Age-Associated Reduction of the Count and Functional Activity of Stromal Precursor Cells Can Be Caused by Both True Reduction (Exhaustion) of Cell Pool and Regulatory Effects of the Organism

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 151, No. 2, pp. 173-177, February, 2011
Original article submitted April 13, 2010

The study was carried out on CBA mice using the method of heterotopic transplantation. A fragment of the femoral bone marrow ($^1/_2$) or spleen ($^1/_5$ of the organ) was transplanted under the renal capsule of a recipient. The following donor–recipient cross-transplantation variants were studied: young→young (Y→Y), young→old (Y→O), old→old (O→O), and old→young (O→Y). Cell suspensions were prepared from 2-month transplants inoculated in monolayer cultures and the cloning efficiency (ECF-F) of stromal precursor cells (CFC-F) was evaluated. The bone marrow transplant ECF-F and the count of CFC-F in the O→O group were 8-fold lower than in the Y→Y group. In the O→Y group, ECF-F was 3-fold higher than in the O→O group, but by 2.5 times lower than in the Y→Y group. ECF-F in Y→O group was 2-fold lower than in Y→Y group. The ECF-F and CFC-F count in spleen transplants in the O→O group were 4- and 6-fold lower, respectively, than in Y→Y group. However, in O→Y group ECF-F was 7-fold higher than in O→O group and higher than even in Y→Y group. The weight of induced ectopic bone tissue after transplantation of the osteoinductor (fragments of the allogenic urinary bladder mucosa) was 2-fold lower in the O→O *vs.* Y→Y group. However, comparison of the ectopic bone tissue weights in different experimental groups showed that osteoinductor activity of the bladder epithelium did not decrease, but increased 3-fold with age (O→Y:Y→Y). A 5-fold reduction of this proportion in groups where the osteoinductor was transplanted from old donors to old and young recipients (O→Y:O→O) could be attributed to age-specific reduction of the count of inducible osteogenic precursor cells (IOPC). The data in general suggest that age-specific reduction of the stromal precursor count and functional activity could be caused by the true reduction (exhaustion) of cell pool (bone marrow CFC-F; presumably, IOPC) and by the regulatory effects of the organism (bone marrow and splenic CFC-F, IOPC). These data seem to be significant for understanding of the role of osteogenic stromal precursor cells in the development of age-associated bone tissue defects, for example, senile osteoporosis.

Key Words: *stromal cells; age-associated changes*

Stromal cells have many functions in a living organism: they are involved in tissue reparation, are responsible for creation of the hemopoietic and lymphoid cell microenvironment, participate in the creation of intercellular matrix, and act as antigen-presenting cells. Aging is associated with bone weight loss and reduction of the reparative capacity of tissues, of CFC-F count and osteogenic activity [2,5,11]. It remains un-

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0007-4888/11/1512-0210

clear to what measure this reduction is caused by true reduction of the stromal stem cell count and to what measure it is a result of the growth factor, hormone, and cytokine activities.

After heterotopic transplantation (*e.g.*, under the renal capsule) of organ fragments, cell suspensions, polyclonal strains, or individual clones of stromal fibroblasts, stromal stem cells present in the transplant reproduce the structure of the initial organ: determined osteogenic precursor cells (DOPC) of the bone marrow form the bone populated by hemopoietic cells, while splenic stromal stem cells form the spleen [6,9,10]. The transplant is a sort of a chimerical organ in which stromal stem cells and their descendants belong to the donor, while the hemopoietic and lymphoid cells, macrophages and the endothelium belong to the recipient [9]. Hence, analysis of the transplant status after cross transplantation of the bone marrow or spleen to old and young animals can show whether the CFC-F pool is really exhausted with age or reduced count of these cells in the corresponding organs is a result of host regulation. Studies of the mechanisms of the formation of ectopic (extraskeleal) bone tissue by inducible osteogenic precursor cells (IOPC) under the effect of specific osteogenesis inductors bring forward similar problems. It is known that osteogenesis induced by decalcified bone matrix, reduces with age in animals [8,12] and that the activity of this osteoinductor is due to morphogenetic proteins [8]. We previously showed a 2-fold age-associated reduction of ectopic osteogenesis under the effect of another osteoinductor (topographically not related to the skeleton), the urinary bladder transitional epithelium [7]. It remains unclear to what measure this reduction was caused by age-associated reduction of IOPC count or sensitivity of these cells to the osteoinductive effect of the transitional epithelium and to what measure it was due to age-associated changes in epithelial osteoinductive activity. In order to answer this question, we performed cross-transplantation of the urinary bladder transitional epithelium from old and young donors (guinea pigs) to old and young recipients with subsequent measurement of induced bone tissue weight.

The study was carried out using the method of heterotopic transplantation of mouse bone marrow and spleen with subsequent evaluation of cloning efficiency (ECF-F) and CFC-F count in the transplants by the monolayer culturing method.

