Apoptotic lymphocytes induce progenitor cell mobilization after exercise

Frank C. Mooren and Karsten Krüger

Department of Sports Medicine, Institute of Sports Sciences, Justus-Liebig-University, Giessen, Germany

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Moenen FC, Krüger K. Apoptotic lymphocytes induce progenitor cell mobilization after exercise. J Appl Physiol 119: 135–139, 2015. First published May 28, 2015; doi:10.1152/japplphysiol.00287.2015.—There is evidence that apoptotic cells and their components have immunomodulatory properties and signaling function. The present study investigated first whether exercise-induced apoptosis and exercise-induced mobilization of progenitor cells are similarly affected by subjects’ training status and, second, whether the appearance of dying cells in the circulation might mobilize progenitor cells. CD1 SWISS mice were subjected to a 10-wk endurance training using free wheel running or served as untrained controls. Mice of both groups performed an intensive exercise test after the training period at a velocity corresponding to 80% maximal oxygen uptake for 30 min. Cells from blood and bone marrow were analyzed, and apoptosis and number of progenitor cells determined via flow cytometry. A second experiment, apoptotic cells were transferred into recipient mice, and mobilization of progenitor cells was analyzed while vital cells served as controls. In untrained animals, the exhaustive exercise was followed by an enhanced rate of annexin V positive CD3+ cells in blood and bone marrow (P < 0.05), whereas no increase was found in trained mice. Similarly, exercise mobilized Sca-1+/c-kit+ and Sca-1+/Flk1+ cells in untrained (P < 0.05) but not trained mice. Furthermore, application of apoptotic cells and their supernatant mobilized Sca-1+/c-kit+ cells into the blood (P < 0.05), whereas Sca-1+/Flk1+ cells were not affected. The present study demonstrated that both lymphocyte apoptosis, as well as mobilization of progenitor cells are similarly related to training status. Furthermore, apoptotic cells seem to induce signals that effectively mobilize hematopoietic progenitor cells. The relevance of this effect for the adaptation to exercise stimuli remains to be shown.

cell death; exercise training; training status; stem cells

STRENUOUS EXERCISE IS WELL KNOWN TO AFFECT LYMPHOCYTE’S LIFE SPAN BOTH IN THE CIRCULATION AS WELL AS IN VARIOUS ORGAN COMPARTMENTS (14, 15, 19). Depending on the duration and intensity of exercise, lymphocyte apoptosis is enhanced in the early postexercise period (16, 20). There is evidence that exercise-associated increases in the concentration of free radicals and/or soluble mediators, such as Fas-ligand, can mediate both intrinsic as well as extrinsic apoptosis-inducing pathways (14, 27). Interestingly, the sensitivity of lymphocytes to apoptotic stimuli seems to be inversely related to the training status. Accordingly, it was found that programmed cell death occurred only in less-trained, but not in well-trained, athletes (21, 25). Lymphocytes seem to adapt to repeated exercise stress by upregulating their cellular defense mechanisms (2). However, there is still a debate about the physiological role of exercise-induced apoptosis (EIA). It has been proposed that EIA may transiently compromise the adaptive immune competence and, therefore, may be responsible, at least in part, for the enhanced rates of upper respiratory tract illness in the postexercise period (23). Others have proposed that EIA may be helpful to avoid autoimmune processes as exhaustive exercise may potentially have uncovered auto-antigens (16, 33). The relation of EIA to training status initiated the discussion about a possible regulatory or signaling function of EIA. Recently, a number of studies have demonstrated that an effective clearance of dying cells by phagocytes, a process known as efferocytosis, plays an important role in tissues homeostasis (11). The ingestion of dying cells or parts of them by phagocytosing cells can have tremendous influence on the course of an immune response by either enhancing or suppressing inflammation (31). In this regard, a number of studies demonstrated that the physiological process of apoptosis is accompanied by the release of small membranous particles from the cell surface, termed apoptotic bodies (ABs) (30). The release of these microparticles is suggested to have a profound paracrine and endocrine signaling function. Beside the role of ABs in modulating immune responses, they might serve as transporters of cell-derived compounds, like DNA, peptides, or oxidized phospholipids, which might stimulate receptors for maturation or mobilization of endothelial or hematopoietic endothelial progenitor cells (40). For some progenitor cells, it was shown that they phagocytosed ABs, followed by an increase of the proliferation rate. Therefore, a horizontal transfer of DNA by the uptake of ABs is discussed (9)

