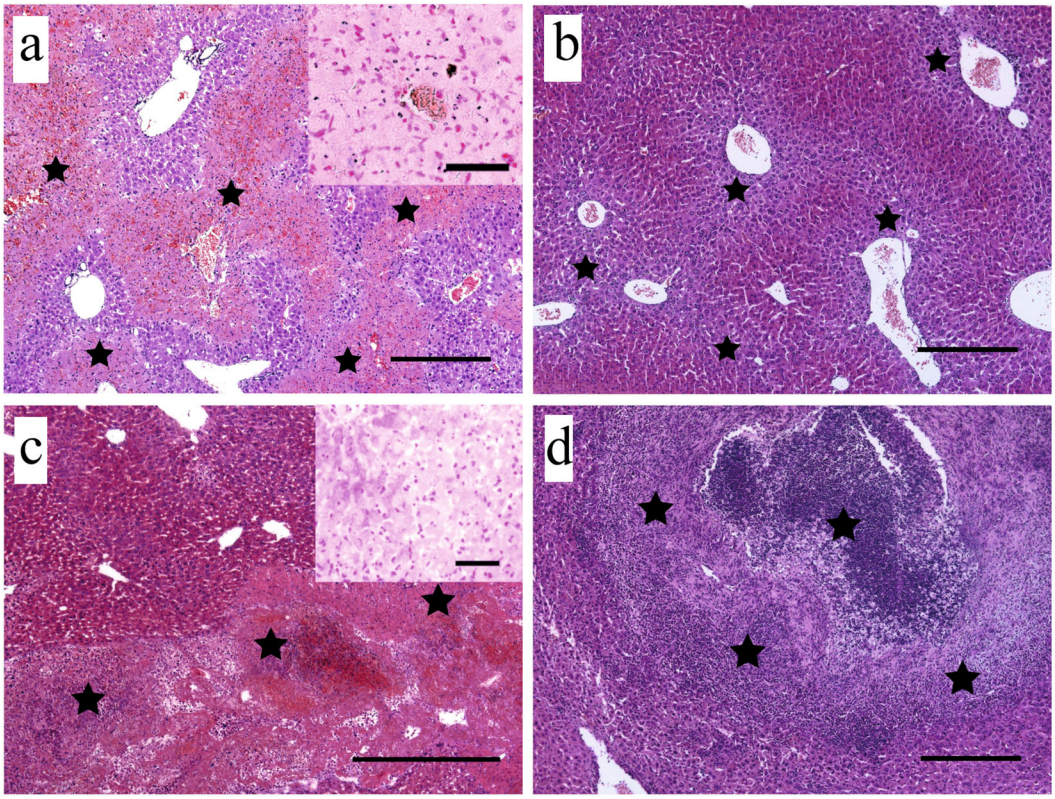

Fig. 1. Development of complete (restitution) or incomplete (substitution) lever regeneration depending on the type of injury. a and b) Regeneration of the C57BL/6 mouse liver after administration of thioacetamide: a) after 48 h (mass apoptosis without inflammation; verified using TUNEL-method (black nuclei in inset)); b) after 12 days (complete regeneration of lobules with signs of hepatocyte hypertrophy). c and d) Regeneration of the C57BL/6 mouse liver after cryodamage: c) after 48 h (necrosis and inflammation, TUNEL-negative nuclei – see inset); d) after 12 days (site of inflammation and fibrosis). In all images damaged regions are shown with black stars. Scale bars and magnification: a-d) 200 μm, magnification – 100×, (inset in panel a, 20 μm, in panel c, 15 μm; magnification – 1000×); staining with hematoxylin and eosin (insets in panels a and c, TUNEL-method). Images made by the author [24].