MATERIALS AND METHODS

Experiments were carried out on 2-24-month-old male CBA mice and 4-5- and 36-month-old male guinea pigs from Kryukovo Breeding Center. For heterotopic transplantation, half of mouse femoral bone marrow (contents of the femoral bone cavity) or $^1/_5$ of the spleen was implanted under the renal capsule of these animals as described previously [1,3] in the following donor-recipient combinations: young→young (Y→Y), young→old (Y→O), old→old (O→O), and old→young (O→Y). At least 8 transplants per group were analyzed. Suspensions of mouse bone marrow and spleen and guinea pig bone marrow were prepared with a syringe as described previously [6]. Suspensions of cells from 2-month transplants of the bone marrow and spleen were prepared as described previously [1,3]. The procedure was as follows: the transplant contents was scraped out with a scalpel into α-MEM with 5% FCS (PanEco), several times passed through a syringe with needles of lesser and lesser diameters, and filtered through four Capron layers. Bone marrow (10^6) and splenic cells (5×10^6) were explanted into 25-cm^2 flasks in 5 ml α-MEM (Sigma) with 5% FCS. After 2 h the medium with free cells was discarded, the cultures were washed twice in α-MEM, and complete culture medium (80% α-MEM, 20% ECS, penicillin and streptomycin, 100 μg/ml each) was added. Bone marrow cells (10^7) from guinea pigs irradiated in a dose of 60 Gy (Co 60, 10 Gy/min) served as a feeder. The cultures were incubated for 12 days in a CO_2 incubator at 37°C, fixed in ethanol, stained with azur-eosin, and colonies consisting of at least 50 fibroblasts were counted. ECF-F (number of stromal fibroblast colonies formed by 10^5 explanted bone marrow or 10^6 explanted splenic cells) was evaluated. In order to evaluate the impact of aging for the intensity of induced osteogenesis, fragments (1/4 from young (4 months) and 1/5 from old (36 months) guinea pigs) of allogenic urinary bladder mucosa obtained as described previously [9] were implanted under the anterior abdominal wall fascia of young and old guinea pigs. The weight of the resultant bone tissue was measured after 19 days, because the ectopic bone organ had formed by this time and immunological rejection of the epithelium had not yet taken place [2,9,13]. Six to eight transplants per group were evaluated.

RESULTS

The counts of CFC-F in hemopoietic and lymphoid organs decrease with age [2,5,7]. ECF-F and the count of CFC-F in mouse bone marrow and spleen decrease with age by 2 and 8 times, respectively.

We previously showed that age-associated reduction of CFC-F counts in the bone marrow, spleen, and thymus of rapidly aging SAMP mice was significantly early, at the age of 9-11 months, compared to only 16-19 months in SAMR mice (control strain with normal aging tempo) [2]. Those data seemed to indicate that stromal tissue was involved in the

TABLE 1. Age-Specific Changes in ECF-F and Count of CFC-F in Mouse Femoral Bone Marrow and Spleen (*M*±*m*)

Age of mice, months	Bone marrow			Spleen		
	count of nuclear cells per organ, $\times10^6$	ECF-F, 10^5	count of CFC-F per organ	count of nuclear cells per organ, $\times10^6$	ECF-F, 10^6	count of CFC-F per organ
2	12.4±2.1	3.7±0.8	467±98	133.3±7.7	1.72±0.18	229±27
5	15.1±2.0	3.4±0.5	525±105	144.8±8.8	0.38±0.05	55±10
10	17.8±1.2	3.1±0.6	532±63	180.4±15.6	0.52±0.24	89±37
24	17.9±1.7	1.4±0.2	242±65	168.0±10.2	0.17±0.04	28±16

total aging process and underwent changes within the framework of this process. It was found that although ECF-F and the counts of CFC-F in the femoral bone marrow of old CBA mice decreased 2-fold in comparison with young animals (Table 1), the decrease in these parameters in bone marrow transplants in the corresponding groups (Y→Y *vs.* O→O) was 8-fold (Table 2). These data seemed to indicate a drastic reduction of the transplantability of stromal stem cells, in other words, their capacity to construct new microenvironment with appropriate counts of CFC-F in old animals. However, when the bone marrow from old mice was transplanted to young recipients (O→Y), ECF-F and the count of CFC-F in the transplants increased more than 3-fold in comparison with the bone marrow transplants in the O→O group, remaining by 2.5 times lower than in the Y→Y group. Hence, the 8-fold age-associated reduction of ECF-F and count of CFC-F in the correspondent bone marrow transplants, judging from the proportion of these values in different experimental groups, was caused by true age-specific reduction (2.5-3 times) of ECF-F and the count of CFC-F (Y→Y:O→Y and Y→O:O→O) and by the regulatory effects of the organism reducing these values 3-fold more (Y→Y:Y→O and O→Y:O→O). The results support the hypothesis about possible exhaustion of osteogenic stromal bone marrow cells with aging. These data seem to be important for the choice of donor age for transplantation of human bone marrow stromal tissue.

