Embryogenesis and Regeneration of the Intervertebral Disk (Review)

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Degenerative processes of the intervertebral disc are shown to represent a diversity of molecular, cellular, structural, and functional alterations, the main clinical manifestation of which is pain syndrome. On this basis, therapy of intervertebral disc degeneration is directed to pain elimination and does not take into consideration real causes of degenerative process development, does not study the feasibility of regenerating the structure and biomechanical function of the disc. A new approach to the study of molecular and cellular mechanisms of intervertebral disc degeneration, examination of the disc degenerative process pathogenesis from the standpoint of its ontogenetic development allows discovery of new links of pathogenesis and suggestion of new promising ways of therapeutic intervention. The developed models of intervertebral disc degeneration, which make it possible to explore comprehensively morphogenesis and associated intracellular signal pathways, as well as early postnatal alterations in the discs, are considered here. Current strategies of biological therapy of degenerative processes, which are directed to the activation of regenerative potentials in the disc and in its self-renewal, are presented. One of the perspective methods of biological therapy of this disease is application of autologous intervertebral disc cells cultured *in vitro* with their subsequent transplantation, which can potentially compensate for cell deficiency and, consequently, disc matrix as well.

Key words: intervertebral disc degeneration; nucleus pulpous; annulus fibrosus; extracellular matrix; aggrecan; models of disc degeneration; embryogenesis; intracellular signal pathways.

Currently, degenerative processes of the intervertebral disc (IVD) continue to remain a burning problem of modern medicine. The most common clinical manifestations of the IVD degenerative processes are pain in the back, which is often associated with early disability [1, 2]. The pain syndrome in the back is experienced by more than 85% of people over the age of 35 [3, 4]. IVD is a partially movable joint connecting vertebral bodies, and providing a uniform distribution of the load and mobility of the entire spinal column. The causes of IVD degenerative process development have not been completely studied but they are certain to be linked with the processes of human body aging [5]. IVD degeneration is a complex cascade of reactions, which evolves primarily in the IVD cells and progressing for decades manifests itself in the form of structural and functional disorders [6, 7].

Present-day methods of treating degenerative IVD processes are directed only to the elimination of the pain syndrome and include, as a rule, administration of anti-inflammatory drugs and physiotherapy. In those cases when operative treatment is indicated, spondylosyndesis with different fixing systems is a golden standard [8]. However, abolition of the pain syndrome only without restoration of the IVD mechanics or structure can result in recurrence of the pain syndrome and, as a consequence, to the progression of IVD degeneration [9, 10]. Recently, arthroplasty (replacement by an artificial disc) becomes a common surgical method of treating degenerative IVD processes aimed at restoration of spine mobility. However, these types of implants have a number of disadvantages, which limit their wide application: they are not able to reproduce the mechanical function of the native IVD, subjected

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to a fast deterioration and fractures [10]. Therefore, the most perspective method of treating degenerative IVD processes is biological therapy based on studying molecular mechanisms of IVD degeneration.

Degenerative IVD processes are known to be not typical for adolescents, nevertheless, microstructural alterations in IVD are found already in the first years of the infant life [11, 12]. Embryology considers IVD as a unique structure originating from the cells of the notochord and somite stalks. It is these cell populations that will form subsequently IVD tissue with its unique microstructural, mechanical, and functional features. And we will consider molecular and cellular mechanisms of degenerative IVD processes and perspective methods of biological therapy of the degenerating IVD from the standpoint of embryology.

The structure and metabolism of the intervertebral disc

IVD is a highly specialized formation consisting of three main structural components: an annulus fibrosus, nucleus pulposus, and endplates [13]. Annulus fibrosus bounds the nucleus pulposus, a gel-like core, consisting of randomly arranged collagenic fibers and radially located elastic fibers immersed in a highly hydrated aggrecan-containing gel. The annulus fibrosus is composed of about 25 concentric rings or lamellae generated by the located in parallel collagen fibrils surrounded by elastic fibers [14, 15]. The highly hydrated aggrecan in the nucleus pulposus maintains osmotic pressure providing the formation of IVD properties.

