Effect of Dexamethasone on Differentiation of Multipotent Stromal Cells from Human Adipose Tissue

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Effect of dexamethasone on differentiation of multipotent stromal cells from human adipose tissue was evaluated. Addition of dexamethasone to growth medium resulted in active adipogenesis. Addition of dexamethasone to the osteogenic medium (containing active vitamin D_3 form as the main inductor) led to simultaneous realization of the adipogenic and osteogenic potencies of multipotent stromal cells of the adipose tissue. Hence, the quality of the transplant on the basis of predifferentiated multipotent stromal cells from the adipose tissue for bone tissue repair can be deteriorated by dexamethasone directing some cells to adipogenic development.

Key Words: *human adipose tissue multipotent stromal cells; osteogenesis; adipogenesis; dexamethasone*

Numerous studies devoted to repair of bone tissue defects in modern dentistry, maxillofacial surgery, and orthopedics are focused on transplantation of tissue engineering constructs to the patient. Bone marrow or adipose tissue multipotent stromal cells (MSC) predifferentiated in the osteogenic direction are most often used as the cell component of these tissue equivalents.

Initially, most differentiation media were developed for bone marrow MSC; later the same differentiation inductors were used for studies of functional activity of adipose tissue MSC. Since the mechanism of action of some inductors is little studied, this extrapolation can lead to ambiguous results. One of these inductors, dexamethasone, is a synthetic fluorine-containing long-acting glucocorticoid without mineralocorticoid effect. The effect of dexamethasone on functional activity of MSC is actively studied, but it can be hardly regarded as strictly specific. Dexamethasone in different concentrations (from 10 to 1000 nM) is a component of media inducing adipogenic, osteogenic, myogenic, chondrogenic differentiation of MSC [16]. It stimulates the expression of NMDA glutamate receptors [2], is involved in triggering of the expression of transcription factors needed for hepatocyte differentiation [7]. As a rule, it is not used as the only or the main inductive agent, but just an accessory one.

Osteogenic differentiation of MSC is possible without dexamethasone [5]. The main inductor in these cases is 1,25-dihydroxyvitamin D_3 (active form of vitamin D_3). [15]. Negative effects of dexamethasone on osteogenic differentiation of MSC were demonstrated. It stimulates the synthesis of alkaline phosphatase without modifying the expression of osteocalcin and suppresses the synthesis of collagen 1 [3,10].

Among the expected complications of long-term corticosteroid therapy are obesity and osteo-

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porosis [1]. Some scientists attribute this fact to the corticosteroid (including dexamethasone) capacity to induce adipogenic differentiation of osteoblasts [9] or their precursors [13]. Clinical results correlate with the findings demonstrating dexamethasone capacity to induce bone marrow or adipose tissue MSC adipogenesis [6,14] without 3-isobutyl-1-methylxanthine, often used for this purpose. Importantly, that these studies were primarily carried out on rat or mouse MSC of early passages. These cultures are characterized by heterogeneity and high probability of spontaneous adipogenesis [12]. In addition, human and animal MSC can differently respond to the same inductors, for example, BMP-2 [11]. Hence, extrapolation of the data on the role of dexamethasone in the realization of the osteogenic potential of MSC from the adipose tissue of laboratory animals to human adipose tissue MSC is not correct. We failed to find published reports about such studies carried out on human cells.

We evaluated the effect of dexamethasone on osteogenic differentiation of human adipose tissue MSC.

