Angiogenesis in Rat Uterine Cicatrix after Injection of Autologous Bone Marrow Mesenchymal Stem Cells

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 150, No. 12, pp. 705-711, December, 2010
Original article submitted December 2, 2009

Results of injection of autologous bone marrow mesenchymal stem cells with transfected GFP gene into the rat uterine horn cicatrix were studied by light microscopy. Large groups of blood vessels with blood cells inside were seen after injection of autologous bone marrow cells into the cicatrix on the right horn, formed 2 months after its ligation; no groups of vessels of this kind were found in the cicatrix in the contralateral horn. Examination of unstained sections in reflected UV light showed sufficiently bright fluorescence in the endothelium and outer vascular membrane in the uterine horn cicatrix only on the side of injection. Hence, autologous mesenchymal stem cells injected into the cicatrix formed the blood vessels due to differentiation into endotheliocytes and pericytes. The expression of GFP gene not only in the vascular endothelium, but also in vascular outer membranes indicated that autologous mesenchymal stem cells differentiated in the endothelial and pericytic directions.

Key Words: *uterine cicatrix; mesenchymal stem cells; angiogenesis; endotheliocytes; pericytes*

Physiological regeneration of tissues in adult body and their repair in case of injury are realized with direct participation of poorly differentiated precursor or stem cells (SC). The bone marrow generating, in addition to its main hemopoietic function, precursor cells for many tissues is the main source of SC.

The bone marrow contains two principal SC types: hemopoietic and mesenchymal [2], which have precursors potentially capable of transdifferentiation into cells of various phenotypes [8].

In addition to the bone marrow, multipotent SC were found in other tissues of an adult body: in adipose, muscle, and nervous tissues, in the peripheral blood and in umbilical/placental blood. Depending on their microenvironment, SC pass through the blood-brain/mesenchymal barrier, *i.e.* are characterized by high plasticity as regards their differentiation and transdifferentiation.

An attempt at using autologous bone marrow mesenchymal SC (AMSC) for stimulation of regeneration of the myometrial cicatrix under experimental conditions was undertaken due to numerous reports about the efficiency of cell technologies in the treatment of coronary disease. Cell-mediated strategies in the treatment of this condition are based on implantation of bone marrow cells (BMC) directly into the ischemic myocardium or into coronary vessels. The two aims of this treatment are revascularization of the myocardium and compensation for deficiency of the myocardial functional cells [6,9,10,14].

Two main hypotheses on the fate of implanted AMSC are as follows: AMSC continuing differentiation in hypoxic tissues are directly involved in neoangiogenesis and revascularization or the efficiency of AMSC is mainly determined by expression of various angiogenesis factors and other cytokines by these cells.

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We studied the possibility of correcting the myometrial cicatrices in rats by cell technologies and studied the fate of SC after their injection.

MATERIALS AND METHODS

Six-month-old female Wag rats (180-200 g) served as the model. Inferior median laparotomy was carried out under aseptic conditions in rats narcotized with ether. The uterine horns (UH) were brought to the wound and thoroughly wrapped with sterile gauze. A catgut ligature was brought under the terminal of each UH near the corpus uteri and tied. The abdominal cavity was then sutured layer-by-layer.

Autologous mesenchymal SC were isolated by washing out the bone marrow from the tibial bone epiphyses collected under ether narcosis from Wag male rats. The resultant cell suspension was put into plastic flasks (Nunk) and 48 h after bone marrow explantation the free cells were discarded. Adherent cells were cultured in α-MEM with 10% FCS (Biolot) at 37°C in a CO_2 incubator with 5% CO_2 at saturating humidity. The medium was replaced every 3 days. Subculturing was carried out by reinoculation of the monolayer culture at a density of 1000-5000 cell/cm^2 (depending on the growth characteristics of FCS) using standard Versen and trypsin solutions.

Passage 2 AMSC were transfected with pEGFP-N1 plasmid DNA (Clontech Laboratories) carrying green fluorescent protein (GFP) gene. Transfection was carried out in the presence of TurboFect transfection reagent (Fermentas) according to manufacturer's instruction. After transfection, the cells were cultured in α-MEM with 10% FCS (Biolot); G-418 (Sigma) in a concentration of 400 ng/ml was used for selection.