special cases, such as nephroblastoma, primitive renal glomeruli [30]. However, at this point the processes of morphogenesis ends, and renal lobules or nephrons are never formed from these cells. Even in the benign teratomas the cited primitive elements are formed, but not the complete organ fragments [31]. Cell-precursors behave similarly also in the cases, when the organ "needs" to enhance its functional capabilities. In particular, in the case of vicarious (replacing) kidney hypertrophy there is no formation of new nephrons, but rather increase of length of the existing tubules occurs [24], which can be seen in histological images as dramatic increase of the surface area occupied by the tubules with sparsely distributed glomeruli (Fig. 2a). In the case of kidney regeneration after acute necrotic nephrosis, the non-damaged basement membranes of tubules served as peculiar "guide rails" along which the proliferating cells could rebuild the destructed elements of nephrons form glomeruli to collecting tubules; that is why tubulorrhexis makes nephron regeneration impossible [25]. Exactly the same situation takes place in the liver lobules, which only increase in size in the case of liver hypertrophy, but are nor formed *de novo*, and in testis, where after damage the existing tubules elongate, but new ones are not formed.

Hence, stem cells present in the regenerating tissues or introduced exogenous stem cells are capable of incorporation into already existing structures (as during physiological regeneration), but they are not capable of building new structures due to the absence of required embryonic morphogenic gradients. Furthermore, the processes preventing regeneration along the organ frame very often occur in the damaged tissues.

ORGAN FRAME ELEMENTS AND THEIR ROLE IN CONSTRUCTION OF ORGAN STRUCTURES

From the abovementioned examples, as well as from many others, it seems obvious that the most important cause explaining impossibility of restitution is disruption of architectonic of organ frame structures. Frame elements include such formations of extracel-

