Structural and Functional State of the Bone Marrow during Its *In Vitro* Interaction with Ferromagnetic Nanoparticles

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Hemopoietic islets, associations of stromal (macrophages, fibroblasts) and blood (including stem) cells, are structural and functional units of the bone marrow. We studied cellular and molecular processes developing following short-term (1 h) contact of hemopoietic islets with ferromagnetic nanoparticles in a multicellular system of the bone marrow *in vitro*. It was established that nanodispersions of magnetite (Fe_3O_4, mean particle diameter 18 nm) and iron coated with carbon (Fe(C), particle diameter 5-10 nm) in a dose of 3 mg/liter had a minor effect on processes of necrotic and apoptotic cell death. Nanodispersion of carbon-coated iron (Fe(C)) most mildly stimulated oxidizing processes recorded by intracellular levels of reactive oxygen species. These nanoparticles, in contrast to magnetite, did not reduce the amount of hemopoietic islets in the bone marrow cell suspension.

Key Words: *mouse bone marrow; hemopoietic islets; apoptosis; necrosis; reactive oxygen species*

Current approaches to systemic and regional therapy imply "targeted" delivery of drugs by using nanocarriers [15] having also magnetic properties [4].

Nanopowders of iron and its oxides or hydroxides often serve as carriers of the magnetic properties [2,10]. It is well known that nanoparticles and nanopowders of metals possess high reactivity and catalytic activity [8]. At the same time, ample data suggest that these nanoferromagnets possess its own toxicity against cells, tissues, and components of biological fluids [14] due to their participation in free radical processes [5].

Targets of nanoparticles are blood system, central nervous system, respiratory and gastrointestinal tracts. It is believed that nanoferromagnets can avoid phagocytosis, circulate in the blood and lymph, penetrate through biological barriers, being systematically distributed in various organs and tissues [13]. Particles with a diameter <50 nm can be toxic for bone marrow cells [12].

Hemopoietic islets (HI), associations of stromal (macrophages, fibroblasts), and blood-forming cells, are structural and functional units of the bone marrow. They regulate functional activity of hemopoietic stem cells and their proliferation and differentiation into mature blood elements [11].

In this regard, we evaluated cellular and molecular processes developing during brief contact of HI with ferromagnetic nanoparticles in the multicellular system of bone marrow *in vitro*.

MATERIALS AND METHODS

Quantitative composition of HI was examined as described elsewhere [11] with some modifications [1]. The bone marrow from BALB/c mice was washed from the femur in tubes with 1 ml of RPMI-1640. Nanodispersions of magnetite particles (Fe_3O_4) and carbon-coated iron (Fe(C)) were obtained immediately

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before addition to cells by ultrasonication in a solvent (isotonic 0.9% NaCl) for 5 min. Then nanodispersdions were added to some tubes to a final concentration of 3 mg/liter (10 maximum tolerated concentrations of iron).

Electron microscopy under a JEM-200CX electron microscope showed that the mean linear size of Fe_3O_4 particles was 18 nm and Fe(C) size varied from 5 to 10 nm. The method of obtaining and physical and chemical properties of nanoparticles are described in [3].

In each of 4 groups (2 groups for control of toxicity and 2 experimental groups with addition of nanoparticles), 5-6 samples were studied. According to ISO 10993-5 requirements, we used negative and positive controls of toxicity in our work. To this end, suspension of myelokaryocytes was incubated with a solvent at 1:10 (100 μl) of the total volume of culture medium (negative control) or H_2O_2 in final dilution of 1 mM producing a cytotoxic effect (positive control) [6]. Components were mixed by pipetting of the cell suspension through a needle 1 mm in diameter and cultured in an incubator for 1 h at 37°C. Then the cell suspension was again mixed, 0.1% neutral red in 0.9% NaCl was added (1:1), and the suspension was transferred to a Goryaev chamber. We counted HI with central parts stained or unstained with neutral red before and after incubation. HI was determined as cell cluster containing at least 3 myelokaryocytes associated with a centrally located monocyte/macrophage or stromal mechanocyte.

