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The Content of Multipotent Stromal Cells in 3-4.5-Month Heterotopic Bone Marrow Transplants of CBA Mice Subjected to a Single Exposure to Osteogenic Stimuli (Curettage, BMP-2) or Antigens (*S. typhimurium* antigenic complex, LPS)

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At the early stages of development (3 months), transplants from bone marrow donors subjected to single *in vivo* stimulation (curettage, administration of BMP-2 or antigenic complex of *S. typhimurium*) 1 day before transplantation were characterized by significantly elevated content of nucleated cells (by 1.4, 1.9 and 2.9 times, respectively), efficiency of cloning of multipotent stromal cells (by 3.8, 3.8 and 7.2 times), and total number of multipotent stromal cells (by 5, 7 and 21 times) and osteogenic multipotent stromal cells (by 5, 9 and 15 times) in comparison with the control (intact donors); more rapid increase in the weight of bone capsules was also noted. At later terms, the difference by these parameters between the control and experimental groups became less pronounced, but even in 4.5 months, the total number of multipotent stromal cells in the transplant in experimental groups exceeded the control values by 1.4-1.7 times and osteogenic multipotent stromal cells by 2 times. In donors exposed to the specified stimulations, the content of multipotent stromal cells in the femoral bone marrow in 1 day increased by 2.1 times (curettage), 2.6 times (administration of *S. typhimurium* antigens), and 3.3 times (LPS); administration of BMP-2 reduced this value by 50%. The content of osteogenic bone marrow multipotent stromal cells at this term increased by 1.7 times (BMP-2) and 5.5 times (curettage), after administration of *S. typhimurium* antigens, this parameter corresponded to the control. The concentration of osteogenic multipotent stromal cells in the bone marrow of intact donors was 22%; the maximum values were observed after curettage (57%) and BMP-2 administration (74%) and minimum after treatment with *S. typhimurium* antigens (8%). However, this parameter in all groups of transplants little differed and leveled as soon as by 3-4 months, which can be due to regulatory influences of the recipient body. The initial advantage in the content of bone marrow multipotent stromal cells in donors exposed to osteogenic stimuli and administration of antigens ensured considerably more rapid growth of the transplants in comparison with the control. These results can be useful for the development of optimal protocols of tissue grafting.

Key Words: *bone marrow stromal cells; osteogenic stimuli; immune response; transplantability*

Multipotent stromal cells (MSC) are increasingly used in the sphere of cellular technologies. Thus, autologous transplantation of human bone marrow stromal cells is successfully used for bone reconstruction [6]. In light of this, it seems important to identify factors increasing and reducing the proliferative and differentiation (in particular, osteogenic) potential of these cells and applicable to the MSC populations both *in vitro* and

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in vivo. It has been shown, however, that in the vast majority of cases (22 of 24 donors) human bone marrow stromal cells grown *in vitro* in media containing and not containing osteogenesis-stimulating supplements (Dex/AscP) formed the same amounts of bone *in vivo* after transplantation [6]. The number of MSC forming stromal colonies in cultures did not increase under these conditions, but the number of their descendants (including osteogenic) increased [6]. It was demonstrated that *in vivo* administration of antigens and BMP-2 increased bone marrow count of MSC and osteogenic MSC, respectively [1]. However, the effects of this treatment on the ability of bone marrow MSC to form transplants remained unclear. We have previously demonstrated that bone marrow transplants taken in 2 months after repeated immunization of the animals with killed group A type 5 *Streptococcus* vaccine contained almost 3-fold lower number of MSC than transplants from intact donors. The number of MSC in these transplants returned to normal only in 6 months after immunization of donors. These experiments demonstrated significant suppressive effect of multiple immunizations with Streptococcal antigens on the bone marrow stromal tissue, in particular, on its transplantability [2]. However, the effect of single injection of antigens on transplantability of the stromal tissue was not studied. It should be taken into account that productive immune response, in contrast to chronic, is not only a favorable, but even necessary conditions of successful tissue repair [5,7,8].

Here we studied changes in the number of nucleated cells, MSC, and osteogenic MSC in the bone marrow of donor mice and in 3-4.5-month bone marrow transplants taken in 1 day after single exposure of the donor mice to osteogenic stimuli (curettage, administration of BMP-2) or administration of some antigens (*S. typhimurium* antigenic complex, LPS).