Since no specific markers of AMSC typical of these cells alone were identified, the presumable population was identified by physical, morphological, and functional characteristics intrinsic of cultured mesenchymal SC *in vitro*: adhesion to flask surface, morphology, proliferation, formation of fibroblast-like colonies, and induced osteogenic differentiation. Nontransfected and transfected cells were studied in order to verify retention of AMSC characteristics after transfection.

Light and fluorescent microscopy and cytological methods showed that cultured nontransfected and transfected BMC adhered to plastic *in vitro*, had fibroblast-like morphology throughout the entire period of culturing, were maintained in the culture during several passages, formed colonies of fibroblast-like cells after low density inoculation, and differentiated into bone tissue cells in the presence of strain-specific factors. Nontransfected cells of passages 0-3 and transfected cells of passages 0-2 were studied.

However, physical, morphological, and phenotypical signs are not specific criteria which can be used for specific identification of AMSC. The capacity of AMSC to induced differentiation *in vitro* into bone, adipose, and cartilage cells is the only critical requirement to identification of the presumable SC populations.

Osteogenic differentiation *in vitro* was induced by adding 0.1 μM deoxymethasone, 50 μM ascorbic acid, and 10 μM β-glycerophosphate (Sigma), inducing osteogenic differentiation.

Osteogenic differentiation was identified by two markers: activity of alkaline phosphatase and mineralization of intercellular matrix with calcium ions. Cytochemical detection of alkaline phosphatase was carried out using nitroblue tetrazolium in the presence of 5-bromo-4-chloro-3-indolyl (alkaline phosphatase substrate). Calcium accumulation in the intercellular matrix was detected by alizarin red staining.

Four hours after transfection, the cells were mixed 1:2.5 with nontransfected cells. Repeated laparotomy was carried out and after removal of nonlyzed fragments of the suture material, 100 μl of cell mixture was injected into the cicatrix 2 months after ligation of the right UH. The remaining cells were cultured for 10 days in order to evaluate the efficiency of transfection and stability of GFP expression.

The expression of GFP by rat AMSC was evaluated visually under a fluorescent microscope by directly examining the culture or in a Goryaev chamber by counting transfected cells 48 h after transfection. The efficiency of transfection was evaluated as the percentage of fluorescent cells from the total cell count in a Goryaev chamber. The relative content of transfected cells in diluted culture reached 3%.

Similarly as in the majority of cases, the use of the technology based on the plasmid DNA transfection led to just temporary expression of GFP in rat AMSC. Culturing of cells transfected with pEGFP-N1 plasmid without selection (no G-418) led to reduction of the percentage of GFP-producing cells because of their substitution with nontransfected cells. However, cells producing GFP were found after 1 week in passage 1 culture of transfected cells inoculated at the density of 5000 cell/cm^2. Hence, similarly as in the majority of cases, the use of the technology based on cationic lipid-mediated transfection with plasmid DNA without subsequent selection for obtaining a stable clone led to derivation of rat AMSC culture with temporary expression of GFP.

Fragments of UH with the cicatrix and synechias collected by biopsy 1, 2, 3, and 4 weeks after injection of AMSC were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for at least 24 h, dehydrated in ascending ethanol concentrations, clarified in xylene,

and embedded in paraffin. Sections without staining (5-7 μ) were examined under an Axioimager M1 light microscope at ×1500 in the fluorescent mode with an Alexa 488 filter. The cicatrix on the left UH during the same periods after injection of AMSC suspension and myometrial cicatrix 2 months after UH ligation without AMSC treatment served as controls. Six animals were examined per term.

RESULTS

The development of a moderate adhesive process in the pelvis without involvement of the upper compartments of the abdominal cavity was observed 2 months after the intervention. Pronounced changes in UH manifested by hydrometra above the site of ligature application reaching the isthmic part of the uterine tube, presumably because of impossibility of discharge into the tube and to the abdominal cavity from the tube. The contents of UH lumen was transparent, serous, without suppurative elements. Encapsulated suture material was present at the site of UH ligation, which disagreed with published data on catgut absorption within 15-20 days in humans. We previously noted late absorption of 'lyzed" suture material [1].