The nucleus pulposus and annulus fibrosus have different cellular composition. The cells of the annulus fibrosus in the external part of the fibroblastoid structure are situated in parallel with the collagenic fibers. In the inner part, they are more oval, chondrocyte-like. The cells of the nucleus pulposus have a chondrocyte-like structure and are located sporadically, about 5,000 in 1 mm^3, are embedded in the matrix and sometimes are encapsulated. Some IVD cells both in the nucleus pulposus and annulus fibrosus have an elongated shape and can reach 30 μm. They are supposed to fulfill a sensory, communicative role in IVD [16, 17]. Two endplates composed of the hyaline cartilage close the disc axially and adjoin the neighboring vertebrae. Their thickness does not exceed 1 mm [18, 19].

IVD of a healthy adult practically lacks blood vessels and nerve fibers. A few nervous fibers are detected only in the outer lamellae of the annulus fibrosus, part of them are the ends of proprioceptors [20]. Already in the first decades of life the disc loses the greater part of its blood supply causing deficiency of nutrients [21, 22]. The secondary relative to the loss of significant part of the blood supply is calcification of the endplate leading to the reduced diffusion of the plastic substances [23]. Without the necessary nutrients cells die, synthesis of the energy substrates, proteins of intercellular substance, diminishes [24]. An adult has half as many IVD cells as a child [25, 26].

Degenerative alterations of the nucleus pulposus are characterized by a progressing decrease of the IVD height. The boundary between the nucleus pulposus and annulus fibrosus becomes more distinct. A brown pigmentation in the IVD tissue intensifies, the tissue becomes more fragile. Degenerative changes in the annulus fibrosus display themselves by disorganization of the regular interlace of the collagen lamellae and elastin, replacement of the gel-like structure by the fibrocartilaginous tissue. In the majority of people the course of aging (degeneration) is slow, gradual, however in some situations it may essentially accelerate, causing the occurrence of the pain syndrome [27, 28].

IVD matrix destruction is performed by specific enzymes. They include aggrecanases, various kinds of matrix metalloproteinases (MMP) and other degenerative enzymes. For example, MMP-3, or stromelysin, destroys types III, IX and X collagens, proteoglycans, fibronectin, MMP-2, or gelatinase, causes degradation of type IV collagen. Some other degenerative enzymes are united in the ADAMTS family [29, 30].

Pathogenesis of intervertebral disc degeneration

Prominent changes of the extracellular IVD matrix take place with age [31, 32]. Reduction of aggrecan synthesis in the nucleus pulposus results in its dehydration and subsequent impairment of its mechanical function [33, 34]. Dehydrated nucleus pulposus is not able to distribute uniformly mechanical pressure over all IVD structures. Due to this reason, the annulus fibrosus experiences an elevated load, which cannot but have an effect on its structure in the form of local damages [35–38]. Annulus fibrosus lesions lead to the formation of IVD protrusions and hernias, causing acute radicular pain syndrome. Besides, the damaged annulus fibrosus reduces markedly the IVD height, which also entails changes of the biochemical parameters of the spine and development of back pain [39].

Several interconnected factors are known to participate in the initiation and progression of degenerative IVD processes: nonuniform distribution of mechanical load over IVD structures, decreased diffusion of nutrients through the endplate, and genetic factors [40]. Age changes of the extracellular IVD substance are associated with alterations in metabolism and cellular population apoptosis [41]. Cellular microenvironment becomes aggressive relative to the IVD structures and is characterized by the increased synthesis of proinflammatory cytokines, mediators and catabolic enzymes [42]. The reason of inflammation activation in IVD is partly linked with the reduction of nutrient diffusion through the endplates, which are subjected to marked thinning and calcification [42]. Mechanical load also play an important role in the progression of IVD degeneration. Thus, Walsh [43]

showed that in case of inadequate mechanical action on IVD tissue, the number of cells and intercellular substance decrease substantially. On the other hand, insignificant mechanical load on IVD stimulates nutrient diffusion through the endplate and protein synthesis of extracellular matrix.

The role of genetic factors in the development of degenerative IVD processes does not raise any doubts [44]. Examination of twins proves the presence of genetic predisposition to the development of IVD degeneration [45].