MATERIALS AND METHODS

The experiments were carried out on 2-3-month-old male CBA mice (Kryukovo Breeding Center). The donor mice were subjected to one of the following stimulations: curettage of the tibial bone marrow cavity [1,4], intraperitoneal injection of recombinant human BMP-2 (10 μg/mouse; obtained at the Laboratory of Bioactive Nanostructures, N. F. Gamaleya Federal Center of Epidemiology and Microbiology), or administration of antigenic complex of *S. typhimurium* (200 μg/mouse) or LPS (10 μg/mouse) (Sigma). On the next day, a half of the femoral bone marrow cavity content from experimental or intact donor mice was transplanted to intact animals under the renal capsule as described previously [1,2]. In some experiments, BMP-2 was added to *in vitro* bone marrow cell culture from intact mice and mice subjected to stimulating influences in 10 min after cell seeding until the end of culturing (10 μg per 1 ml culture medium). The suspensions of mouse bone marrow and bone marrow transplants were prepared as described elsewhere [1,2]. Bone marrow cells from donor mice on the next day after stimulating exposures and cells of bone marrow transplants (in 3, 3.5, 4, and 4.5 months after transplantation) were explanted into 25 cm^2-flasks ($1\text{-}3\times10^6$ cells per flask) in 5 ml α-MEM supplemented with 20% fetal calf serum (FCS) (PanEco) and antibiotics (penicillin and streptomycin, 100 μg/ml). The cultures were grown in a CO_2 incubator at 37°C for 12 days, then fixed in ethanol, stained with azure and eosin, and colonies containing >50 fibroblasts were counted.

The content of MSC in bone marrow cell suspensions was evaluated by the number of stromal fibroblast colonies formed by MSC after explantation of these suspensions in monolayer cultures; the content of osteogenic MSC was assessed by the number of stromal fibroblast colonies positively stained for alkaline phosphatase (P^+ colonies). Efficiency of cloning (ECF-MSC) was evaluated by the number of colonies formed by 10^5 explanted cells. Osteogenic activity of MSC was evaluated by alkaline phosphatase activity in stromal colonies measured using C-86 kit (Sigma).

The data are presented as the mean±standard error of the mean ($M\pm m$) from at least 3 experiments. Significance of difference was evaluated by Student's *t* test; the differences were significant at $p<0.05$.

RESULTS

In 3-month transplants from donors subjected to *in vivo* stimulation (curettage, BMP-2, *S. typhimurium* antigens), we observed a significantly increased number of nucleated cells (by 1.4, 1.9 and 2.9 times, respectively), higher ECF-MSC (by 3.8, 3.8 and 7.2 times), number of MSC (by 5, 7, and 21 times) and osteogenic MSC (by 5, 9 and 15 times) in comparison with the transplants from intact donors (Table 1); more rapid increase in the weight of bone capsules was also noted (Table 2). Thus, the rate of transplant formation during the first 3 months was normally considerably lower. However, at later terms (by the 4th month) as the number of nucleated cells in the transplant of the experimental groups approached their maximum values, the increase of these parameters slowed down (more drastically after administration of *S. typhimurium* antigens and less markedly after BMP-2 injection), while in the control, the rise of these parameters became more pronounced. Thus, the difference by ECF-MSC and by the content of nucleated cells and MSC between the control and experimental 4.5-month transplants was leveled, but the total number of MSC

TABLE 1. MSC Content in the Bone Marrow Transplants of CBA Mice after Exposure to Osteogenic Stimuli or Antigens (*M*±*m*)