MATERIALS AND METHODS

Primary MSC cultures were isolated from adipose tissue specimens from the anterior abdominal wall, obtained by cosmetic liposuction from donors (2 women and 1 man, mean age 47 years). Biopsy specimens were delivered in a transport container to the laboratory within 1 h after surgery. Adipose tissue was repeatedly washed in Hanks solution (PanEco) and treated with sterile collagenase-1 solution (250 U/ml; PanEco) at 37°C for 2-3 h. Isolated cells were precipitated by centrifugation (1100 rpm for 10 min) and transferred into culture flasks (NUNC) with complete growth medium (DMEM/F12 1:1; PanEco) supplemented with FCS (HyClone-Perbio) to a concentration of 10%, L-glutamine (4 mM; PanEco), and amikacine (500 mg/liter; Sintez AKO). The flasks were incubated in a CO_2 incubator under standard conditions (37°C, 5% CO_2). The medium was replaced after 24 h. Free cells were removed.

The MSC culture formed a subconfluent monolayer within 1-1.5 weeks, after which it was reinoculated 5-7 times; cells of passages 4-5 were used in experiments. Immunophenotype of MSC was evaluated on a FACS Calibur flow cytofluorometer (BD Biosciences) using mouse monoclonal antibodies to CD13, CD29, CD44, CD63, CD73, CD90, and CD166 (BD Pharmingen).

For directed differentiation the cells were inoculated in 90-mm Petri dishes or 6-well plates and after attaining confluence the growth medium was replaced for differentiation medium (growth medium with 1,25-dihydroxyvitamin D_3 (10^8 M), L-ascorbic acid (50 mg/liter), and β-glycerophosphate (10^{-2} M); Sigma).

In order to detect mineralization foci, the cells were washed twice in PBS (PanEco), fixed in cold 70% ethanol, and stained with 40 mM Alizarine Red S (pH 4.1, Mosreaktiv) for 5 min. Free stain was washed with PSB.

Immunocytochemical analysis was carried out using mouse monoclonal antibodies to alkaline phosphatase and osteocalcin and rabbit antimurine antibodies conjugated with tetramethylrhodamine isothiocyanate (Abcam). Staining was carried out according to manufacturer's instruction.

In order to evaluate the effect of dexamethasone on differentiation course, dexamethasone (Sigma) was added to the growth or differentiation media to a concentration of 100 nM.

Lipid incorporations were stained with Oil Red O (Bio-Optica). The cells were fixed in 4% neutral formalin, washed in PBS, and incubated with the stain for 10 min. After washing of free stain the cells were poststained with hematoxylin (PanEco).

RESULTS

Passage 4 human adipose tissue MSC cultures were presented by homogeneous actively proliferating elongated fibroblast-like cells. Immunophenotypical analysis showed that the greater part of cells expressed stromal markers CD13, CD29, CD44, CD63, CD73, CD90, and CD166 (data not presented).

Even after long-term (more than 3 weeks) culturing of MSC after formation of a confluent monolayer we detected no mineralization foci or lipid incorporations in the cytoplasm in the control cultures (cells cultured in growth medium without inductors; Fig. 1, *a*).

Addition of 100 nM dexamethasone to the growth medium resulted in the appearance of typical phase-contrast lipid incorporations in cells on days 7-10. Staining with Oil Red O showed that these incorporations contained neutral lipids (Fig. 1, *b*). At the early stages, adipocytes appeared in areas with greater density of cells; lipid vacuoles were not large. By days 12-14, the percentage of mature adipocytes (with large or even fused lipid vacuoles, moving the nucleus towards the cell periphery) increased significantly, while their distribution became more even (Fig. 1, *c*, *d*).

Replacement of the growth medium for differentiation one resulted in significant changes in

