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Simultaneous Administration of NOD-2 (MDP) and TLP-4 (LPS) Ligands to Bone Marrow Donors 24 h before Transplantation Increases the Content of Multipotent Stromal Cells (MSCs) in Bone Marrow Grafts in CBA Mice Compared to the Total Result of Their Isolated Administration

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In 3-month bone marrow transplants of CBA mice from bone marrow donors receiving single injections of TLR-4 ligand (LPS) or NOD-2 ligand (muramyl dipeptide, MDP) 24 h before transplantation, an increase in the total number of MSCs (by 2.6 and 1.9 times, respectively), as well as a slight increase in the number of nuclear cells and the mass of bone capsules (by 1.3 and 1.2 times) were observed. After combined administration of MDP and LPS to donors, the total content of MSCs in the grafts was higher by 1.6 times in comparison with the total result of their isolated administration (and by 7.2 times in comparison with the control). At the same time, the concentration of osteogenic MSCs in the grafts of all groups was almost the same and corresponded to the control level. The number of nuclear cells and the mass of bone capsules of the grafts after combined administration of LPS and MDP were close (~80%) to the sum of the results of their isolated administration. These findings suggest that activation of the stromal tissue and the success of bone marrow transplantation depend on the intensity of innate immune responses. These data can be useful for the development of optimal methods of tissue transplantation.

Key Words: *transplantation; MSCs; immune response; NLR and TLR ligands*

Multipotent stromal cells (MSC) are widely used in cell technologies for reparation of bone and tissue defects. For instance, autologous transplantation of human bone marrow (BM) stromal cells was used successfully for bone reconstruction [11]. The anti-inflammatory and immunomodulating potencies of MSC are well known and used in clinical practice [11]. Importantly, MSC play a role of tissue-protective agents by inhibiting apoptosis, restricting the oxidative damage, and promoting tissue regeneration [13]. In light of this, it is important to identify the factors that augment the proliferative and osteogenic and differentiation potencies of these cells and efficiency of their transplantation.

We have previously shown that at the early developmental terms (3.0-3.5 month), BM transplants derived from donor mice subjected to single osteogenic stimulus (curettage or administration of *S. typhimurium* antigens) 1 day prior to transplantation were characterized by elevated content of nucleated cells, higher content of total and osteogenic MSC,

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and increased mass of bone capsules in comparison with the control group [5]. The content of MSC in BM of the donor mice increased by 2.1 or 2.6 times in 24 h after curettage or administration of the antigens, respectively [5]. Probably, initially greater content of MSC in experimental donor mice accelerated the growth of their transplants in comparison with the control. It is known that single administration of antigens and ligands of the pattern recognition receptors (PRRs) *in vivo* can increase MSC content in BM [1-3]. We have previously demonstrated that injection of NLR2, TLR3, TLR4, and TLR5 ligands to CBA mice increased cloning efficiency, proliferative activity, and total MSC content in hematopoietic and lymphoid organs as soon as in 1 h postinjection [1-3]. Thus, the population of MSC (specifically, BM MSC) that contains a large number of osteogenic progenitors is activated as early as during the innate immune response. Hence, activation of several types of PRRs can trigger the immune response, which would dramatically differ from the response induced by individual PRRs. For instance, combined stimulation of NLR and TLR pronouncedly up-regulates NF-κB activity and augments the innate immunity responses; moreover, it essentially strengthens the antimicrobial resistance, *e.g.* against *Salmonella* infections [14]. We have previously demonstrated that combined administration of muramyl dipeptide (MDP) and TLR-4 (LPS) synergistically elevates MSC content in bone marrow and peritoneal exudate in comparison with the sum of their individual injections as early as 24 h after administration to CBA mice [6]. This finding put the questions: *1*) how this pronounced elevation of total MSC content in the transplanted BM relates to its transplantability, *2*) can this transplantability be inhibited in parallel with elevation of MSC content, and *3*) whether transplantability can be improved by simultaneous administration of ligands of different PRRs to BM donors.