Examination of UH structures at a 6-10-fold magnification showed the formation of synechias at the site of ligation, hypertrophy and stretching of the uterine wall, and formation of complete obstruction. Endometrial atrophy was found in the stenosed zone.

One week after injection of AMSC into the cicatrix, large groups of blood vessels with blood cells inside were seen in the cicatrix in the right horn (Fig. 1, *a*). No vessel groups of this kind were found in the cicatrix in the left horn (Fig. 2, *a*).

Examination of unstained sections of the uterine cicatrix in reflected UV light showed sufficiently bright fluorescence in the endothelium and outer membrane of the vessels in the right UH cicatrix (Fig. 1, *a*). Slight edema and numerous small fluorescent objects, most likely capillaries, were also found in the endometrium and myometrium (Fig. 1, *b*).

Very bright fluorescence in the endothelium and adventitia of the large vessels in the right UH cicatrix was seen 2 weeks after AMSC injection. The endothelium and outer membrane of these vessels presented as fluorescent clear-cut lines (Fig. 1, *c*). No fluorescent structures were found in the right UH myometrium and endometrium during this period (Fig. 1, *d*).

During week 3, weak fluorescence (virtually at the basal level) was seen in the walls of just some blood vessels (Fig. 1, *e*). By week 4, the vascular wall fluorescence intensity and the number of vessels with fluorescent structures decreased still more (Fig. 1, *f*).

Vascular membrane fluorescence on the contralateral side was at the basal level throughout the entire period of observation; no groups of vessels were detected. No small fluorescent objects were found in the myometrium and endometrium, but, on the other hand, there were no signs of edema (Fig. 2, *a*-*e*). No fluorescent objects were found in vessels, cicatricial tissues, and adjacent tissues in the UH cicatrices of animals receiving no AMSC (control; Fig. 2, *f*).

The injection of AMSC did not stimulate the absorption of the cicatrix and arrest of the hydrometra phenomena. However, this pilot study was carried out on just few animals. In order to make better based conclusions on the efficiency of this procedure for the status of the cicatrix, the study should be repeated on a greater number of animals during longer periods after AMSC injection.

The rat uterine horn is a thin tubule, one terminal of which is connected to the corpus uteri cavity, the other to the uterine tube opening to the peritoneal cavity at the ovarian surface. Normally the uterine tube contents in mammals is discharged into the abdominal cavity and is then absorbed by the peritoneum. UH ligation leads to ischemia of this zone with subsequent necrosis and formation of a cicatrix. These changes were clearly seen in micropreparation at a slight magnification.

Vascular network developed at the site of AMSC injection (Fig. 1, *a*, *c*, *e*, *f*). Fluorescence of some sites of vascular walls proved that these groups of vessels formed as a result of AMSC injection and from these injected cells (Fig. 1, *a*, *c*, *e*, *f*). The GFP gene inserted in the AMSC DNA was transmitted intact to the daughter cells and cells of next generations. Those cells and the structures formed from them fluoresced just the same way in reflected UV light.

It was assumed up to recent time that the angiogenic response during the postnatal period developed during the growth of preexisting capillaries. However, it was persuasively proven that a small, but biologically significant portion of endothelial cells participating in the formation of new capillaries were of bone marrow origin [3,12,13].

Blood vessels are formed by two interacting cell types: endothelial cell lining the inner surface of a vessel and perivascular cells (pericytes) encapsulating the outer surface of the vascular tubule. Pericytes are not only involved in the hemodynamic process, they also play an active role in vessel formation. Some scientists hypothesized that endothelial cells and pericytes have a common precursor with hemopoietic cells. This hypothesis is based on the fact that developing hemopoietic and endothelial cells have common surface markers and that hemopoietic cells can develop from cells of the main fetal blood vessels [4,5,11].