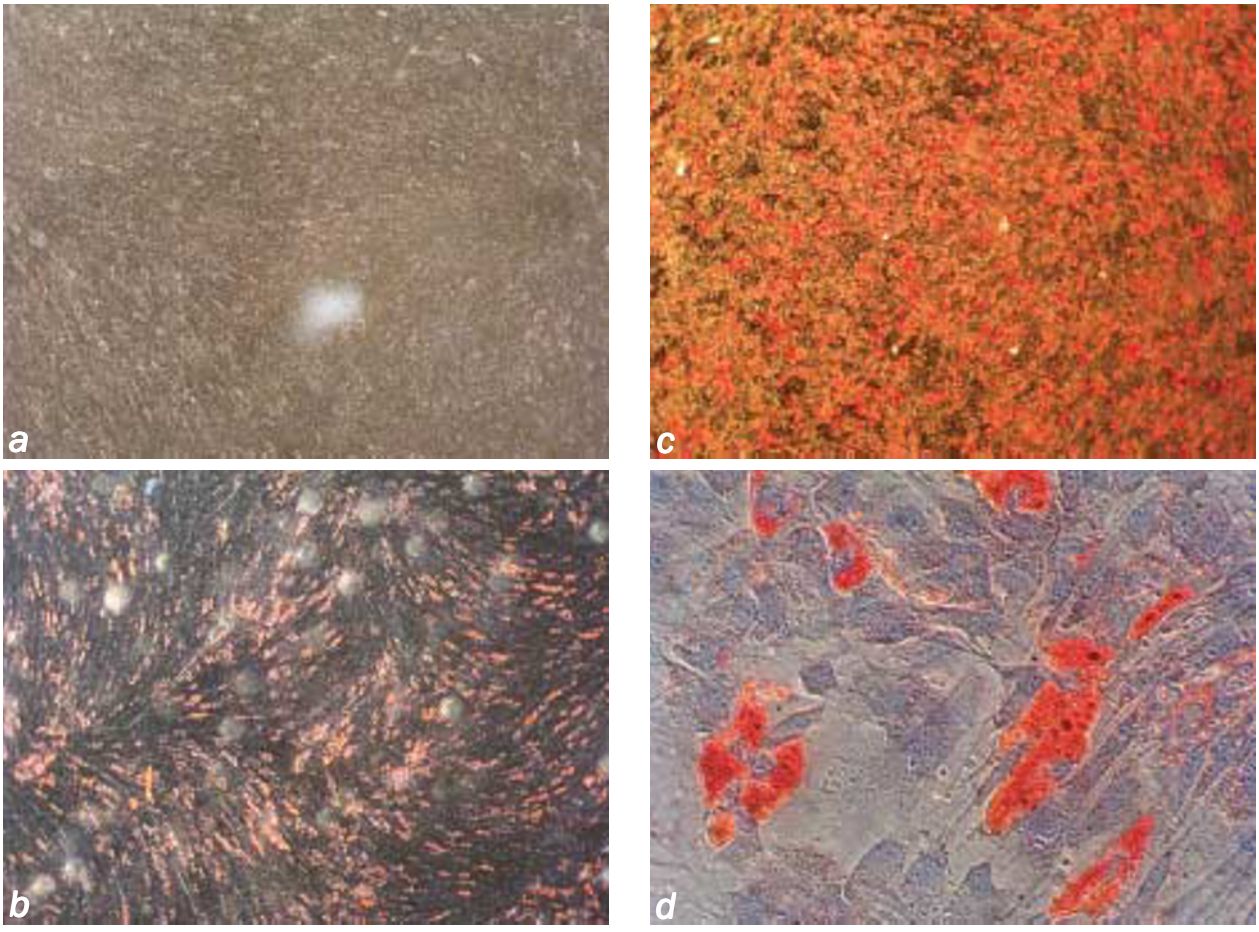

Fig. 1. Dexamethasone-induced adipogenesis in human adipose tissue MSC culture. *a*) control (Oil Red O staining; ×40); *b*) day 7 (Oil Red O staining; ×40); *c*) day 14 (Oil Red O staining; ×40); *d*) day 14 (Oil Red O and hematoxylin staining; ×400).