Models of intervertebral disc degeneration

Creation of the IVD degeneration models in order to study the links of pathogenesis and to develop methods of therapy was rather a difficult problem. It was connected with time characteristics (several years are needed to gain an understanding of a real picture of the degenerating IVD), and with the ways of initiation of IVD degeneration in experiment [46]. However, low availability of IVD tissue at the initial stages of degeneration and almost complete absence of unaltered IVD for comparison *in vitro* means that such models of IVD degeneration, despite their drawbacks, are necessary for the study of signal pathways of the degenerating IVD. IVD of many animals have anatomical and biological properties similar to human making them suitable for creating models of degeneration [47]. Trauma of the annulus fibrosus, nucleus pulposus, mechanical overload as well as enzymatic treatment of nucleus pulposus glycosaminoglycan are distinguished among the methods of degenerative IVD process initiation in models [48–51]. Models with spontaneous IVD degeneration have also been developed [52, 53].

Development of intervertebral discs: embryogenesis and postnatal period

Axial string from the cells having initially thickness equal only to one cell called a notochord is present in embryos of all chordates and gives them the pertinent name. In lancelets and tunicates the notochord remains a single axial skeleton for the whole life of the organism. In vertebrates, the notochord disappears at the early stage of embryonic development and is replaced by a cartilaginous or bony spinal column [54]. Only several weeks after conception an embryo (12 mm long from the cephalic pole to the sacrum) has a spinal column with distinct vertebrae and IVD. They are pierced by a notochord, which is still preserved over the full length of the spine [55]. Later on, the notochord is displaced from primordial vertebral bodies by the growing cartilaginous cells and remains only in the form of drop-like inclusions in the center of IVD designating the location of what later will become a nucleus pulposus. The outer zone will subsequently change into the annulus fibrosus [56, 57]. Even at an early stage of development it already contains longitudinal fibers extending to the cartilaginous layer of the primordial vertebral body. They are precursors of Sharpey's fibers in the transition zone between the disc and the vertebra. There are many fibers and few cells in the outer zone. It smoothly goes over to the loose inner zone around the notochord with a less number of structures [57]. Nucleus pulposus develops from the parachordal inner zone and somewhat eccentrically located remnant of the notochord. While the center of the vertebra is being gradually ossified, a cartilaginous plate is formed at the vertebral body-disc interface. Later on a bony edge of the vertebral body develops from the cartilaginous edge of this plate [57].

All structures of the disc involved in the biochemical function of the spine are already present at birth. During embryonic development and at a very young age a growing IVD still has its own system of blood supply. These blood vessels evolve from the vascular network situated immediately outwards from the vertebral column especially in the intervertebral foramens, and directly enter the annulus fibrosus radially piercing its layers (lamellas) and forming interlamellar capillary plexuses. Two types of vessels, peripheral and central axial, supply blood to IVD. They never go either to the inner layers or nucleus pulposus [58, 59]. Thus, from the very beginning of its development, the central part of IVD is nourished only by diffusion. Formation of vertebral bodies and IVD is finally completed at preadult age. Vertebral bodies grow from the zones of proliferation in the cartilaginous endplates. In the region of the endplate facing the bone marrow there is a typical zone of cartilage growth and decay, which is preserved till about 20 years of age [59].

Inside the cartilaginous ring at the edge of the endplate regions of ossification appear, they coalesce by 12 years of age to form a bony ring, which then merges with the vertebral body. Annulus fibrosus lamellas are fixed to this bony marginal ring by Sharpey's fibers. The annulus fibrosus and nucleus pulposus enlarge in size by interstitial appositional growth. In the outer disc layers dense lamellar bundles are generated, which go between the bodies of two vertebrae in the form of twisted spirals [60]. The farther from the IVD periphery the lamellas of the annulus fibrosus are located, the weaker they are and less tightly grouped. Even in a fully mature IVD, its center, the nucleus pulposus, consists mainly of a structureless matrix [60]. Connective tissue cells located in the annulus fibrosus and producing fibers and matrix are established to be supplied with blood only till the age of about 2 years [61]. Then the supplying vessels undergo a regress, and by the age of 4 the annulus fibrosus appears to be avascular. It would seem vice versa, a human IVD, being so rich in cells and fibers, needs a plentiful blood supply due to a continuous synthesis and decay of macromolecules going on in it. IVD blood supply starts to regress at the age of about a year, when a baby begins to walk upright. There may

be supposed the association between IVD avascularity and mechanical load, which it constantly endures during standing and walking.