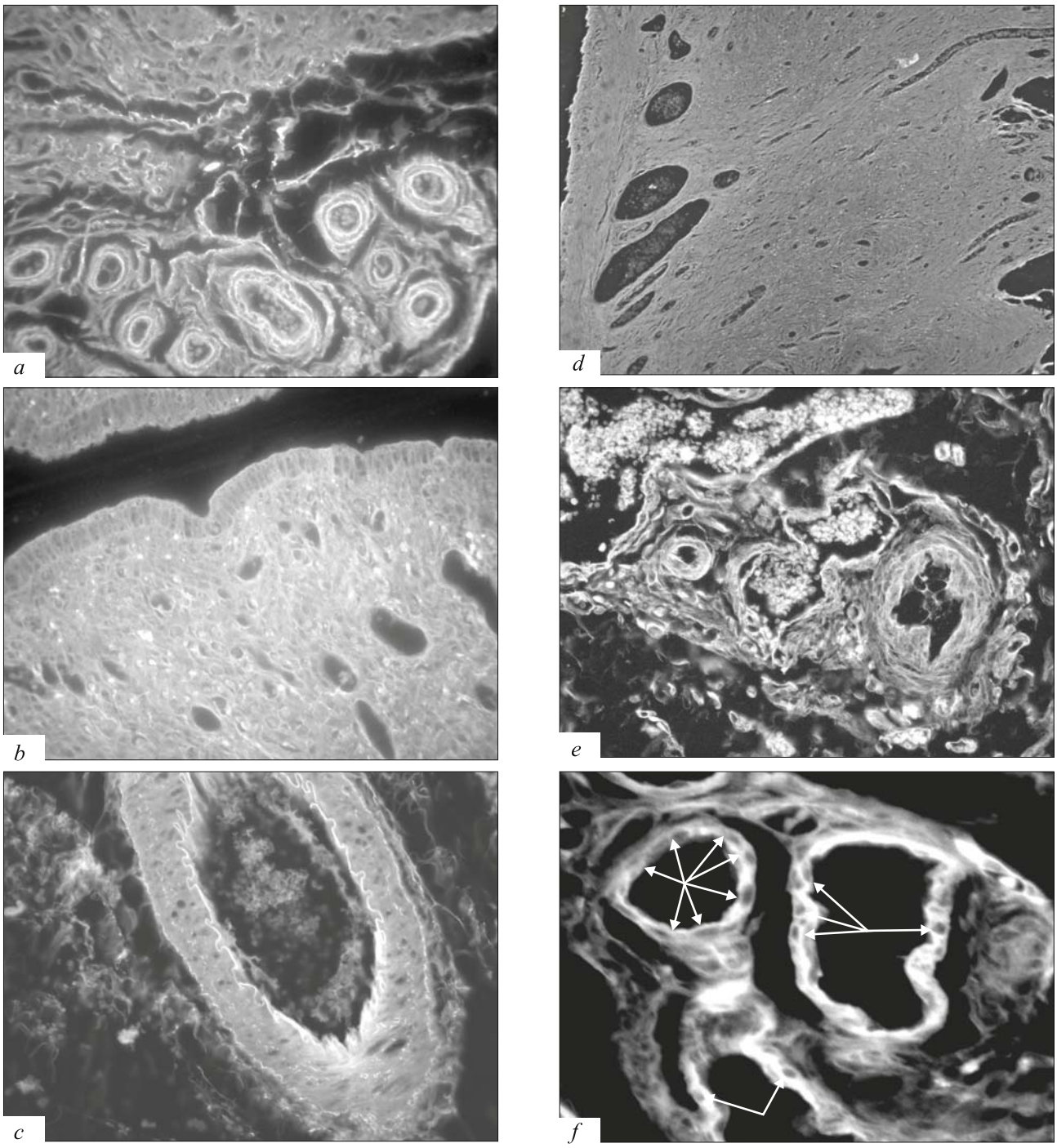

Fig. 1. Cicatrix and tissues of right rat UH (on the side of injection of AMSC with transfected GFP gene) at different terms after injection. Unstained sections in reflected UV light with Alexa 488 filter. *a*) groups of blood vessels in the cicatrix 1 week after AMSC injection. Clearly seen endothelial lining and outer membrane fluoresced in the majority of vessels; *b*) numerous small fluorescent objects (capillaries) in the endometrium and myometrium and interstitial edema 1 week after AMSC injection; *c*) very bright fluorescence in endothelium and adventitia of large cicatricial vessels 2 weeks after AMSC injection. Fluorescent vascular membranes present as clear-cut lines; *d*) no fluorescent structures and signs of edema in the myometrium and endometrium 2 weeks after AMSC injection; *e*) slight fluorescence (virtually at the basal level) in walls of some vessels 3 weeks after injection of AMSC; *f*) weak fluorescence is retained in the walls of just solitary vessels 4 weeks after AMSC application. ×240 (*a*, *b*, *d-f*); ×630 (*c*).

Fluorescence of the endothelium and outer membrane of the vessels observed in our study (Fig. 2, *a*) indicated that the injected AMSC were directly (but not indirectly, *i.e.* through cytokines or other cell signals) involved in the vessel formation, presumably, due to the multipotent cells stimulated to differentiation