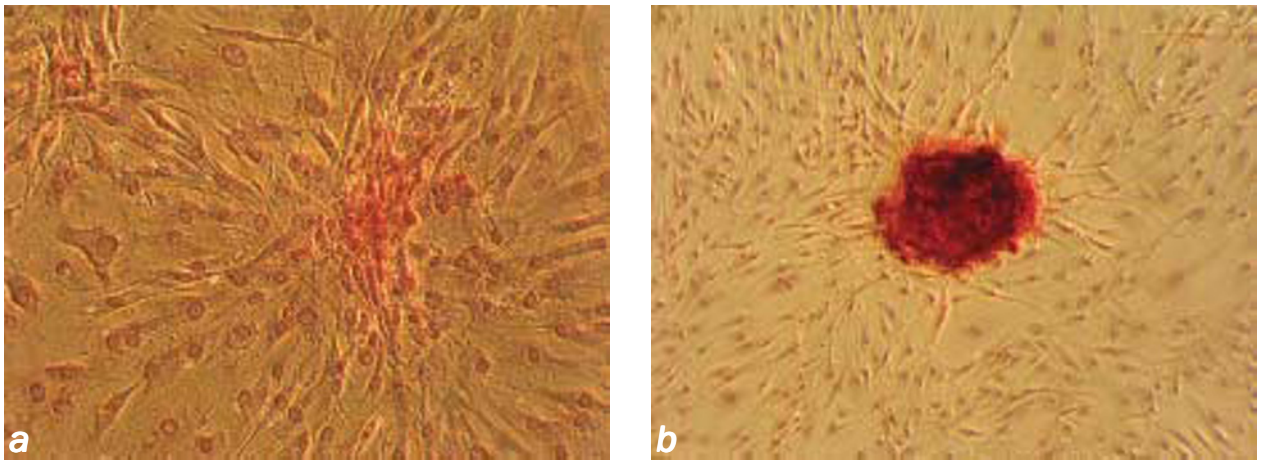

Fig. 2. Vitamin D_3-induced osteogenesis in human adipose tissue MSC culture. *a*) mineralization foci, day 10 (Alizarine Red staining; ×200); *b*) mineralization foci, day 14 (Alizarine Red staining; ×100).

MSC morphology: the orderliness of cell distribution (the so-called waves) disappeared, the cells grew larger and the number of processes increased. By day 10 of culturing, there were characteristic sites where the cells formed 3D structures stained with Alizarine Red (Fig. 2, *a*). During further culturing (14 days) mineralization foci became larger and more compact (Fig. 2, *b*). Immunocytochemical staining revealed accumulations of osteocalcin (the main component of noncollagen bone matrix)

