Angiomatosis and Ectopic Ossification in the Dog Myocardium after Intramyocardial Implantation of Autologous Bone Marrow Mononuclears in Experimental Coronary Disease

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Disseminated ossification of the myocardium and severe angiomatosis were detected in sites of implantation of unseparated bone marrow mononuclear fraction cells in dogs with experimental chronic coronary disease. Minor immunocytochemical differences in cells of the mononuclear fraction adhering and not adhering to plastic were found. Significant differences in the expression of mRNA of chondro-osteogenesis genes (aggrecan, lumican, and osteopontin) in adherent and nonadherent cells were detected. The expression of aggrecan gene mRNA was 3-fold lower, of lumican gene 6-fold, and of osteopontin gene 11-fold lower in nonadherent cells compared to adherent fraction.

Key Words: *simulation of coronary heart disease; intramyocardial implantation of bone marrow cells; angiomatosis; ectopic ossification of dog myocardium; morphology*

The use of cell technologies for stimulation of the regeneratory potential of the myocardium in chronic coronary heart disease (CCHD), especially aggravated by the development of progressive heart failure, necessitates identification of cell types capable of transdifferentiation into cardiomyocytes after transplantation to the focus of lesions and induction of angio- and neovasculogenesis [4]. Cell populations with presumable cardiogenic and angiogenic potentialities should be used for more effective cardiomyoplasty. These potentialities are intrinsic of bone marrow cells. The population of adherent cells isolated from the bone marrow exhibited *in vitro* characteristics of undifferentiated mesenchymal stem cells, *i.e.* multipotent cells capable of differentiating into several cell strains [8-10].

The use of the autologous bone marrow mononuclear fraction (BM MNF) for "biological (cellular) shunting" was proposed in 1997 [7] as an alternative to direct revascularization methods [1,3]. At present BM MNF is used for cell therapy in mono- and multicenter clinical trials in many cardiological centers (http://www.clinicaltrial.gov, key words: bone marrow heart). At the same time, the probability of osteogenic differentiation of BM MNF cells in the myocardium was demonstrated in experimental studies of indirect revascularization [1,2, 5,13], which is regarded as a serious complication of cell therapy. It is therefore important to evaluate the intensity and dissemination of ectopic calcification of the apatite type with consideration for immunophenotypical and molecular biological characteristics of implanted bone marrow cells.

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We studied postinfarction morphogenesis after implantation of autologous BM MNF cells into the periinfarction zone and evaluated the molecular and phenotypical characteristics of these cells.

MATERIALS AND METHODS

The study was carried out on 11 mongrel dogs (15-20 kg). CCHD was induced in all animals by ligation of the interventricular coronary artery and collateral branches of the first diagonal artery under conditions of intubation narcosis. The formation of myocardial infarction was confirmed by electrocardiography and visually. All animals received analgesics and antibacterial therapy during the postoperative period. Two animals died from acute heart failure. After 3 months (stage II) implantation of unseparated BM MNF cells was carried out in 4 animals.

The bone marrow was obtained by puncture of the spina ileaca posterior superior 24 h before repeated intervention. The BM MNF cells were separated in Ficoll-urograffin density gradient (1.077 g/ml) at 400*g* for 30 min. Cell suspension was then washed 3 times in buffered saline. Before implantation BM MNF cells were diluted in saline to a concentration of 5×10^6 cell/ml and 2 ml of this suspension was implanted into the myocardium by 5-10 perifocal injections in the cicatricial area.

The animals (n=9) were sacrificed 4 weeks after repeated intervention. Specimens of the anterolateral and apical left-ventricular myocardium were collected for microscopy. Serial cryostat sections (7 μ) were sliced; microtomy was carried out perpendicularly to the transmyocardial channel axis. The sections were stained with hematoxylin and eosin and by the method of van Gieson. The sections were sliced on a Microm HM-550 cryostat (Carl Zeiss) using BioVitrum materials. Morphological analysis was carried out using Axioskop FL-40 microscope with AxioCam MRc camera and AxioVision 3.1 software.