We previously showed [3] that if the volume of transplanted splenic tissue was sufficiently low ($^1/_{15}$-$^1/_5$ of the organ), the size of the transplanted fragment of the spleen, counts of CFC-F and nuclear cells in the transplant formed a linear relationship, while the ECF-F value in the transplants remained unchanged. This suggests that the method of heterotopic transplantation of the spleen can be used for evaluation of age-associated changes in CFC-F count in this organ.

The counts of nuclear cells in splenic transplants changed little in all the studied groups (Table 3). The reduction of ECF-F and CFC-F count in the spleens of old mice in comparison with young animals (Table 1) in general corresponded to reduction of these values in splenic transplants in the correspondent groups (Y→Y and O→O). The ECF-F value in splenic transplant cell cultures and count of CFC-F in transplants of the O→O group reduced 4- and 6-fold, respectively, in comparison with Y→Y group. However, if the spleen from old mice was transplanted to young recipients (O→Y), the ECG-F in the transplants increased almost 7-fold in comparison with the ECF-F in the O→O group transplants and was even 1.3 times higher than in the Y→Y group. Hence, when the stromal tissue from old donors was transplanted to young recipients, the effects of young recipient organism determined the size of CFC-F population in the transplant territory.

TABLE 2. ECF-F and Count of CFC-F in Bone Marrow Transplants from Mice of Different Age (*M*±*m*)

Transplant type	Count of nuclear cells per transplant, 10^6	ECF-F, 10^5	Count of CFC-F per transplant
Y→Y	3.5±0.7	6.2±1.3	217±43
Y→O	3.2±0.6	2.6±0.6	79±16
O→O	3.0±0.7	0.9±0.2	27±5
O→Y	2.9±0.3	2.8±0.6	84±14

TABLE 3. ECF-F and Count of CFC-F in Splenic Transplants from Mice of Different Age (*M*±*m*)

Transplant type	Count of nuclear cells per transplant, 10^6	ECF-F, 10^6	Count of CFC-F per transplant
Y→Y	4.7±1.0	3.2±0.1	15.0 ±4.2
Y→O	5.2±0.8	2.0±0.3	10.4±3.4
O→O	2.9±0.1	0.8±0.1	2.5±0.1
O→Y	3.7±1.0	5.4±1.1	19.0±1.3

TABLE 4. Weight of Induced Ectopic Bone Tissue after Implantation of Allogenic Urinary Bladder Mucosa Fragments from Young and Old Guinea Pigs to Young and Old Guinea Pigs (*M*±*m*)

Transplant type	Mean weight of induced bone tissue per transplant, mg
Y→Y	0.29±0.07
Y→O	0
O→O	0.18±0.03
O→Y	0.88±0.20

Therefore, the type of age-specific shifts in mouse splenic stromal tissue differed from that in the bone marrow, in which some presumable age-associated stromal tissue defect prevented recovery of old donor bone marrow CFC-F population in young recipients.

Two types of osteogenic precursors in the body are known [10]: DOPC, forming the bone tissue without special exogenous inductors, and IOPC, needing osteogenesis inductors for bone tissue formation. In contrast to DOPC present only among bone marrow cells [9], IOPC are widely presented in the body [9]. For example, they are present in the subcutaneous and intermuscular connective tissue, and therefore implantation of specific osteogenesis inductors (urinary bladder transitional epithelium or decalcified bone matrix) in an open system under the skin or fascia leads to the formation of ectopic bone tissue [9,13]. The volume of new bone in those cases was in direct linear relationship with the volume of implanted osteoinductor [9]. The weight of induced ectopic bone tissue was 2-fold lower after implantation of mucosa fragments of the autologous [7] and allogenic (Table 4) urinary bladder from old donors to young recipients (O→O) in comparison with the group of young donors and recipients (Y→Y). However, comparison of the ectopic bone proportions in different experimental groups (Table 4) suggested that osteoinductive activity of the bladder epithelium did not decrease with age and even increased more than 3-fold (O→Y:Y→Y). Presumably, factors preventing ectopic osteogenesis were present in old animals and there were no factors stimulating this process. However, the 5-fold reduction of the ectopic bone weight proportion in the groups with the osteoinductor from old donors implanted to old and young recipients (O→Y:O→O) could be explained by a significant (up to 5-fold) age-associated reduction of IOPC count with this threshold sensitivity to the transitional epithelium osteoinductive factor. As the osteoinductor from old donors amplified the bulk of induced ectopic bone tissue in old and young recipients, it seemed that the IOPC sensitivity to the osteoinductor changed little with age.

Hence, age-associated reduction of CFC-F count and their functional activity could be caused by reduction of the count (exhaustion) of the cell pool (bone marrow CFC-F, DOPC, presumably IOPC) and regulatory effects of the host (bone marrow and splenic CFC-F, IOPC).

These data seem to be significant for understanding of the role of osteogenic CFC-F in the development of age-associated bone tissue defects, for example, senile osteoporosis.

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