The absolute number of HI, total number of karyocytes (TNK), and number of dead cells in the femoral bone marrow stained with 0.4% trypan blue were evaluated as described previously [1] before and after adding nanodispersions. The initial (before culturing) percent of dead cells was 9.10±0.42%.

There were individual variations of the initial levels of HI and TNK in the femur. Therefore, the experimental data were converted to percentage from the baseline (before culturing) to simplify the analysis.

The production of reactive oxygen species (ROS) in cells was evaluated by laser flow cytometry using dichlorofluorescein diacetate, a dye with blocked fluorescence. To this end, the isolated cells were cultured for 24 h in 96-well plates in 200-μl medium. After culturing, 90 μl cell suspension containing 10^6 cells/ml was transferred into flow cytometry tubes and 10 μl dichlorofluorescein diacetate working solution was added. After 20-min incubation at 37°C, 11 μl 0.2% EDTA was added for 30 min at 37°C, the mixture was centrifuged for 1 min at 500*g*, and the supernatant was removed. The reaction was terminated by adding 200 μl lysing solution, the cells were centrifuged for 1 min at 500*g*, and the supernatant was removed. The cells were washed once with 200 μl phosphate-buffered saline (pH 7.4) and resuspended in 400 μl phosphate-buffered saline (pH 7.4). Analysis of cell samples was performed using FL-1 histograms reflecting the intensity of cell fluorescence in the green spectrum, and the corresponding statistic windows for each sample that described the geometric mean of the emission intensity of labeled cells [7]. The results were expressed in arbitrary units.

To evaluate apoptosis, the cells were transferred into flow cytometry tubes, and centrifuged for 3 min for their precipitation. The supernatant was removed, and 1 ml of chilled phosphate-buffered saline (pH 7.4) was added. The mixture was vortexed and then centrifuged for 3 min at 500*g*, then the supernatant was removed. The washed cells were resuspended (10^6 in 1 ml) in annexin buffer containing FITC-labeled annexin V and propidium iodide and incubated for 15 min in the dark at room temperature. Analysis of samples was carried out on a Beckman-Coulter Epics XL flow cytometer. Cell population was examined for the presence of fluorescence using different coordinate systems [16].

For evaluation of experimental data, methods of descriptive statistics and verification of statistical hypothesis were used. For the analysis of available data samples, Kolmogorov–Smirnov test (hypothesis of distribution normality) was used. If the data distribution in the studied samples corresponded to the normal distribution law, the hypothesis on equality of the means of two samples was verified using Student's *t* test. To evaluate the significance of differences between the samples not fitting the normal distribution, nonparametric Mann–Whitney *U* test was used. For evaluation of the relationship between the studied parameters, Spearman rank correlation coefficient (*r*) was calculated. The differences were significant at $p<0.05$.

RESULTS

The experiments revealed no significant differences in morphological parameters during bone marrow culturing *in vitro* (Table 1). Under conditions of short-term exposure of cultured cells with Fe_3O_4 and Fe(C) nanoparticles, TNK and necrotic myelokaryocyte death varied within the range of negative and positive controls for toxicity.

Structural and functional activity of bone marrow cells decreased by almost 1.5-2 times after adding H_2O_2 to the culture (Table 2); the number of HI with neutral red-positive central element most markedly decreased (by 45% compared to negative control).

It should be noted that the action exerted by Fe_3O_4 nanoparticles on HI was similar by its amplitude to the effect of H_2O_2: the total content of HI decreased to 70% (p=0.031) of the level of negative toxicity control.

On the other hand, direct contact of Fe(C) nanoparticles with hemopoietic cells did not break their associations playing an important role in hemopoiesis. HI content varied within the range of negative toxicity control and exceeded the corresponding parameters (stained HI, amount of HI) of the positive toxicity control (Table 2).

We studied intracellular concentrations of ROS and the number of apoptotic cells in cell-cell associations (in HI) simulating the behavior of hemopoietic elements *in situ* after *in vitro* exposure to nanodispersions.