The BM MNF cells were incubated on plastic for 20 min in RPMI-1640 with 10% human albumin. Nonadherent cells were collected and concentrated by centrifugation in buffered saline. Adherent cells were first harvested by short-term incubation in Versen solution with trypsin and then processed as nonadherent cells. The next step was immunocytological analysis of rapidly adhering and nonadherent BM MNF cells on a FACSCalibur flow cytofluorometer (Becton Dickinson). The immunophenotypical characteristics of BM MNF were analyzed using the following markers: CD34 (hemopoietic stem cells), CD45 (lymphoid cells), CD31 (endothelial cells), and CD73, CD90, and CD105 (mesenchymal stem cells).

The expression of chondro-osteogenesis genes (aggrecan, lumican, osteopontin) mRNA in BM MNF was studied by reverse transcription semiquantitative real time PCR. This method specifically amplified cDNA fragments of these genes and β-actin gene (reference gene). Intercalating SYBR Green I stain, materials from Axygen Company, and Bio-Rad equipment (iQ5 Real-Time PCR Detection System) were used.

RESULTS

After 4 months, the morphological changes in the myocardium of dogs receiving no BM MNF consisted in the formation of extensive transmural cardiosclerosis (large cicatricial fields) located distally from the site of coronary artery ligation, assumed as the main morphofunctional criterion of postinfarction remodeling of the heart. An important morphofunctional characteristic of the large connective tissue cicatrices was devastation of the vascular network involving both large arteries and veins and arterioles and venules. The devastation process was realized by obliteration of the vascular lumen at the expense of intimal cell hyperplasia and largely at the expense of tunica media smooth muscle cell hyperplasia (Fig. 1, *a*). Remodeling of the vascular network detected in dog myocardium during the postinfarction period consisted also in recalibration and/or formation of multi-luminal vessels (Fig. 1, *b*, *c*).

Another persistent morphological characteristic of the postinfarction heart was replacement of muscle fibers with adipose tissue progressing mainly from subepicardial to the middle layer of the myocardium. This peculiar postinfarction substitution of the myocardium manifested in the formation of large groups of muscle fibers enveloped in adipose tissue (Fig. 1, *d*). Outside the cicatrices, changes in the vascular network consisted also in the appearance of closed vessels. Important pathomorphological characteristics of postinfarction myocardium were pronounced edema, focal lymphocytic infiltration in the cicatricial zone and intact myocardium, and hypertrophy of some muscle fibers.

Morphological study of the myocardium after CCHD simulation and implantation of BM MNF showed diffuse ossification of the epicardium and subepicardial layer of the myocardium (Fig. 2, *a*, *b*, *c*). Cartilaginous plates could be differentiated in some cases (Fig. 2, *d*); focal calcification without formation of bone or cartilage structures was noted. The next (by significance) obligatory manifestation of atypical morphogenesis was angioma-