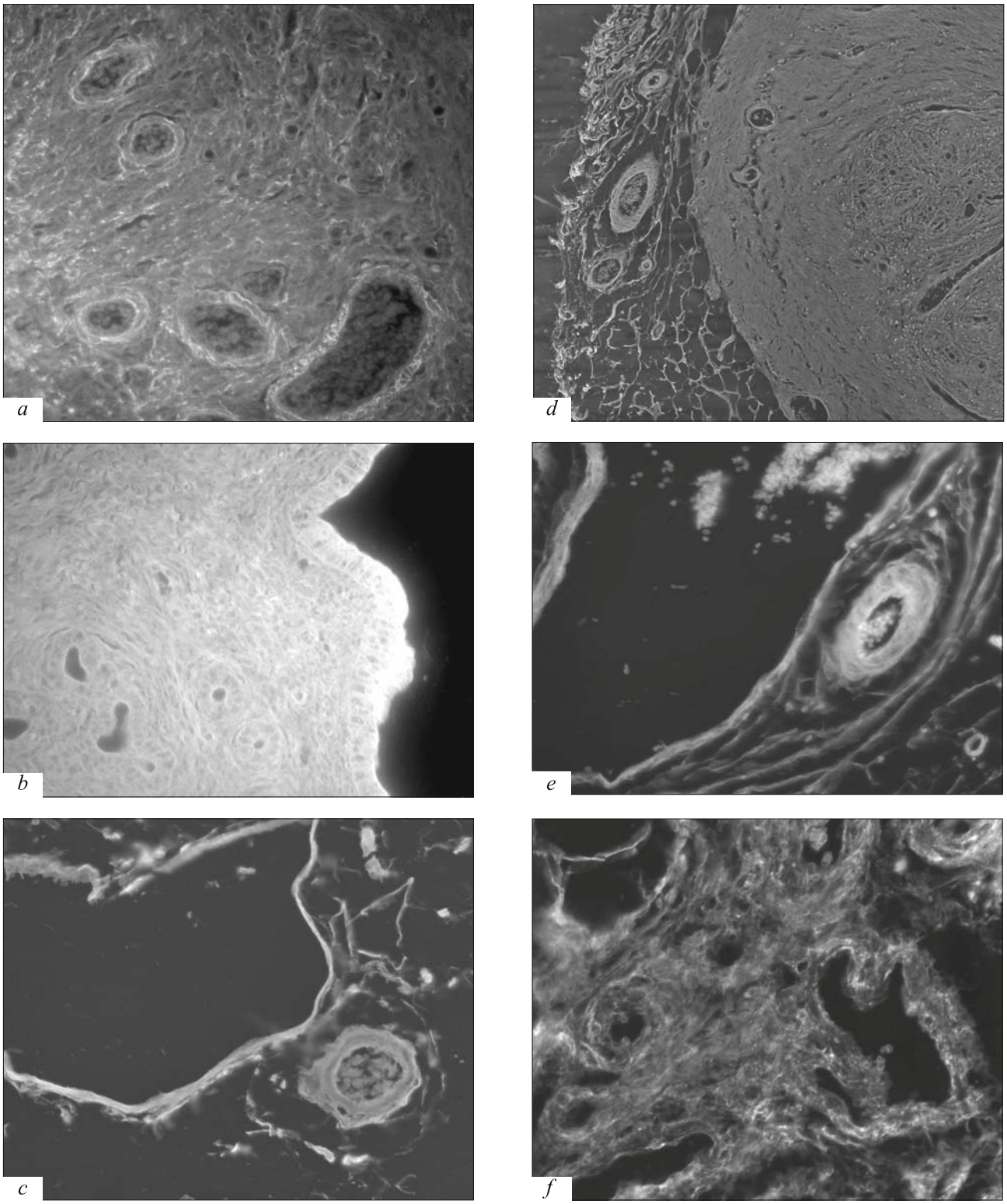

Fig. 2. Results of AMSC injection: control specimens (left UH). Unstained sections in reflected UV light with Alexa 488 filter. *a*) number of vessels in the cicatrix 1 week after AMSC injection is significantly lower, their structures fluoresces virtually at the basal level, ×320; *b*) no fluorescent objects and signs of edema 1 week after AMSC injection, ×240; *c*) no fluorescent structures and signs of edema in cicatricial cells 2 weeks after AMSC injection, ×240; *d*) no fluorescent objects in UH structures and adjacent tissues 3 weeks after AMSC injection, ×180; *e*) no fluorescence in cicatricial vessels 4 weeks after AMSC injection, ×480; *f*) control animals (no AMSC injection); no fluorescent objects in cicatricial structures, ×480.

in the endothelial or pericytic directions. It was also possible that tissue hypoxia caused by ligation of the structures together with the vessels also stimulated differentiation of injected AMSC into endotheliocytes [7].

One more evidence of angiogenesis as a result of AMSC application was the presence of groups of vessels only in the right UH (on the side of their injection; Fig. 1, *a-f*), but not in the contralateral zone (Fig. 2, *a-e*) or control (Fig. 2, *f*).

The expression of transfected GFP gene not only in the vascular endothelium, but also in the outer membranes (Fig. 1, *a*, *c*) most likely indicated that endotheliocytes and pericytes had the same precursor cell or that AMSC could differentiate in both endothelial and pericytic directions. Many scientists suggested that endotheliocytes and pericytes have a common precursor with hemopoietic cells [4,5,11].