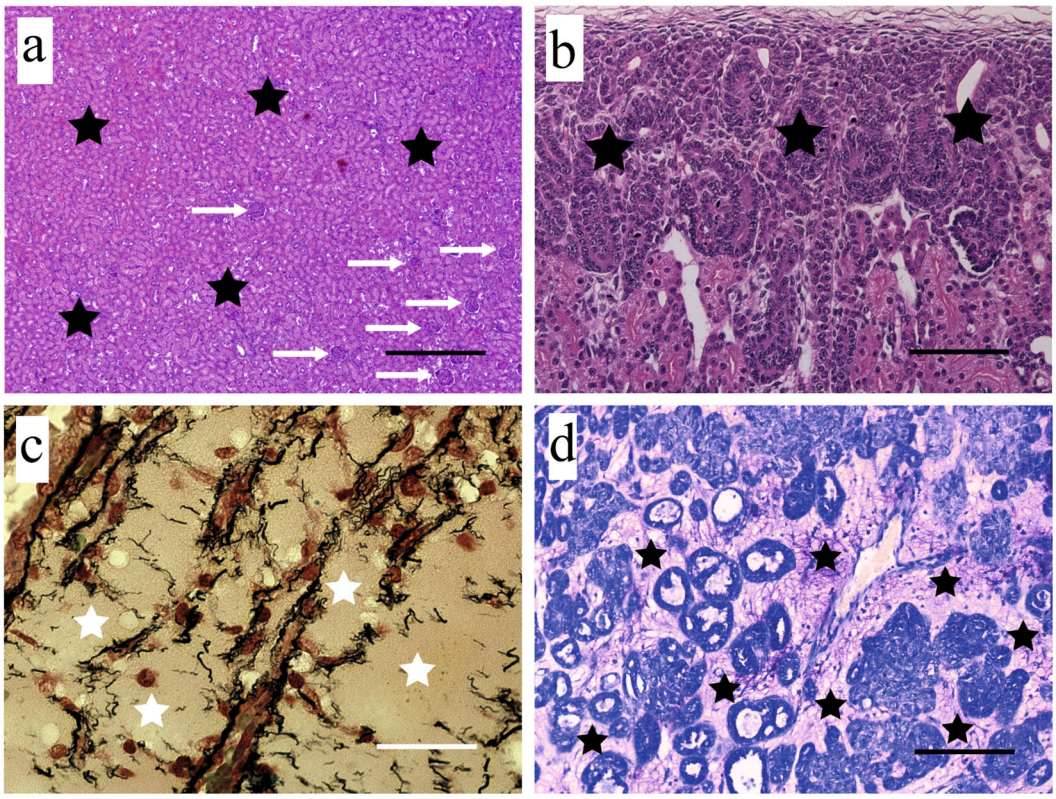

Fig. 2. Tissue responses upon hypertrophy and formation of new organ structures. a) BALB/c mouse kidney in the case of vicarious hypertrophy after ischemia of the second kidney (relative decrease of the number of glomeruli (shown with arrows) on the background of the large surface area occupied by only tubules in the slice, marked with stars); b) kidney of a newborn Chinchilla rabbit, subcapsular area of the cortex (metanephrogenic blastema and forming new nephrons are marked with stars); c) "embryonic" connective tissue in the kidney medulla of the newborn Chinchilla rabbit (sparse amount of collagen and loose reticular stroma; main unstructured substance of the connective tissue (marked with stars) fills the space between the forming tubules); d) spontaneous adenocarcinoma of the Chinchilla rabbit uterus (metachromatically stained myxoid stroma resembling embryonic connective tissue marked with stars). Scale bars and magnification: a) 200 μm, magnification – 100×; b) 50 μm, magnification – 400×; c) 20 μm, magnification – 1000×; d) 100 μm, magnification – 200×. Staining techniques: a and b – hematoxylin and eosin staining; c – impregnation according to Gordon–Sweets followed by staining with Twort mixture (neutral red – light green); d – thionine staining.

lular matrix that not only ensure mechanical stability, but also have ordered structure corresponding to architectonic of the organ, and it is exactly this architectonic is provided by these components. At least three types of such structures could be mentioned.

1) Complex three-dimensional structure comprising totality of basement membranes (mostly based on type IV collagen in epithelia, and types III and V in muscles, as well on perlecan, laminin, and numerous components [31-34]), which together form a contour basis of an elemental unit of a certain organ (hepatic lobule, nephron, etc.); such frame structures are essential for the organs with parenchyma made of epithelial tissues and for muscle-based formations; role of these structures is not only to provide stability, but also to ensure ordered arrangements (for epithelia) or support for combining elements into one mechanical structure (for muscles).

2) Three-dimensional network formed by reticulin fibers (based on type III collagen), which comprise frame structures in hematopoietic organs and organs of immunogenesis (thymus, spleen, lymph glands, bone marrow) or filling the space between the organ structures separated by basement membranes (in kidneys, liver, etc.); reticulin formation allow compartmentalization of an organ (for example, they comprise the basis of spleen follicles and lymph nodes) and at the same time, they do not prevent locomotion of mobile cells and even support them [31, 32].

3) Special types of frame structures such as myelin sheaths of axons in nerve cells, elastic membranes, and various collagen fibers in vessel walls, main substance in eye cornea consisting of the type I collagen fibers, and others.

It is very important to mention that the frame structures arise during embryogenesis as a one system for the whole organism simultaneously with formation of all its compartments, which provides corresponding morphogenic positioning effect on all differentiating cells. Such positioning information is maintained

in an adult organism likely with the help of described frame elements; that is why destruction of these elements prevents rebuilding of organ structures.

It must be noted that the frame structures of extracellular matrix undoubtedly could affect intracellular signaling pathways and most important cellular reactions (prevent anoikis, induce specific cell differentiation, etc.) [34, 35]. In particular, it is known that the composition of laminins in the basement membrane determines direction of differentiation of epithelial cells (for example, laminin composition determines what epithelia would develop in a particular section of intestine) [36], development of reticulin fibers and elastic framework predetermines compartmentalization of lungs in embryogenesis [37], and reticulin fibers and even type I collagen fiber bundles predetermine branching of the developed ducts in mammary and salivary glands [38-40]. It seems, however, that these and similar effects are only an important addition to the role of "guiding rails" during reconstruction of organs in regeneration.