Spearman correlation analysis revealed no statistically significant relationship between the fractions of cells stained with trypan blue (indicator of necrosis) and subjected to apoptosis. This suggests the possibility of studying the effect of nanodispersions on the two forms of cell death. The results showed that the tested toxicants (H_2O_2 and nanodispersions) did not affect significantly the processes of necrosis (Table 1) and apoptosis (Table 3).

At the same time, artificial nanomaterials significantly increased (by 320-450%) intracellular concentration of ROS, which reached the level of positive toxicity controls (Table 3). However, we found no correlation between intracellular ROS concentration and the increase in the ratio of apoptotic cells (r= -0.075; p>0.74; n=21).

On the other hand, we found a positive correlation between the intracellular level of ROS and TNK (r=0.41; p<0.048; n=21) and a negative correlation with total HI content (r=-0.43; p<0.037; n=21). Identified correlations enabled us to consider the intracellular level of ROS to be the one of the most important indicators according to which the most mildly oxidizing processes (Table 3) were stimulated by Fe(C) nanodispersions. These findings are consistent with its weak catalytic activity realized by the free radical mechanism [3]. At the same time, these nanoparticles did not reduce the number of HI in bone marrow cell suspension (Table 2).

TABLE 1. Number of Stained Nucleated Cells and TNK after Addition of Ferromagnetic Nanodispersions to Myelokaryocyte Culture from BALB/c Mice (X±m)

Experimental condition	Number of stained cells, % of baseline	TNK
Negative toxicity control	138.73±24.92	81.25±7.73
Fe_3O_4	120.72±12.16	93.62±11.19
Fe(C)	137.77±16.09	97.37±10.29
Positive toxicity control (1 mM H_2O_2)	157.82±11.59	81.96±9.97

From the standpoint of basic science, ferromagnetic nanoparticles in the studied doses had minor effects on the molecular mechanisms underlying the processes of cell death. The observed increase in intracellular ROS level is apparently insufficient to induce apoptosis, but can disturb intercellular contacts, which is accompanied by a decrease in the quantity of structural association between stromal and hemopoietic cells (mainly HI with stained central element).

Supravital staining of nuclei with neutral red increased with increasing the degree of maturity [9]. We can assume that the target cells are mature cells (most likely macrophages) positively stained with neutral red [1,11]. In turn, this may indirectly disrupt functional activity of hemopoietic stem cells associated with their proliferation and differentiation into mature blood elements in HI.

From the standpoint of applied science, Fe(C) nanoparticles, due to lower toxicity compared to nanosized Fe_3O_4 can be used for creation of magnetic delivery systems, including nanoparticles, drug molecules, and bioactive substances.

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TABLE 2. Content of HI (% of Initial Amount) after Addition of Ferromagnetic Nanodispersions to Bone Marrow Culture from BALB/c Mice (X±m)

Experimental condition	Stained HI	Unstained HI	Total HI
Negative toxicity control	126.15±15.27	224.38±28.27	161.06±14.42
Fe_3O_4	91.68±11.30	148.18±24.22	113.45±11.08*
Fe(C)	137.35±22.35$^+$	175.17±8.66	153.30±15.20$^+$
Positive toxicity control (1 mM H_2O_2)	69.03±16.21*	142.18±30.51	92.78±10.74*

Note. Here and in Table. 3: p<0.05 compared to *negative toxicity control, $^+$positive toxicity control (Mann–Whitney U test).

TABLE 3. Number of Nucleated Cells Entered Apoptosis and Intracellular Level of ROS after Addition of Ferromagnetic Nanodispersions to Bone Marrow Culture from BALB/c Mice ($X \pm m$)

Experimental condition	Aapoptotic cells, %	Intracellular level of ROS, arb. units.
Negative toxicity control	6.73±2.72	0.04±0.02
Fe_3O_4	9.37±3.73	0.21±0.06*
Fe(C)	10.68±3.58	0.13±0.03*+
Positive control of toxicity (1 mM H_2O_2)	11.64±1.93	0.22±0.03*

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