Arteries and veins of the vertebrae lie in the interstices left by a robust trabecular bone system, and therefore are not exposed to the axial mechanical load. Physically loose homogeneous tissue of the IVD lower part, on the contrary, resembles a liquid [62]. Thus, depending on the body position pressure inside the IVD can be high enough to cause a prolonged compression of the intradiscal vessels resulting in metabolism disturbance of the IVD tissue. Insufficient delivery of nutrients in IVD has a detrimental effect on the quantity and quality of intradiscal connective tissue. In subsequent years, the annulus fibrosus and nucleus pulposus enlarge in volume due to appositional growth of interstitium but their increase lags behind the growth of vertebral bodies. So, the ratio of IVD height to the vertebral height gradually increases from 1:1 at birth to 3:1–5:1 by the completion of the axial growth phase.

In a qualitative sense, IVDs of adolescents are also found to be involutionally changed, which speaks of preliminary aging, these changes are mainly associated with a rapid fall of the water content level. Alterations of the consistency and color of the IVD tissue in the first years of life is easy to see by the naked eye on fresh sections of autopsy specimens. In newborns and infants, IVD surface looks hyaloid, gelatinous and semi-liquid. For example, even in a 2-year child this semi-liquid central part can be removed with a cotton tampon, while it is already impossible to do in adults [63]. Even after the completion of the axial growth, IVD is subjected to further regressive changes, which are noticeable on its appearance. At a mature age, the tissue in the IVD central part is not already homogeneous, gelatinous and looks dry and fibrous. If a motor segment is immobilized by spondylosis spurs, IVD connective tissue may undergo reorganization so that blood vessels begin to grow into IVD again [63].

Cellular signal pathways in intervertebral disc embryogenesis

Development of IVD in ontogenesis wholly depends on the coordinated work of molecular signals originating in the notochord and nervous tube plate [64]. Shh protein (sonic hedgehog) is a signaling molecule, which performs an important function of tissue morphogenesis regulation, presenting information about location and degree of cell differentiation [65, 66]. Somite stalks develop under minimal effect of Shh and Wnt signaling pathways, whereas a sclerotome tissue develops only under the activating effect of the Shh pathway [67]. A specific feature of the Shh intracellular cascade work is a synergism with Noggin-cascade, which is a direct antagonist of the BMP pathway in the induction of sclerotome growth [67]. Noggin molecules are at first actively expressed by the notochord cells blocking BMP signaling from the developing vertebral bodies till the formation of the annulus fibrosus [68].

Genes of the *Pax* family encode transcription factors regulating the processes of proliferation, differentiation, apoptosis and migration of polypotent cells in embryogenesis. It is the expression of these genes that plays a primary role in differentiation of cellular populations, from which later IVD and vertebral bodies will originate [69–71]. It is proved that exactly the *Pax1* and *Pax9* genes participate in the IVD development. When these genes are deleted, IVD and vertebral bodies do not develop, and a cartilaginous core of the irregular shape is formed at their place [72]. Expression of *Pax1* gene in the sclerotome tissue is mediated by the action of Shh and Noggin signaling pathways from the notochord cells [73, 74]. Till the completion of IVD and vertebral body formation, almost all cellular population of the sclerotome expresses *Pax1* gene. After IVD formation, *Pax1* expression occurs only in the tissue of IVD primordium (precursor of the annulus fibrosus) surrounding the notochord. Evidence exists that *Pax1* gene is able to influence indirectly the notochord development via the sclerotome tissue: in *Pax1* mutant cells the notochord is essentially enlarged in size due to a marked cellular proliferation [74]. Thus, *Pax1* gene influences the notochord development by activating cellular proliferation till the latter will turn to the nucleus pulposus.

Also important is a family of genes participating in the development of the spinal column structures represented by *Sox* genes [75, 76]. *Sox5, Sox6,* and *Sox9* genes are of special significance for IVD tissue development. *Sox5* and *Sox6* express both in the cells of sclerotome and the notochord [77]. In the experiment on mice deprived of *Sox5* and *Sox6* genes, formation of notochordal membrane was impaired. It was connected with the fact that these genes are responsible for the synthesis of intercellular substance components (aggrecan and type II collagen) [77]. Absence of notochordal membrane leads to apoptosis of the notochordal cells and disturbance of further development of all IVD components. Gene *Sox9* expresses initially in the notochord and sclerotome, participating mainly in the synthesis of type II collagen [78]. In the cells with deleted *Sox9* gene, an adequate notochord is initially formed, but later on it degrades due to the absence of notochordal membrane matrix. Underdevelopment of notochords and, as a consequence, a mediated cellular signaling affects detrimentally further formation of the sclerotome [79].