Moreover, unilateral development of vessels and presence of fluorescent objects in their walls (Fig. 1, *a*, *c*, *e*, *f*) suggest that the injected AMSC did not migrate from the site of injection, were not destroyed, and did not serve just as the "construction material" or "signal" for angiogenesis for cells of the donor organism. The release of fluorescent protein and transfected GFP gene from destroyed AMSC and their absorption by adjacent cells are possible in all these cases. However, proteins and DNA fragments entering the cells as a result of phagocytosis or pinocytosis were subjected to degradation with loss of the capacity to fluorescence and integration into the genome. In other words, in case of injected AMSC destruction (release of fluorescent GFP protein from AMSC), fluorescence could be the minimum and very rapidly disappearing; and surely, there could be no clear-cut well delineated structures in that case.

Gradual decrease in the fluorescence intensity in vessels of UH cicatrix on the side of AMSC injection was presumably caused by gradual recovery of the genome of transfected cells or substitution of autologous, but however foreign (collected from a donor) red bone marrow cells with recipient cells.

The number of objects synthesizing GFP also decreased during culturing of cells transfected with pEGFP-N1 plasmid without selection, because of their substitution with nontransfected cells.

The metabolic processes in tissues with cicatrix improved due to the increase in the number of vessels, particularly "young" ones with thin walls. Improvement of vital activity conditions and functioning of the fibroblasts can lead to more intense exchange of the intercellular matrix components in the cicatricial connective tissue, rejuvenation of collagen and elastin fibrils, which would further manifest in the appearance of more fine structures, their more orderly disposition, and, presumably, would result in recovery of UH patency with formed synechias.

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