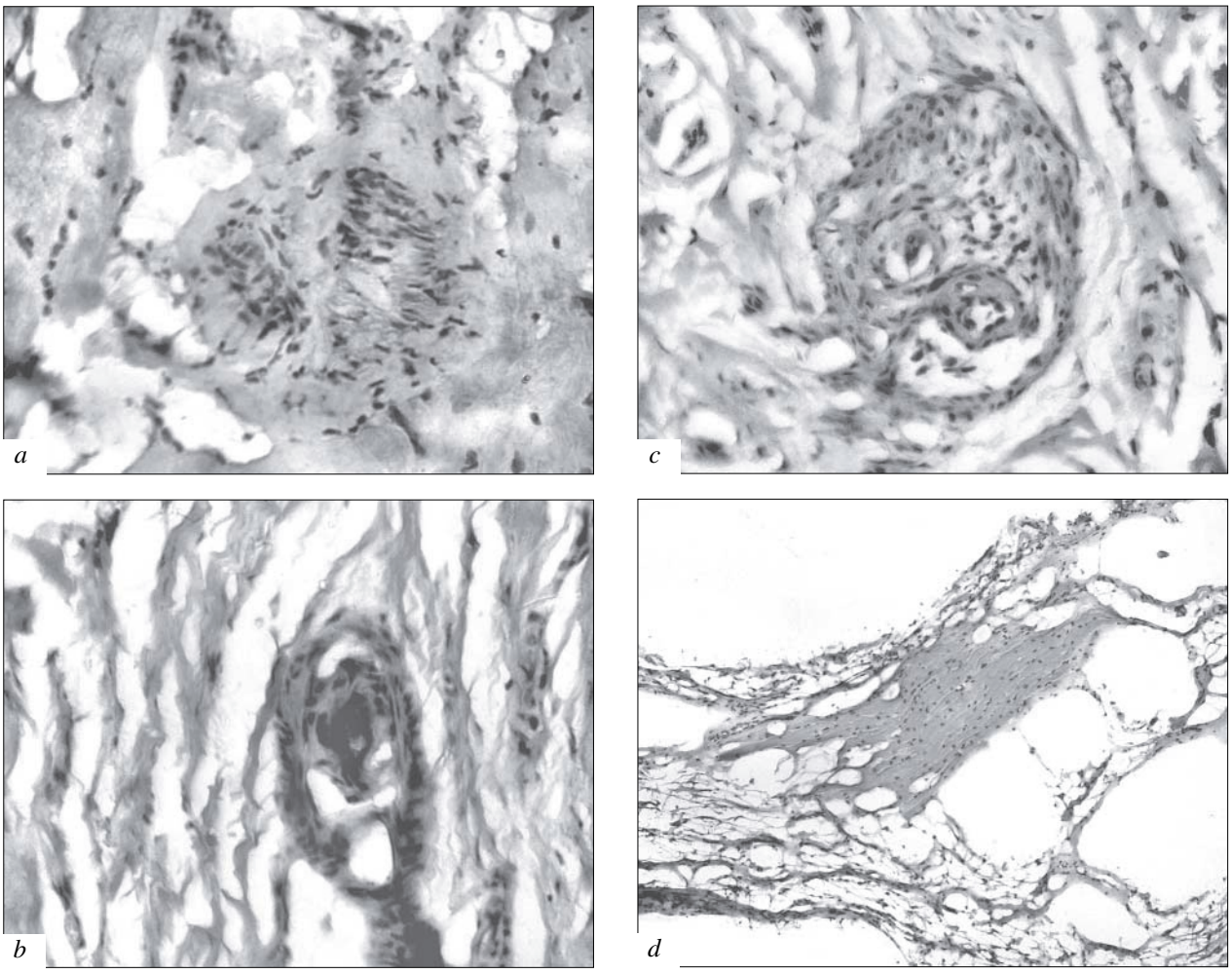

Fig. 1. Morphological changes in dog myocardium 4 months after simulation of myocardial infarction. *a*) thickened tunica media of the intramural artery, ×160; *b*) degenerative changes in endothelial lining, accumulation of cell detritus in vascular lumen, ×180; *c*) formation of multi-layer vessels, ×180; *d*) progressive fatty degeneration of the subepicardial layer, ×40. *a*, *d*: hematoxylin and eosin staining; *b*, *c*: van Gieson staining.

tosis. Importantly, the cells forming angiomatous structures often retained proliferative activity. Formation of "vessel in vessel" structures characteristic of angiomatosis was observed (Fig. 3, *a*, *b*, *c*). In addition, diffuse growth of small monomorphic ("stamped") vessels with hyperchromatic endothelium was seen in the epicardium and subepicardial layer of the myocardium in zones of BM MNF implantation (Fig. 3, *d*); in addition, "vascular buds" were observed here.

One more permanent morphological characteristic of BM MNF implantation zones was focal infiltration with blast cells, often with proliferative activity. Moreover, the stage of capillary formation could be traced: from large cell with hyperchromatic eccentric nucleus and weakly oxyphilic cytoplasm resembling plasma cell (Fig. 3, *e*, *f*) to the next stage, *i.e.* elongation of nuclei and appearance of a peculiar fission with uneven condensation of chromatin. The next stage was the formation of

TABLE 1. Distribution (in %) of Summary Gates of BM MNF Cells

Cell fraction	CD $31^-/34^-$	CD $31^-/34^+$	CD $31^+/34^+$	CD $31^+/34^-$	CD $34^-/45^-$	CD $34^-/45^+$	CD $34^+/45^+$	CD $34^+/45^-$
Nonadherent	51.46±4.62	0.74±0.11	0.56±0.18	47.17±4.72	11.61±4.15	87.41±1.72	0.81±0.18	0.20±0.08
Adherent	55.26±5.33	0.87±0.18	0.29±0.06	43.34±5.32	20.56±1.71	78.56±4.04	0.79±0.19	0.22±0.07