A concomitant phenomenon during exercise is the exercise-induced mobilization of progenitor cells. Both acute as well as chronic exercise training mobilize hematopoietic and endothelial progenitor cells (HPCs and EPCs) into the peripheral blood (1, 22, 29, 30). It has been proposed that HPCs are involved in cell-mediated hematopoiesis and myofiber formation in response to exercise (22, 24). Regarding EPCs, there is some evidence for their involvement in endothelial regeneration and the formation of new vessels termed postnatal vasculogenesis (1, 18, 41). The mechanisms by which exercise mediates progenitor cell mobilization are intensively discussed. It has been assumed that exercise induces the expression of potential progenitor cell-mobilizing signaling molecules (17). Moreover, there are references indicating that exercise-induced mobilization of progenitor cells is similarly regulated as the induction of lymphocyte apoptosis, which raises the question whether both processes might be connected. Together with recent findings that ABs might have the potential to affect proliferation and differentiation of progenitor cells, we propose that there might be a role for dying cells/ABs to stimulate the mobilization of progenitors (42). Such a link would couple exercise-induced tissue destruction and regeneration, which is fundamental for an adaptation to training stimuli.

Therefore, in a first step, we aimed to compare the induction of EIA and the release of progenitor cells after exercise with respect to the training status. As the adaptational response to physical training is related nonlinearly to the subjects’ training status, we expected that a similar relative exercise stimulus...
should induce less apoptotic cells and mobilize less progenitor cells in the trained organisms. Furthermore, we hypothesized that the application of apoptotic cells and/or their supernatant would be able to induce the release of progenitor cells, which might he helpful for the adaptational response to training.

**METHODS**

**Mice.** This work was performed on male SWISS mice, age 8-12 wk and body mass 29.5 ± 2.5 g. Mice were randomized into training groups and control groups (n = 6-8). All animals had ad libitum access to food and tap water. All experiments were approved by the Local Animal Care and Use of Münster (G78/2005).

**Determination of training status.** By using a treadmill and spiro-ergometry equipment (custom made), maximal oxygen uptake (V\textsubscript{O}\textsubscript{2} max) and maximal running speed of mice were tested before and after training and analyzed using the calculation of Weibel and Hoppeler (39). The treadmill was placed in a metabolic chamber, where air was led through at a rate of 0.5 l/min. Samples of 200 ml/min of gas were extracted to the paramagnetic oxygen analyzer (type 1155, Servomex) and the carbon dioxide analyzer (Lair 12, M&C Instrument). All animals were acclimatized to the treadmill once before V\textsubscript{O}\textsubscript{2} max and maximal running speed was tested during a continuous, progressive test on the treadmill ergometer until exhaustion. After 10 min of acclimatization in the treadmill chamber, the test uptake started at 0.15 m/s, increasing every 3 min with 0.05 m/s.

**Exercise protocols.** Endurance training was performed using free wheel running for 10 wk. After the training period, groups of trained mice and sedentary controls performed an exhaustive exercise test at 80% of individual V\textsubscript{O}\textsubscript{2} max, corresponding to a continuous treadmill speed of ~0.36 ± 0.03 m/s. Running wheels were removed at least 4 days before trained mice performed their acute exercise test to avoid acute effects on immune changes. Similarly, untrained mice performed a running test at a speed of 0.33 ± 0.06 m/s until exhaustion. Mice of the control groups were exposed to treadmill noise without running.

**Cell isolation, apoptosis induction, and cell transfer.** For adoptive cell transfer, mice splenic lymphocytes were used. The spleen was removed, and a cell suspension was created by pressing tissues through a sterile nylon mesh (100-µm pore size) by using a stopper of a syringe. After centrifugation at 2,000 rpm for 10 min, cells were counted and showed a viability of 97% (Trypan blue exclusion). Next, lymphocytes were incubated for 4 h with either phosphate-buffered saline (PBS) or 100 µM hydrogen peroxide to induce cell apoptosis. Apoptosis rate was 9.66 ± 4.56% in the control treatment and 51.78% ± 7.64% in the apoptosis group. Next, control (“vital cells”) and apoptotic cells were harvested and washed two times with PBS. Finally, cell suspensions of either control or apoptotic cells (2 × 10\textsuperscript{6} to 4 × 10\textsuperscript{6} cells) were injected intravenously into the lateral tail vein of mice. Three hours after injection, blood samples were taken for analysis of progenitor cells. In a further approach, the effect of the supernatant, which was collected after apoptosis induction, on progenitor cell mobilization was elucidated. Hydrogen peroxide was neutralized by addition of catalase in excess. Subsequently, the supernatant, which might include ABs or other signaling molecules that were expressed by the dying cells, were separated by centrifugation and selectively injected into mice.