FACTORS PREVENTING RESTORATION OF NORMAL ORGAN STRUCTURE IN THE PRESENCE OF PROLIFERATING PARENCHYMAL CELL-PRECURSORS IN THEM

If we accept the hypothesis that maintenance of frame structures is the main condition for complete regeneration of a damaged organ in adult mammals, we can identify the factors preventing such regeneration. Typical in fibrosis transformation of fibroblasts into myofibroblasts actively secreting collagen and associated with this formation of scar tissues in mammals should be mentioned in the first place [33, 34]. On the one hand, it appears that such tissues fill the space of the organ making development of parenchymatous elements impossible (due to mechanical and metabolic reasons such as avascularity), and, on the other hand, frame components of the organ are damaged in the process. Fibroblasts and myofibroblasts are significantly less demanding on conditions for survival and proliferation in comparison with parenchymatous elements, and they fill the space of defect in tissues with collagen much faster than the latter ones [25, 41]. They secrete matrix metalloproteases, which destroy collagen of the organ frame structures and basement membranes [33, 34], and produce a large amount of fibrotic collagen, which eventually leads to disruption of architectonics of the organ frame. It should be mentioned that the key role of preserved guiding frame elements has been recognized for the case of regeneration of conductive structures in nervous system (nerves, spinal cord) [42-44] and as inducer of bone tissue differentiation [45-47], but, obviously, it has not been fully considered in the cases of regeneration of parenchymatous organs (liver, kidney, myocardium, various glands). On the other hand, this particular principle is used nowadays for creation of artificial organs *in vitro*, when for their construction decellularized collagen scaffolds are used, which are seeded with stem cells [48-52].

Another negative condition revealed through the further analysis of available information is inflammation, which often leads to fibrosis even without morphological signs of damage in the tissues [25]. This is facilitated by the so-called M2-macrophages, or macrophages of the second phase of inflammation, cytokine profile of which (primarily secretion of TGF-β) provides conditions for incomplete reparative regeneration of the substitution type (scar) [53-55]. Moreover, under conditions of inflammation the cells-effectors, neutrophils and macrophages, could themselves destroy frame structures by the secreted proteases (collagenases), and in some cases the caused damage could be even more significant than the damage causing tissue necrosis. For example, it was shown in our studies that in the photothrombosis-induced kidney damage argyrophilic structures and basement membranes of the affected renal tubules do not have clearly pronounced changes, and fibrosis development in this model is associated with the following inflammatory infiltration of the zone of necrosis [56].

In its turn, one of the conditions of inflammation development after injury is release of the ligands of Toll-like receptors and other damage-associated molecules (DAMPs) from the cells subjected to necrotic death [25, 28, 57-59]. Although, in the case when phagocytosis of apoptotic bodies is delayed for some reason (for example, when there is at very large number of apoptotic cells), there is a possibility of their secondary necrosis accompanied by the development of inflammatory response [60], and, consequently, of fibrosis. Hence, the type of cell death and fate of the dead cell residues represent another factor determining completeness of regeneration. And in the case of necrosis the process usually ends with the development of fibrous tissues, rather than regeneration of initial organ structure.

It must be emphasized that in the case of preservation of the organ frame structure, organ regeneration in many cases does need neither exogenous stem cells, nor even stimulation with growth and differentiation factors, although these are exactly the key elements considered essential in the majority of studies devoted to stimulation of reparative regeneration [16, 18-22]. It must be noted that contrary to the conditions associated with the state of proliferating parenchymatous elements, existing *in vivo* frame structures have not been discussed in detail in the literature devoted to reparative regeneration.