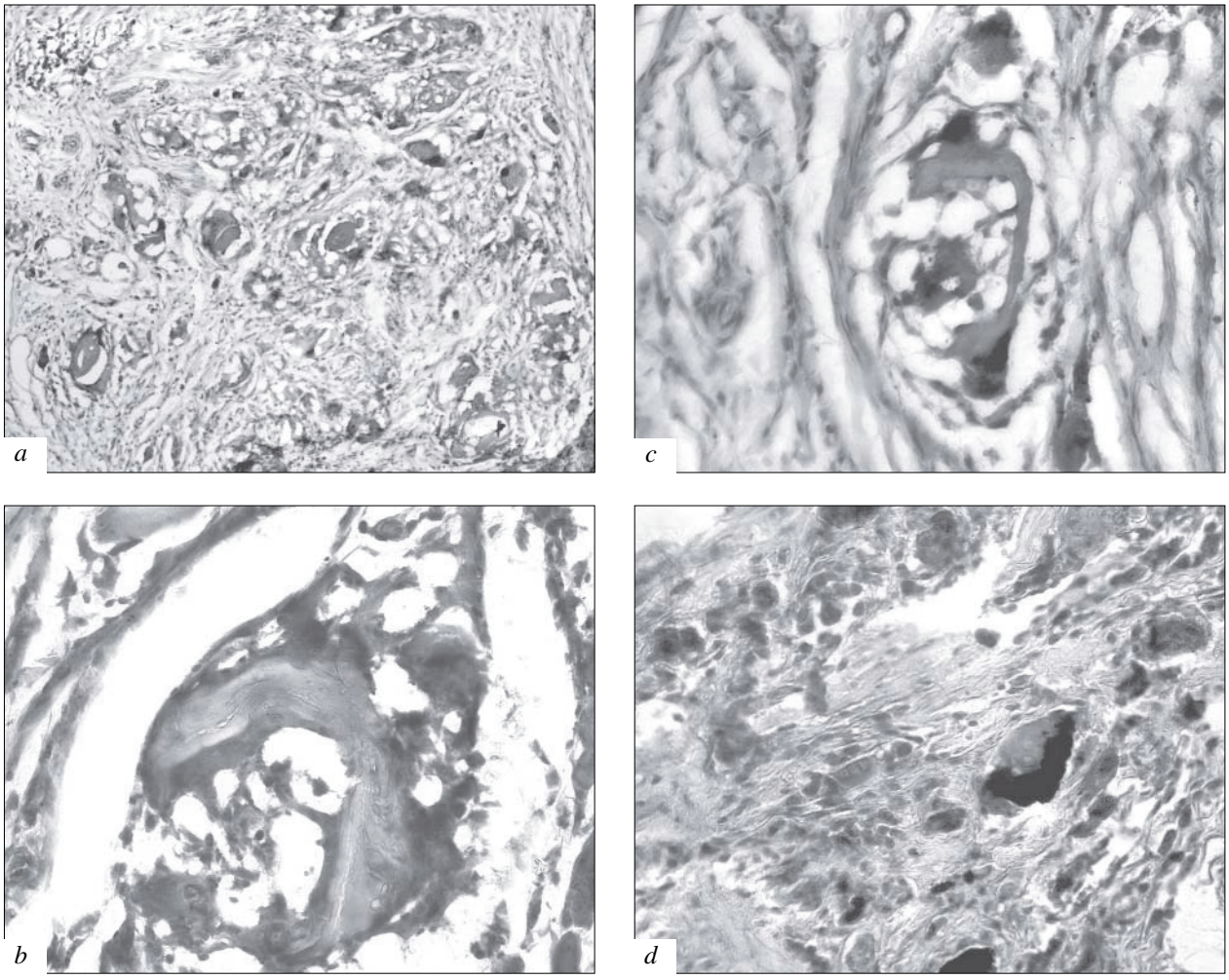

Fig. 2. Ectopic ossification of dog myocardium after implantation of unseparated bone marrow mononuclear cells into the peri-infarction zone. *a*) diffuse ectopic ossification of cardiosclerosis focus, ×40; *b*) formation of a bone bar in the epicardium, ×320; *c*) formation of a bone bar in the subepicardial layer, ×400; *d*) formation of small cartilaginous structures, ×160; *a-c*: van Gieson staining; *d*) hematoxylin and eosin staining.

vascular bud without clear-cut lumen, and then appearance of vascular bud with clearly formed lumen. Importantly, these formations were also observed in infiltrations often organized in a chain.