Age of the transplant	Number of nucleated cells per transplant, ×10^6	ECF-MSC, ×10^5	P^+ colonies, %	Number of MSC per transplant	Number of osteogenic units per transplant	MSC multiplication factor	Osteogenic MSC multiplication factor
3 months							
normal	1.0±0.3	0.8±0.1	25±1	8±1	2±0	1	1
curettage	1.4±0.2	2.9±0.3	25±1	40±5	10±1	5	5
BMP-2	1.9±0.4	2.9±0.4	32±4	55±8	18±2	6.9	9
S. typhimurium	2.9±0.2	5.8±0.2	18±2	168±11	30±2	21	15
3.5 months							
normal	2.1±0.4	0.6±0.1	25±0	12±3	3±1	1	1
curettage	2.9±0.6	1.5±0.1	36±3	44±5	16±2	3.7	5.3
BMP-2	2.8±0.2	1.9±0.3	34±2	53±4	18±2	4.4	6
S. typhimurium	4.8±0.2	2.7±0.2	45±6	130±9	58±5	11	19.3
4 months							
normal	3.0±0.7	2.5±0.1	28±1	74±15	21±4	1	1
BMP-2	4.8±1.1	3.3±0.7	30±5	159±26	48±8	2.1	2.3
S. typhimurium	4.2±0.4	5.4±1.0	24±5	229±43	55±10	3.1	2.6
LPS	6.0±1.8	3.2±0.2	23±3	189±26	43±6	2.5	2
4.5 months							
normal	4.6±1.1	4.8±0.3	29±3	222±41	54±10	1	1
BMP-2	6.3±0.5	5.0±0.7	39±1	311±28	121±11	1.4	2.2
S. typhimurium	4.6±0.3	8.1±0.9	30±4	372±18	112±5	1.7	2.1
LPS	7.6±1.3	4.5±0.3	32±2	344±56	110±18	1.6	2.0

TABLE 2. Dry Weight of Bone Capsules in Bone Marrow Transplants of CBA Mice after Administration of BMP-2 and *S. typhimurium* Antigens to Donors (*M*±*m*)

Treatment of bone marrow donors	Age of the transplant			
	3-3.5 months		4-4.5 months	
	weight of bone capsules, mg	experiment/ control	weight of bone capsules, mg	experiment/ control
Control (intact donors)	0.5±0.1	1	1.2±0.3	1
BMP-2	1.0±0.1	2.0	1.6±0.2	1.3
S. typhimurium antigens	1.2±0.2	2.4	1.1±0.2	0.9

per transplant at this term in the experimental groups surpassed the control values by 1.4-1.7 times and osteogenic MSC by 2 times.

At the early stages (3-3.5 months), more rapid increase in the weight of bone capsules was observed after administration of BMP-2 and *S. typhimurium* antigens (Table 2). At later terms (4-5.5 months), when the weight of bone capsules in the control increased by 2.4 times, the differences observed after administration of introduction of *S. typhimurium* antigens were leveled, while in the group with BMP-2 stimulation, the weight of bone capsules surpassed the control by 1.3 times. Thus, at later terms, the specified influences had either no (antigens *S. typhimurium*) or less pronounced effect (BMP-2) on the weight of bone capsules than on MSC content in the transplants. Thus, the formation of the bone marrow organ with bone capsule occurred more rapidly under the influence of BMP-2 and *S. typhimurium* antigens. The most rapid growth of transplants (judging from number of nucleated cells) was observed after administration of antigens (*S. typhimurium* antigens and LPS), while curettage and BMP-2 treatment were less effective. The concentration of osteogenic MSC was maximum after curettage (57%) and BMP-2 administration (74%) and minimum after stimulation with *S. typhimurium* antigens (8%) in comparison with 22% in the bone marrow of intact donors (Table 1). However, in all groups of transplants, the concentrations of osteogenic MSC little differed and leveled as soon as by 3-4 months due to the decrease in the content of osteogenic MSC after curettage and BMP-2 administration and increase in this parameter in the control group and after administration of antigens). This can result of recipient body control over the development of the transplants or influence of osteogenic stimuli released from the transplant during their formation.