STEM CELLS CAN BUILD ORGAN STRUCTURES IN THE FREE "MORPHOGENIC SPACE"

Alternative path for building new organ structures seems also possible, which does not require the pre-formed organ frame. As is well-known, practically complete regeneration of organ structures occurs in newborn animals in the case of non-traumatic kidney injury (for example in experimental ischemia) [60]. Examination of morphology of neonatal kidney reveals that new nephrons are still actively forming from the subcapsular blastema (Fig. 2b). What attracts attention in this case is the cortical area densely filled with non-differentiated metanephrogenic tissue containing forming elements of nephrons, as well as special character of the connective tissues filling the space between the still sparsely located tubules growing in the direction from cortex to pelvis. This tissue consists of mesenchymal-like cells with outgrowths between which there is almost no collagen but only very sparse network of reticulin fibers (Fig. 2c). The main fraction of this tissue comprises a structure-less main substance, which is clearly visible during staining with neutral red, thionine, or fast green-FCF (Fig. 2c). It is worth mentioning that very similar tissues have been observed in the larva of tail-less amphibia, which apparently provides the possibility of different rearrangements during metamorphosis, and also allows regeneration of lost limbs [61] (it cannot be ruled out that such differentiation of fibroblasts is indeed a critical condition providing the possibility of complete (epimorphic) regeneration of organs and limbs from blastema in the tailed amphibia). Hence, formation of new nephrons in kidneys of newborn animals occur not only in the presence of non-differentiated blastema, but also in the presence of particular embryonic connective tissues, which is similar to gelatinous connective tissue within the umbilical cord – Wharton's jelly. This tissue featuring abundance of the main substance and very few rigid fibrous structures, on the one hand, provides wide-volume space of the organs (otherwise, in the case of rigid capsule, there would be mechanical obstacles for formation of new structures), and on another hand creates three-dimensional frame, which allows branching and growing in different directions ends of collecting tubules to reach metanephrogenic blastema without any hurdles, and increasing length of renal tubules of the new-formed nephrons [62]. Hence, in this case morphogenesis is realized not in the rigid pre-existing morphogenic frame, but in a specific free 'matrix', which provides possibility of three-dimensional growth of new elements of parenchyma. Very similar situation is observed *in vitro* during recreation of organ structures in Matrigel [63]. In the post-fetal period volume of a kidney is filled, instead of matrix, with tubules and with rigid collagen structures, which makes formation of new nephrons impossible.

PATHWAYS OF COMPLETE REGENERATION: PRESERVATION OF FRAME STRUCTURES AND CREATION OF MORPHOGENIC SPACE

Based on the information presented above several possibilities facilitating complete regeneration (restitution) of the damaged organ but not substitution (development of fibrous tissue) could be suggested.

First of all, these possibilities include all measures targeting preservation of the organ frame structure.

1. Switch the type of cell death from necrotic to apoptotic (using even paradoxical proactive method such as addition of apoptosis inducers triggering death of the cells that under normal conditions would die by necrosis). Unfortunately, in this respect there are no particular instructions to certain therapeutic approaches that would be effective.

2. Activation of phagocytosis of apoptotic bodies by macrophages and parenchymatous cells with the goal of their fastest resorption and prevention of secondary necrosis and inflammation development. Removal of dead cells occur rather efficiently in kidney tubules, but is very problematic in liver, where masses of dead cells could accumulate together with calcium deposits, which prevents regeneration. Removal of dead cells is especially important in the case, when it becomes possible to switch cells from necrotic death to apoptotic pathway, and the latter one would be massive. Unfortunately, this approach, as well as the first one, should be considered at present only as hypothetical. It must be taken into account that fast resorption of dead tissues could result not in regeneration but atrophy of the organ part, if this process is not accompanied with just as fast regeneration or filling of the free space with matrix.

3. Inhibition of inflammatory response. For this purpose, there is a wide range of pharmaceutical preparations that have been used in clinical practice for a long time including the cases of alternative pathologies such as coronary heart disease [64]. First of all, these include new generation of non-steroid anti-inflammatory drugs, as well as inhibitors of proinflammatory signaling pathways and cytokines such as TNFα [64-66]. Although these preparations have not been investigated with regard to their effects on preservation of frame structures, there are no directions for the optimal combination or regime of administration; at the same time, it is known that some of them could stimulate fibrosis as a side effect [66].