The results of immunophenotyping of BM MNF cells by flow cytofluorometry were as follows. Distribution of differentiation markers in adherent and nonadherent MNF fractions reflected predominance of cells with the phenotype of BM hemopoietic stem and mature blood cells ($CD34^{+/-}/45^{+}$; Table 1). The percentage of cells with the "endothelial" phenotype $CD31^{+}$ was also high. Mesenchymal stem cells (connective tissue cell precursors [9]) were present in adherent and nonadherent BM MNF (Table 2).

Since endomyocardial implantation of nonseparated BM MNF into the periinfarction zones of the myocardium led to such a side effect as ossification, we analyzed the chondro-osteogenic potential of rapidly adherent and nonadherent cells of the BM MNF by evaluating the expression of aggrecan, lumican, and osteopontin genes mRNA.

The expression of chondro-osteogenesis genes in the supernatant cells was significantly lower than

TABLE 2. Percentage of Mesenchymal Stem Cells in BM MNF

Cell fraction	CD73	CD90	CD105
Nonadherent	23.43±3.74	36.43±6.77	34.43±8.31
Adherent	34.00±5.89	31.57±8.03	27.86±7.78

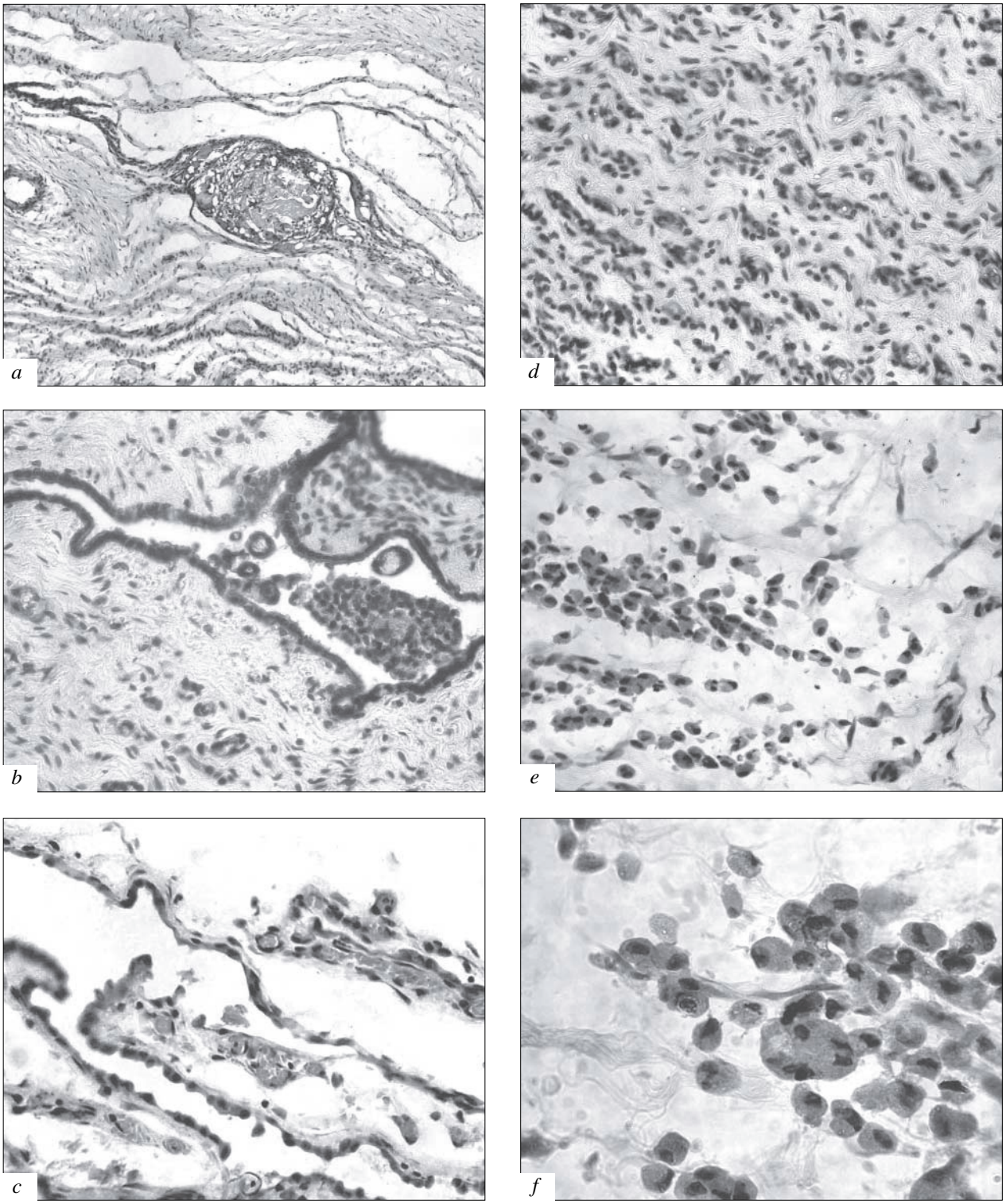

Fig. 3. Angiogenesis morphology in dog myocardium after implantation of nonseparated bone marrow mononuclear cells into the periinfraction zone. Hematoxylin and eosin staining. *a*) formation of *vasa vasorum* in periinfarction zone, ×40; *b*) appearance of cubical endotheliocytes in venous sinuses, ×160; *c*) formation of "vessel in vessel" structures, ×400; *d*) monomorphic capillaries with hyperchromatic nuclei in endotheliocytes, ×160; *e*) numerous chains of blast cells in subepicardial zone, ×160; *f*) blast cells with proliferative activity and oxyphilic cytoplasm, ×400.

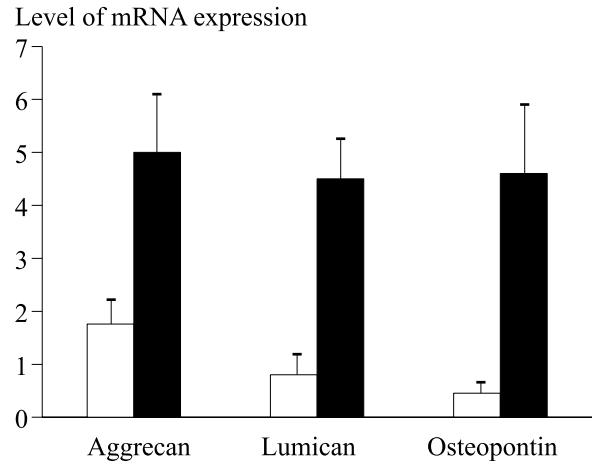

Fig. 4. Expression of aggrecan, lumican, and osteopontin genes mRNA in nonadherent (light bars) and adherent (dark bars) fractions of bone marrow mononuclear cells. The values are presented in arbitrary units. $p<0.05$ for paired comparison of all transcripts.