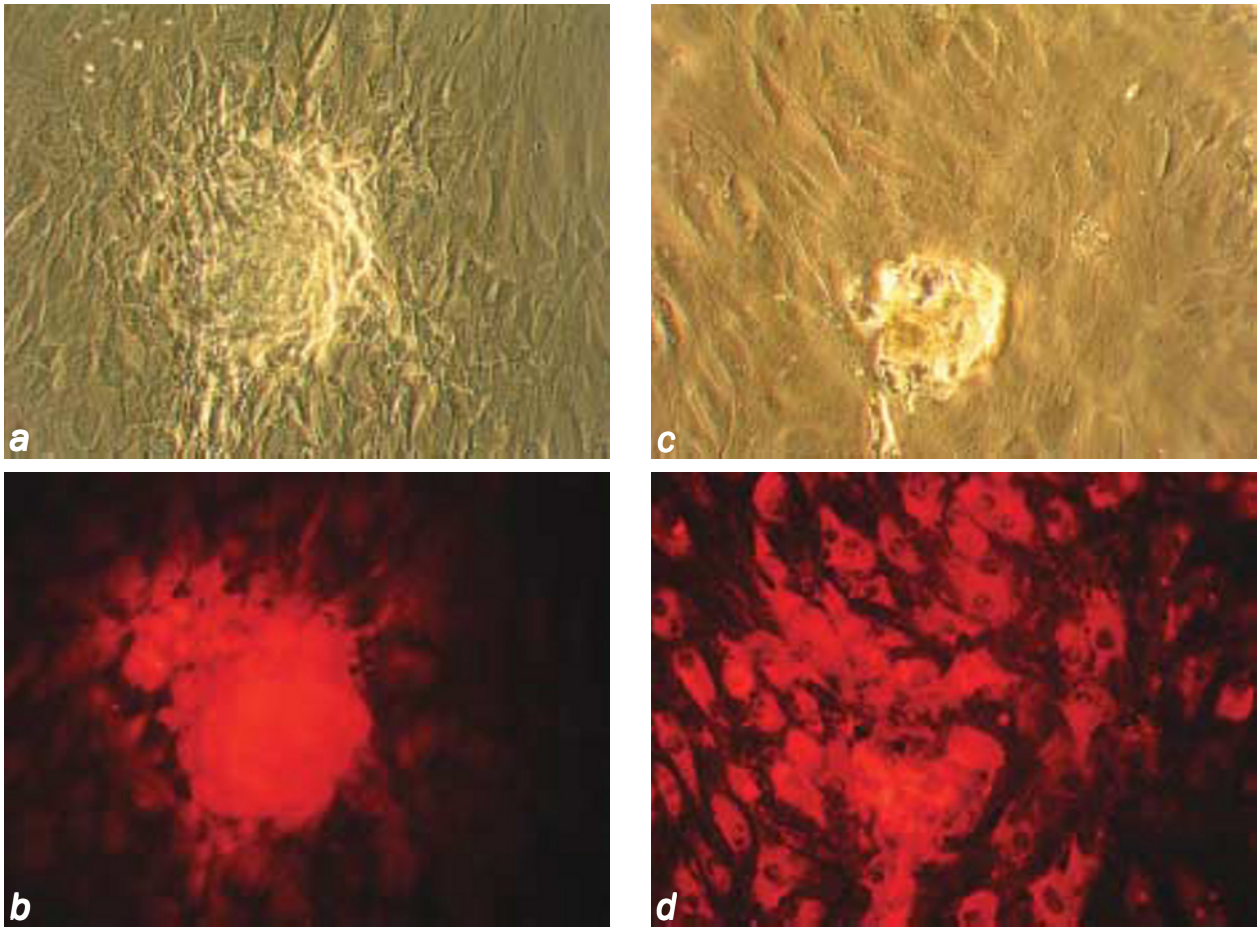

Fig. 3. Osteocalcine synthesis in foci of MSC mineralization (*a*, *b*) and alkaline phosphatase in MSC culture (*c*, *d*) on day 14. *a*, *c*: ×200; *b*, *d*: immunofluorescence (×200).

in these foci (Fig. 3, *a*, *b*). In addition, the level of alkaline phosphatase increased significantly in mineralization foci and in cells adjacent to them (Fig. 3, *c*, *d*).

Cell culturing in differentiation medium with dexamethasone did not modify much the capacity of multipotent cells to form mineralization foci: the time of their appearance, their number and size, and the level of extracellular matrix secretion remained virtually unchanged (data not presented). However, on days 7-10 the development of osteogenic differentiation was paralleled by the appearance of adipocytes in the culture, which were located near and far from the mineralization foci (Fig. 4). By day 14, the number of mature adipocytes increased (Figs. 5, 6); though the level of adipogenesis in general was lower than in MSC culture with dexamethasone in the growth medium (presumably because vitamin D_3 is an inhibitor of adipogenesis [4]).

Our results indicate that dexamethasone can really induce adipogenic differentiation of adipose tissue MSC within short time, being the only inductor (without 3-isobutyl-1-methylxanthine, insulin, indomethacin, or rosiglythasone; Table 1). Importantly that the study was carried out on human MSC of passages 4-5 (the period when the stromal fraction is sufficiently homogenous and free from cell admixtures characteristic of the earlier passages [8]).