A signaling TGF-β pathway actively participates in the development of IVD and vertebral bodies as well. TGF-β cascade regulates cellular proliferation, differentiation and synthesis of IVD intercellular substance components [80]. There are several tissue-specific types of TGF-β. TGF-β_3 is actively synthesized in the perichordal membrane and promotes the development of the annulus fibrosus and vertebral bodies. Blockage of the TGF-β_2 receptors

responsible for the synthesis of type II collagen results in incomplete formation of the nucleus pulposus, the outer part of the annulus fibrosus and also to partial IVD mineralization. This confirms the fact that TGF-β_2 receptors took part in the differentiation of IVD tissue and vertebral bodies forming an adequate spine [80].

Current methods of biological therapy of degenerative intervertebral discs processes

To activate a regenerative potential of IVD, various approaches are used: introduction of therapeutic agents, proteins-activators, different types of cells or cellular populations influencing the biosynthesis and degradation of diverse components of extracellular matrix as well as methods of gene engineering.

Introduction of biologically active substances in the degenerating intervertebral disc. Single or repeated direct injections of TGF-β into the tissue of the human degenerating intervertebral disc *in vitro* promoted the increase of proteoglycan synthesis and reduction of tissue resorption due to the decrease of MMP-2 secretion; a periodic proliferative effect was also observed [81]. Similarly, IGF-1 facilitates increase of proteoglycan synthesis and slowdown of IVD resorption decreasing the level of active MMP-2 [82]. IGF-1 elevates also the viability of the cells acting antiapoptotically [83]. An interesting fact is that the level of IGF-1 reduces with age. At present, there have been published the results of the investigation, in which IVD degeneration of the lumbar part of spinal column was modeled in rabbits. Direct injections of osteogenic protein-1 (OP-1), a growth factor belonging to TGF-β family, contributed to the increase of proteoglycan synthesis and restoration of the IVD height. The obtained result was stable for 8 weeks after injections [83]. After direct injections of OP-1 to the rats, in which IVD degeneration was modeled, inhibition of pain-determined behavior was observed [84].

Methods of gene engineering therapy for IVD regeneration. Delivery of biologically active substances to the degenerating IVD is possible using genetically modified IVD cells expressing the necessary gene product. Owing to the advances in molecular genetics it is possible to introduce the necessary genetic element practically into any cell. Isolated cells from the IVD of a bull or rat were transformed with the help of a retroviral vector containing gene-encoding antagonist of IL-1 receptor (IL-1RA) [85, 86]. These gene constructs were used for *in vivo* injections in the experiments on rabbits with modeled IVD degeneration, as well as transfection of human IVD cells *in vitro* [86]. These experiments showed that retrovirus-based vector is capable to transfect effectively the cells of various kinds of mammals. Later, adenovirus-associated vector (AVV) was developed to overcome the danger of immune reaction to the retroviral vector [87]. The authors [50] have demonstrated that AAV effectively transfects IVD cells of a man and rabbit *in vivo* generating humoral rather than a cellular immune response, and leads to an active transgenic expression.

Despite a sufficient number of investigations proving the flexibility of the direct delivery of genes into IVD cells using virus-based vectors, the question about a gene that is to be delivered is still open. Among the candidates are anabolic factors TGF-β1, LMP-1, *Sox9,* and anticatabolic factor MMPI-1. The first researches on exogenous gene delivery *in vivo* were performed by Nishida et al. [88] using adenoviral vector carrying TGF-β1 in rabbits. The authors established a significant increase of TGF-β1 expression as well as proteoglycan in IVD. When LMP-1 was injected *in vivo* into the disc tissue of rabbits, expression increase of anabolic cytokines BMP-2, BMP-7 and aggrecan was observed, which confirms the expediency of using this factor as a therapeutic means [89]. *Sox9* does not influence the synthesis of proteoglycan but at the same time it increases synthesis of type II collagen if transferred to the cells of the human degenerating IVD. Transfection of *Sox9* by the adenoviral vector to the degenerating disc of a rabbit provides preservation of the structure inherent to the unaffected disc, whereas typical degenerative changes in IVD were observed in the control group of animals [89, 90].