4. Inhibition of fibrosis. This pathway has been considered for a long time, and several approaches have been suggested including inhibition of fibroblast

proliferation and their differentiation to myofibroblasts (for example, by affecting signaling pathways associated with TGF-β), slowing down differentiation of M2-macrophages, and suppression of collagen synthesis. This seemingly attractive approach is extremely difficult to implement due to physiological versatility of molecular mechanisms, which should be suppressed for this purpose. However, relatively recently first experimental data have been reported on pharmacological agents form this group capable of suppressing development of fibrosis under experimental conditions [67-69].

A different way could be associated with creation of a morphogenic space in which the exogenously introduced (such as in the case of myocardium) or endogenous (in kidney and in liver) stem cells could recreate organ structures. Such approach could have at least two strategies.

1) Creation of artificial cavities and columns with gelatinous matrix and differentiation factors in the organ itself. The experimentally tested scaffolds and 'bioreactors' with hyaluronic acid could be considered as close examples (although primitive) of such approach [52, 67-73], which resulted, for example, in successful regeneration of limbs in the adult tail-less amphibia (in which it does not occur naturally, contrary to the tailed amphibia) [70].

2) Strategy associated with changing differentiation of stromal fibroblasts in such a way that they produce not the fibrous collagen but a 'matrix' embryonic connective tissues similar to the type of stroma in the liver of a newborn animal or Wharton's jelly. The possibility of using the cells of the latter for the purposes of regenerative medicine have been considered, but only from the point of view of production of pluripotent stem cells [74, 75]. It would be very important to identify factors inducing differentiation of such cells and ways of its pharmacological regulation. Existence of the possibility of "Wharton's" differentiation in the post-fetal tissues is corroborated by the fact of its appearance under condition of tumor growth – in the case of myxoma and myxoid stroma of tumors (Fig. 2d). The latter case is especially interesting, because in this case changes in differentiation occur in non-tumor cells, and this is often observed exactly in the differentiated tumors of the adenocarcinoma type, which grow via elongation and formation of new tubules (while the tumors with fibrous stroma – scirrhous tumors – usually are formed from the poorly-differentiated tumor cells with extremely primitive morphogenic processes) [76, 77]. These types of scenarios could serve as models for investigation of factors inducing 'myxoid' stroma in the post-fetal organism. Induction of differentiation of embryonic stroma, on the one hand, would allow filling the space of resorbed dead tissues with the matrix and to preserve this part of the organ from collapse and atrophy, and on another hand, to create a foothold for revealing morphogenic potential of local or introduced stem cells.

CONCLUSIONS

Information presented in this paper allow suggesting that the general reason of failed attempts to regenerate organ structure after injury by introducing stem cells or growth factors is destruction of organ frame elements, which in the majority of vertebrate animals develop only at the stage of embryonic ontogenesis. Preservation of these structures is a sufficient condition for the organ cells capable of proliferation to rebuilt the organ structure without any external factors. Another path could be creation of the space in the organ filled with gelatinous matrix, similarly to the way it occurs during embryogenesis, by either artificial introduction or by changing differentiation of stromal cells and switching their metabolism to synthesis of respective components of intercellular substance. Creation of organ structures without the presence of organ frame could be expected in this space, similarly to the processes occurring in embryogenesis or reconstruction during amphibia metamorphosis. However, while the strategies for preservation of organ frame structures seem quite achievable via the already known methodological approaches, realization of the second suggestion is at the stage of planning future research.

Development of approaches suggested in this review could likely allow to achieve not only complete regeneration of organ structures from the organism's own proliferating and stem cells, but also to succeed in the cases (heart, nerve tissues), when the necessary introduction of exogenous stem cells did not yet result in clinically significant outcomes.

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