Several teams of scientists observed adipogenic and osteogenic differentiation of adipose tissue MSC [6,9,14,16], but the probability of the response of two cell subpopulations to opposite inductors was never demonstrated in the same experiment.

Two hypotheses on the choice of adipogenic or osteogenic development of adipose tissue MSC were proposed [6]. According to one hypothesis, MSC culture is initially heterogeneous and is presented by two populations: unipotent precursors of osteoblasts and adipocytes. According to the other hypothesis, multipotent precursors, initially present in the tissue, develop into two strains of progenitor cells, capable of responding to inductors. Our results indicate the possibility of simultaneous existence of two progenitor cell subpopulations. Parallel activity of two opposite inductors in one experi-

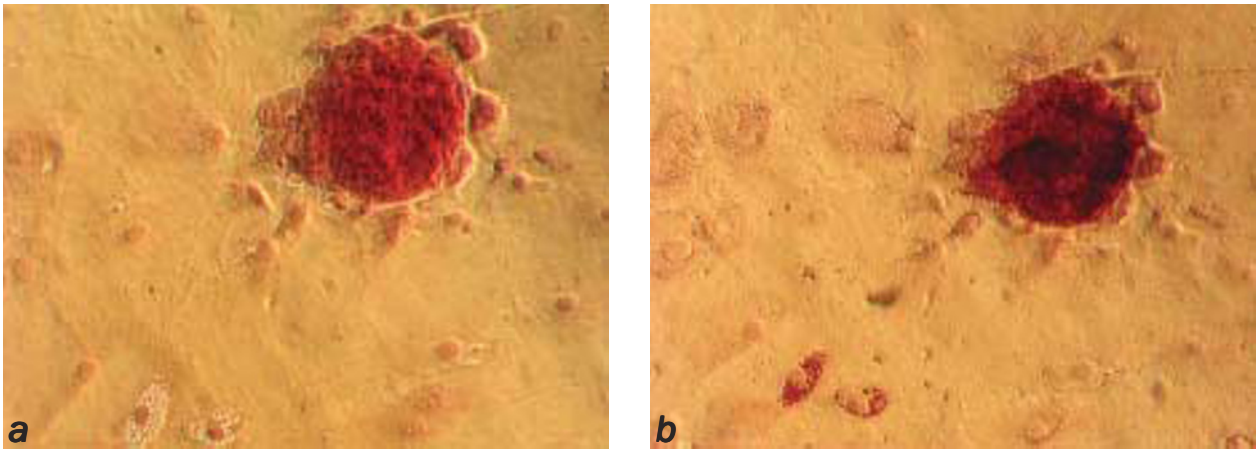

Fig. 4. Simultaneous realization of osteogenic and adipogenic potential of human adipose tissue MSC (×200). *a*) Alizarine Red staining; *b*) Oil Red O poststaining.