Thus, increase of the synthesis not only of proteoglycan but type II collagen as well can prevent degenerative IVD changes. It may also be supposed that a combined application of various factors will be much more effective and physiological. Simultaneous application of TGF-β1, IGF-1, and BMP-2 is known to have a synergetic effect on the synthesis of useful intercellular proteins. An alternative to the anabolic factors may be the application of anticatabolic factors, which will enable deceleration of the degradation process without the need of synthesis increase in IVD cells. Delivery of MMPI-1 into the human degenerating disc using adenoviral vector contributed to the growth of proteoglycan content in the culture [90].

Application of stem cells in the therapy of IVD degeneration. Achievements in the field of mesenchymal stem cell (MSC) applications make it possible to consider them as a source of cells for gene therapy with subsequent implantation. MSCs are uncommitted multipotent stem cells, which are found in different tissues. They are characterized by a high plasticity and ability to multilinear differentiation. They are available and easy to be manipulated. Several vector systems are known to be introduced into MSC, which demonstrated a high activity of expression [91, 92]. However, transformation is not the only problem that is to be solved. MSC are not differentiated, they should be differentiated into chondrocyte-like cells before implantation. For this purpose, growth factors of BMP family were used [93]. A more specific differentiation factor from the Sox family is being studied. A family of Brachyury transcription factors promotes the necessary cell adhesion [94–97]. Nevertheless, it has recently been

established that it is enough to cultivate MSC with IVD cells for discoid phenotype induction [98, 99].

Cultivation in 3D system also promotes formation of a chondrocyte-like phenotype. When MSC system incorporated in the collagen gel was implanted into the degenerating IVD of rabbits, preservation of the nucleus pulposus and annulus fibrosus structure, prevention of proteoglycan synthesis reduction, increase of IVD height were noted [100]. Implanted cells survive and express genetic markers of the nucleus pulposus and annulus fibrosus. Similar results were obtained injecting a MSC suspension into rabbit IVDs, and suspension incorporated into the gel into IVD of the rat coccygeal part [101–103].

Application of MSC gave a new impetus to the development of autotransplantation methods in degenerative processes in IVD. But there are still a lot of questions to be answered, for example, whether chondrocytes originated from MSC are identical or similar to the cells of the nucleus pulposus. Though the results of the last investigations show the affinity of the cells, nobody knows how long they will preserve the phenotype in such microenvironment in the degenerating IVD, and whether new cells will also be subjected to degeneration or malignization. Unstudied is the question about biomechanical properties of a newly synthesized matrix.

Conclusion

Despite significant advances in the study of molecular and cellular mechanisms of intervertebral disc degenerative processes, the etiology of this disease remains insufficiently explored. Therapeutic methods currently employed in clinical practice are not able to restore the structure and biomechanical function of intervertebral discs. It is important to remember that the development of intervertebral discs in embryogenesis is a complex process of coalescence of several cellular populations by a variety of molecular interactions. The structures of intervertebral discs generated in ontogenesis must function in a strict synergism, especially when exposed to unfavorable endogenous and exogenous factors. Changes of intervertebral discs in the postnatal period (angiogenesis regression, decrease of cellular populations and extracellular matrix) are most likely represent the next stage of intervertebral disc "development", the result of which is a gradual destruction of its structures. Considering the degenerative processes of intervertebral discs in the context of embryogenesis, novel methods of biological treatment of this disease can be suggested. Application of autologous intervertebral disc cells cultured *in vitro* with their subsequent implantation is a promising method, which can compensate for cell deficiency, and, consequently, disc matrix. But to realize this approach, it is first of all necessary to study the viability and activity of the implanted material under the influence of various factors for a long period of time. Thus, a complex approach to the study of molecular and cellular mechanisms of intervertebral disc degeneration pathogenesis may help to discover new methods of biological therapy directed towards the regeneration of microstructure and biomechanical function of intervertebral discs.

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