The number of nucleated cells in the donor bone marrow intended for transplantation little varied, and therefore, the same number of bone marrow cells was placed under the renal capsule of the recipients (Table 3). However, all the examined influences significantly increased ECF-MSC of the bone marrow and MSC content per femur, except BMP-2 that reduced these parameters by 50% (which agrees with previous [1]). Hence, the bone marrow used for transplantation in our experiments contained either increased number of MSC (after administration of *S. typhimurium* antigenic complex by 2.6 times and LPS by 3.3 times), or increased number of MSC and osteogenic MSC (curettage; by 2.1 and 5.5 times, respectively), or increased content of osteogenic MSC (administration of BMP-2; by 1.7 times). As the most rapid growth of transplants (judging from the number of nucleated cells) was observed after administration of antigens, but not osteogenic factors (antigens *S. typhimurium* and LPS, but not curettage and BMP-2), accelerated growth of the transplant was most likely determined by increased total amount of MSC in the donor bone marrow giving rise to the adequate pool of osteogenic MSC; however, increased content of osteogenic MSC alone also provided accelerated growth of the transplant. Indeed, the number of osteogenic MSC can be increased, if necessary (here by adding BMP-2 *in vitro*), in all groups, except the group with *in vivo* BMP-2 stimulation, where it appears to provide the maximum content of osteogenic MSC.

Addition of BMP-2 *in vitro* (Table 3) dramatically increased the number of osteogenic MSC in bone marrow cultures from donors of all groups, including group treated with *S. typhimurium* antigens, in which the numbers of MSC increased at the expense non-osteogenic MSC [1]. Thus, in the group stimulated with *S. typhimurium* antigen, non-osteogenic differentiation of MSC in 1 day after single immunization was most likely reversible, and this effect was also observed after *in vivo* BMP-2 stimulation [1]. Indeed, in 1 day after successive (with 3-h interval) injection of *S. typhimurium* antigen complex and BMP-2, the number of osteogenic MSC in femoral bone marrow increased by 2.7 times in comparison with intact animals, by 3.7 times in comparison with administration of *S. typhimurium* antigen complex, and by 1.6 times in com-

TABLE 3. ECO-MSC in Cultures of Bone Marrow Cells from CBA Mice 1 Day after Administration of BMP-2, Curettage, Administration of *S. typhimurium* Antigen Complex against the Background of BMP-2 *in vitro* (50 μg/ml)

Treatment	ECF-MSC, $\times10^5$	P^+ colonies, %	Number of nucleated cells per femur, $\times10^7$	Number of MSC per femur	Experiment/ control	Number of P^+ colonies per femur	Experiment/ control
Intact	3.3±0.4	22±2	1.20±0.12	396±51	1	87±11	1
Intact+BMP-2 *in vitro*	3.1±0.4	54±4				214±28	
BMP-2 *in vivo*	1.6±0.1	74±4	1.21±0.15	194±22	0.5	144±16	1.7
BMP-2 *in vivo*+BMP-2 *in vitro*	1.5±0.3	70±6				136±15	
Curettage	6.2±0.9	57±3	1.35±0.20	837±118	2.1	477±71	5.5
Curettage+BMP-2 *in vitro*	6.4±1.1	82±2				686±96	
S. typhimurium antigen complex	8.0±0.7	8±1	1.30±0.17	1040± 93	2.6	83±7	1.0
S. typhimurium antigen complex+BMP-2 *in vitro*	8.0±1.3	22±4				228±23	
LPS	10.4±0.8	13±3	1.25±0.1	1300±102		169±15	1.9
LPS+BMP-2 *in vitro*	9.8±1.1	32±5				416±34	

parison with administration of BMP-2 [1]. It should be taken into account that bone marrow transplantation that forces MSC to form a new bone marrow organ in the body is a powerful stimulus of osteogenesis capable (at least in short term after administration of antigens) of mobilizing all existing MSC in the donor bone marrow to the formation of transplant, even if they were committed to non-osteogenic differentiation.

This, however, is observed only after single administration of the antigens. Repeated administration of the antigens (reproducing chronic immune process) was followed by a sharp decrease in the number of MSC and bone tissue in the transplants for up to 6 months, probably due to exhaustion of MSC pool after repeated immunization. Similar changes were described for skin fibroblasts: local application of *S. typhimurium* LPS on the wound defect stimulated proliferation of fibroblasts and their regeneration capacity, in contrast to long-term chronic infections [3]. Thus, our findings suggest that the initial benefit of increased MSC in donors subjected to osteogenic stimulation or administration of antigens ensured considerably more rapid growth of the transplants in comparison with the control. These results can be useful for the development of optimal protocols of tissue grafting.

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