in adherent fraction ($p<0.05$; Fig. 4). The expression of aggrecan gene mRNA was 3-fold lower, of lumican gene 6-fold, and of osteopontin gene 11-fold lower in the nonadherent compared to adherent fraction. These data indicate different levels of osteogenic potential of BM MNF adherent and nonadherent cells.

Hence, the use of BM MNF cells not separated on plastic during the postinfarction period promotes induction of angiogenesis in the pericicatricial zone of dog myocardium. Diffuse vascular growth in the subepicardial layers of the myocardium and angiomatosis detected after 1 month form the morphological basis for the improvement of cardiac function observed during this period. These data are in line with the results of other research groups demonstrating that implantation of bone marrow mononuclear cells to patients with CCHD generally promoted improvement of myocardial perfusion, but had virtually no effect on the cicatrix size [12,14]. In parallel, we revealed high probability of myocardial ossification, which was a complication of cell therapy. Transdifferentiation of BM MNF cells implanted into the periinfarction zone, into osteoblasts and chondroblasts is a variant of ectopic regeneration, presumably caused by the absence of factors needed for tissue-specific differentiation in the BM MNF cells microenvironment or by molecular biological characteristics of cells committed for differentiation into connective tissue cells in the implant [8]. This latter hypothesis is confirmed by intensive expression of mRNA of genes involved in chondro- and osteogenesis in adherent BM MNF cells.

These results confirm the need in obligatory conditioning of the microenvironment for implanted cells in cell therapy, for example, by adding growth factors needed for cardiomyogenic differentiation [6,11]. Importantly that blast forms usually characterized by high proliferative activity are retained for a long time in zones of BM MNF implantation. This necessitates long-term monitoring of the morphogenetic events in zones of autologous cell implantation and in zones distal from myocardial infarction focus.

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