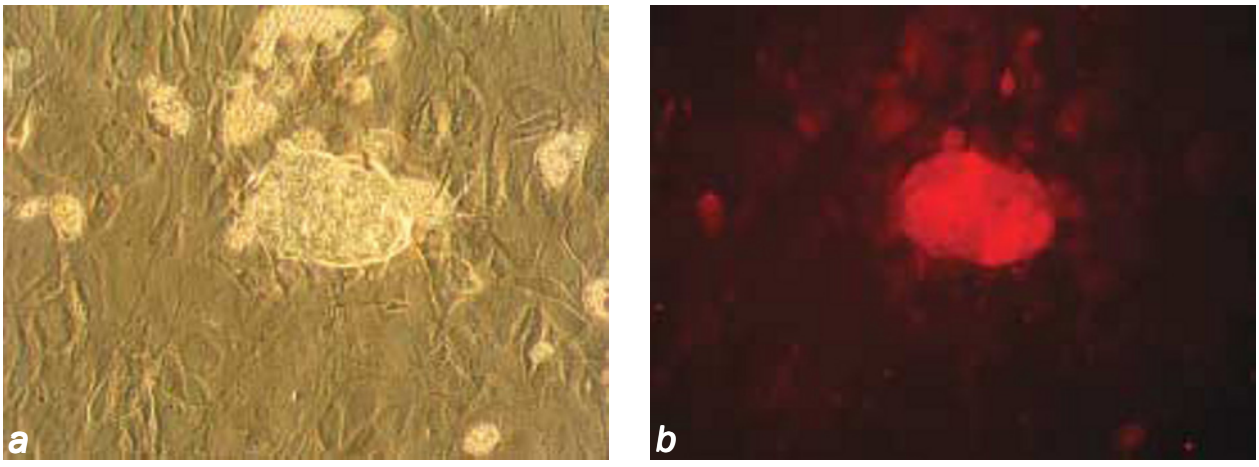

Fig. 5. Osteocalcin synthesis in MSC mineralization foci under the effect of osteogenic medium with dexamethasone (day 14). *a*) ×200; *b*) immunofluorescence (×200).

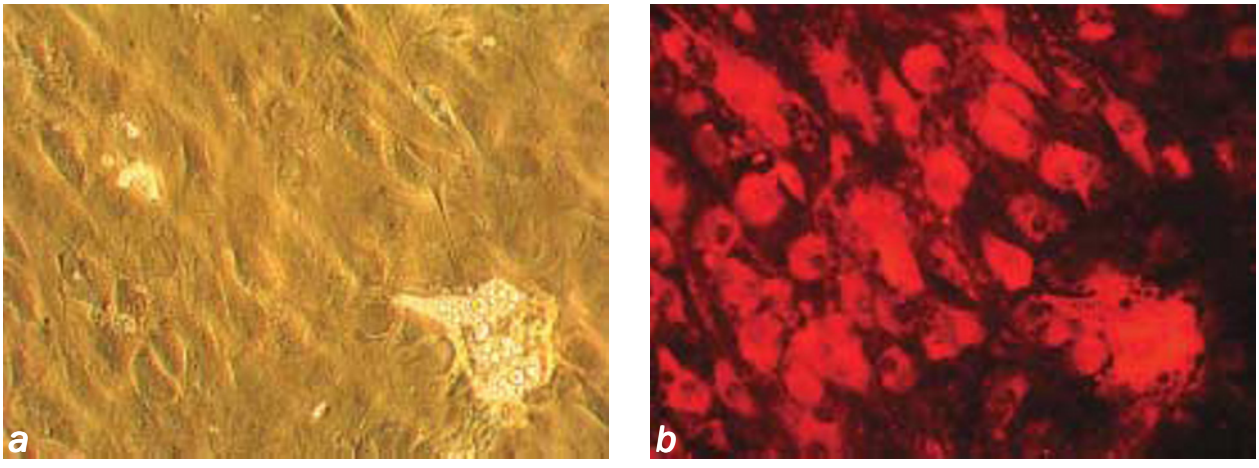

Fig. 6. Alkaline phosphatase synthesis in MSC culture under the effect of osteogenic medium with dexamethasone (day 14). *a*) ×200; *b*) immunofluorescence (×200).

ment leads to simultaneous realization of the MSC osteogenic and adipogenic potentials.

Hence, osteogenic predifferentiation of human adipose tissue MSC is really possible without dexamethasone. Moreover, dexamethasone can appreciably deteriorate the quality of the transplant (MSC-based tissue-engineering construct) by inducing adipogenic development of some cells.

TABLE 1. Effect of Dexamethasone on Realization of Adipogenic/Osteogenic Potential of Human Adipose Tissue MSC

Differentiation direction	Growth medium		Differentiation medium	
	without dexamethasone (control)	with dexamethasone	without dexamethasone (control)	with dexamethasone
Adipogenesis	—	++	—	+
Osteogenesis	—	—	++	++

Note. "-": zero; "+" moderate; "++" significant.

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