This work was designed to determine colony-forming efficiency (CFE-MSC) and the number of MSC (the osteogenic cells included) in to-be-transplanted BM 24 h after individual or combined injections of MDP and LPS to donor mice; in addition, the study aimed at evaluation of the content of nucleated cells, total and osteogenic MSC, as well as the mass of bone capsules in 3-month-old BM transplants in CBA mice, which had been isolated 1 day after individual or combined injections of MDP and LPS to the donors. The content of MSC in BM was evaluated by the number of colonies of stromal fibroblasts formed by MSC after explantation of BM cells into monolayer cultures; the content of osteogenic MSC was determined by the number of colonies with alkaline phosphatase activity (P^+ colonies).

MATERIALS AND METHODS

The experiments were carried out on CBA mice (n=140) weighing 18-20 g obtained from Andreevka Central Animal Breeding Department. The mice received intraperitoneal injections of TLR-4 ligand LPS (Sigma-Aldrich) or NOD-2 ligand (MDP; Sigma-Aldrich) individually or in combination in a dose 10 μg/mouse in 0.4 ml physiological saline. The control group consisted of intact mice.

On postinjection day 1, half the content of BM in femoral cavity was placed under the renal capsule of intact mice [1-3]. To determine CFE-MSC, $1\text{-}3\times10^6$ BM cells of intact mice and those taken 1 and 24 h after immunization as well as the cells of 3-month-old transplants were explanted into 25 cm^2-culture flasks [1-3,5] with α-MEM (Sigma) supplemented with 15% fetal bovine serum (PanEco) and cultured in an incubator at 37°C and 5% CO_2. On days 10-12, the cultures were presented by discrete colonies formed by stromal fibroblast with macrophage admixtures. The cultures were fixed with ethanol and stained with azure and eosin; then, the colonies with no less than 50 fibroblasts were counted. CFE-MSC was determined by the number of colonies formed from 10^5 BM transplant cells. The osteogenic potency of MSC was determined by activity of alkaline phosphatase assayed using a C-86 kit (Sigma-Aldrich).

The data were analyzed statistically using Statistica 10.0 software (StatSoft, Inc.); the Student's t test was applied. The results are presented as $M\pm m$ (by the results of at least 3 experiments). The differences were significant at $p<0.05$.

RESULTS

It is known that simultaneous activation of transmembrane TLR-2,3,4,5 and cytosol NLR-1,2 receptors dramatically (by 5-10 times) increases secretion of cytokines such as IL-1, IL-8, IL-6, IL-10, IL-12, and TNFα by BM macrophages and peripheral blood mononuclears; in addition, it synergistically increases the levels of proinflammatory cytokines in blood serum *in vivo* in response to application of the corresponding ligands [15]. Previously we demonstrated that in 3 h after combined application of LPS and MDP *in vitro* to primary 12-day culture of BM stromal cells derived from CBA mice, a synergistic elevation of TNFα concentration by 32 times relatively to the control value was observed; this elevation surpassed the total effect of individual applications of LPS and MDP [6,8]. At the same time, IL-10 level corresponded to the control, which attested to proinflammatory character of the process and the absence of immunosuppression [6]. Thus, the results of combined use of LPS and MDP

ligands attested to potentiation of the innate immunity response in comparison with the total effect of individual application of these agents, which is reflected by the state of stromal tissue in BM [6].

In 24 h after combined administration of LPS and MDP to donor mice, we observed a synergistic elevation of CFE-MSC and total MSC content (by 9.2 times in comparison with the control level) in BM intended for transplantation in comparison with the total result of individual administration of these agents (by 3.5 and 2.5 times above the control) (Table 1), which agrees with our previous observations [6]. Importantly, the content of nucleated cells in donor BM little changed after individual or combined administration of LPS and MDP. Thus, in both cases, equal numbers of BM cells were implanted under the renal capsule. In contrast, the content of osteogenic MSC decreased in 1 day after administration of LPS, MDP, or LPS+MDP by 1.5, 1.6, and 2.2 times, respectively, in comparison with the control (18%), but never dropped below 8%. This fact agrees with our data on elevation of total number of BM MSC and progressing decline of osteogenic MSC level during up-regulation of innate immunity response, which probably is associated with appearance of a large number of non-osteogenic MSC under these conditions [1-3].

Injections of LPS, MDP, or LPS+MDP increased the content of osteogenic MSC in BM by 2.4, 1.5, and 4.1 times, respectively, in comparison with the control; the most pronounced elevation was observed after combined administration of both ligands. Apparently, the concentration of osteogenic MSC (that must not drop below a certain level), rather than their content plays a role in the maintenance of the bone tissue and its function. Thus, up-regulation of innate immunity response potentiates activation of the stromal tissue, which is manifested by elevation of the total content of BM MSC and the number of osteogenic MSC in parallel with the drop in concentration of the latter. Evidently, alternative ligand combinations can produce other effects on osteogenic MSC than that exerted by LPS+MDP administration. Actually, we demonstrated that activation of TLR-3 *in vivo* by individually administered Poli I:C maintains concentration of osteogenic MSC at the control level, but combined administration of Poli I:C with LPS or *S. typhimurium* antigens augments osteogenesis in population of BM MSC [7].

In 3-month BM transplants of CBA mice injected with a single dose of LPS or MDP 1 day prior to transplantation, an increase in the number of nucleated cells (by 1.3 times), total MSC (by 2.6 and 1.9 times, respectively), and osteogenic MSC (by 2.4 and 1.7 times, respectively) was observed in comparison with the control values (Table 2). However, combined administration of LPS or MDP increased the number of nucleated cells, total MSC, and osteogenic MSC by 1.9, 7.2, and 7.4 times, respectively (Table 2). Thus, in comparison with the total results of individual injections of LPS or MDP, their combined administra-

TABLE 1. MSC Content in BM Cultures of CBA Mice in 1 Day after Individual or Combined Injection of LPS and MDP to BM Donors (*M*±*m*)

Group	CFE-MSC, $\times10^{-5}$	Number of MSC per femur	Increase on MSC content, fold change	Content of P^+ MSC colonies, %	Number of P^+ MSC colonies per femur	Increase on the number of P^+ MSC colonies, fold change
Intact	1.5±0.2	183±24	1.0	18±1	33±4	1.0
LPS	5.4±0.9	648±108	3.5	12±3	78±13	2.4
MDP	3.8±0.7	456±84	2.5	11±1	50±2	1.5
LPS+MDP	12.9±2.0	1677±260	9.2	8±2	134±21	4.1

TABLE 2. MSC Content in 3-Month BM Transplants from CBA Mice Receiving Individual or Combined Injection of LPS and MDP 1 Day prior to Transplantation (*M*±*m*)

Group	Number of nucleated cells per transplant, $\times10^6$	CFE-MSC, $\times10^{-5}$	MSC content per transplant	Increase in MSC content, fold change	Content of P^+ MSC colonies, %	Number of P^+ MSC colonies per transplant	Increase on the number of P^+ MSC colonies, fold change
Intact	1.8±0.2	2.3±0.4	42±9	1	22±2	9±2	1
LPS	2.3±0.4	4.8±0.9	110±20	2.6	20±3	22±4	2.4
MDP	2.3±0.5	3.4±0.5	79±12	1.9	19±4	15±2	1.7
LPS+MDP	3.4±0.7	8.9±1.7	302±61	7.2	22±4	67±13	7.4

tion increased the content of total and osteogenic MSC by 1.6 and 1.8 times, respectively. Thus, combined administration of the ligands produced synergistic rather than additive effects.

Paradoxically, the concentration of osteogenic MSC in the transplants of all groups were virtually identical and corresponded to the control level (Tables 1, 2), despite it was pronouncedly different in various BM samples used for transplantation (Table 1). Similar phenomenon was observed with the use of curettage, BMP-2, or *S. typhimurium* antigens. The concentrations of osteogenic MSC in corresponding experimental groups were 57, 75, and 8%, but their in the transplants was similar in all groups [5]. This fact can be explained by the control of recipient organism of the transplant formation or by the effect of osteogenic stimuli released by the transplants themselves during their development. Probably, the above data indicate that important condition needed for accelerated growth of the transplants is the total number of MSC in donor's BM, which in the following would become the progenitors of osteogenic MSC needed to form the bone capsule. It should be taken into consideration that BM transplantation *per se*, which promotes MSC to build a novel BM organ, is a potent stimulus for osteogenesis, which can be used to mobilize available MSC in donor's BM in order to develop the transplant even if these cells were diverted to the pathway of non-osteogenic differentiation. Actually, in 1 day after successive injections of *S. typhimurium* antigen complex and BMP-2 to mice performed at the interval of 3 h, the count of BM osteogenic MSC increased in comparison with intact control (by 2.7 times), the level attained after individual injection of *S. typhimurium* antigen complex (by 3.7 times), and the count observed after individual injection of BMP-2 (by 1.6 times) [3].

Similarly, the combined administration of LPS and MDP elevated the number of nucleated cells and the mass of bone capsules in the transplants, which attained 80% of the corresponding total values observed after individual injections of these ligands (Table 3). Thus, there were no linear relationships between these parameters and total MSC content (or number of osteogenic MSC) indicating a more intricate association between these indices. It should be stressed that elevation of BM transplantation efficiency was achieved only by a single injection of the ligands. Importantly, we showed that multiple injections of killed type 5 group A streptococcus vaccine to CBA mice, which can be viewed as a kind of chronic immune process model, provoked a dramatic drop in MSC count and a decrease in mass of bone capsules in the transplants aging up to 6 months, which probably resulted from depletion of MSC pool caused by repetitive immunization [4]. A similar effect was observed with cutaneous

TABLE 3. Effects of Individual and Combined Injections of LPS and MDP to Donor Mice 1 Day prior to Transplantation on the Mass of Bone Capsules in 3-Month BM Transplants of CBA Mice (*m*±*SE*)

Group	Mass of bone capsules, mg	Mass of bone capsules relatively to control
Intact (control)	0.6±0.1	1.0
LPS	0.7±0.2	1.2
MDP	0.7±0.1	1.2
LPS+MDP	1.1±0.3	1.8

fibroblasts: in contrast to chronic infections, the local acute application of LPS to the wound region up-regulated their proliferation and regeneration ability [9]. An alternative view on this paradoxical difference can be based on the fact that chronic inflammation activates p53 protein known to moderate proliferation and differentiation needed to repair the tissues by corresponding stem cells [10,12].

It should be noted that not all combinations of NLR2 and TLR4 ligands augment the innate immunity response. Actually, in 3 h after combined application of LPS and Poli I:C *in vitro* to 12-day-old culture of primary stromal cells of mouse BM, concentration of TNFα was intermediate between those observed after their individual use. Interestingly, in 1 and 24 h after administration of this combination *in vivo*, the content of femoral BM MSC was also intermediate between the levels resulted from individual injections of LPS and Poli I:C [7]. Probably, the effective combination of multifarious ligands aimed to increase the level of BM MSC should augment the innate immune response.

This study showed that initial advantage in the content of donor BM MSC secures a more successful growth of the transplants in comparison with the control; the greater advantage, the stronger the effect at least within the observed range of MSC content. It seems possible to enhance BM transplantability by combined administration of various PRR ligands such as LPS and MDP employed in this study. Overall, our data also indicate dependence of stromal tissue activation and effectiveness of BM transplantation on the strength of innate immune response. Evidently, the present data can be useful in the development of optimal methods of tissue transplantation.

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