**Collection of tissue samples and flow cytometry.** Mice were anesthetized by isoflurane and sacrificed by cervical dislocation immediately/24 h after exercise. Cardiac blood was collected immediately by using a heparinized syringe. For preparation of bone marrow (BM) cells, skin and tissues of legs were removed. Hip and knee joints were cut carefully to expose the femur bone. After the muscles were removed, bone ends were cut, and bone was flushed with 1 ml of PBS using a syringe and a 27-gauge needle. BM pieces were homogenized by pulling through the needle repeatedly.

For apoptosis detection, lymphocyte suspensions were incubated with monoclonal antibodies anti-CD3 [phycoerythrin (PE)] and annexin V antibodies (FITC-conjugated; Immunotools, Friesoythe, Germany). Cells were centrifuged again for 10 min at 1,200 rpm. After preparation for flow cytometry using an EPICS XL Flow Cytometer (Beckman Coulter). A minimum of 10,000 events were obtained for all samples.

Peripheral blood mononuclear cells and total BM cells were incubated with specific antibodies against Sca-1 (PE; BD Sciences) and c-Kit (CD117, FITC; Immunotools, Friesoythe, Germany) to detect HPCs or Sca-1 (PE) and Flk (FITC; BD Sciences) to detect EPCs, respectively. All samples were analyzed by flow cytometer (Beckman Coulter EPICS XL).

**Statistical analysis.** Data are means ± SE, unless indicated otherwise in the figure legends. Differences between the groups were compared with two-way ANOVA. If significant main effects were observed, post hoc analysis was conducted by using the Bonferroni test. In all cases, P < 0.05 was accepted as being significant. Data were analyzed using the SPSS statistical analysis program.

**RESULTS**

**Exercise-induced lymphocyte apoptosis depends on training status.** Free wheel running for 10 wk resulted in a significant increase of V\textsubscript{O}\textsubscript{2} max from 130.4 ± 8.9 to 166.1 ± 8.9 ml·min\textsuperscript{-1}·kg\textsuperscript{0.85} body mass\textsuperscript{-1}, while no changes of V\textsubscript{O}\textsubscript{2} max were observed in untrained mice (126.2 ± 7.3 to 129.4 ± 8.1 ml·min\textsuperscript{-1}·kg\textsuperscript{0.85} body mass\textsuperscript{-1}). In untrained animals, exhaustive exercise was followed by a significant enhanced apoptosis rate of CD3\textsuperscript{+} cells in both blood and BM (P < 0.05). Apoptotic cells in BM appeared, however, delayed 24 h after exercise. In contrast, no exercise-induced increase of apoptotic CD3\textsuperscript{+} cells was observed in any compartment of endurance-trained mice (Fig. 1).

**Exercise-induced mobilization of progenitor cells depends on training status.** Acute exhaustive exercise was able to mobilize both hematopoietic (Sca-1\textsuperscript{+}/c-Kit\textsuperscript{+}) as well as endo-

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**Fig. 1. Effect of acute exercise on percentage of annexin V positive CD3 cells in blood (A) and bone marrow (B) of trained and untrained mice. Pre, before exercise; post, after exercise; 24 h, 24 h after exercise. Values are means ± SE. *P < 0.05.**
Apoptotic cells mobilize progenitor cells into blood. As both apoptotic and progenitor cells were affected similarly by an acute bout of exercise, we questioned whether these changes could be linked. Therefore, we injected apoptotic CD3⁺ cells in different concentrations into sedentary mice, while injection of PBS and living cells served as controls. While injection of PBS and vital CD3⁺ cells had no effect on the percentage of progenitor cells in the blood of recipient mice, application of apoptotic cells dose-dependently mobilized Sca-1⁺/c-kit⁺ cells into the blood 3 h after injection (P < 0.05) (Fig. 3). No significant effects were observed on Sca-1⁺/Flk⁺ cells. In a second step, it was analyzed whether progenitor cell mobilization signals derived from the supernatant of apoptotic cells. Here, a significant increase of Sca-1⁺/c-kit⁺ cells was observed (P < 0.05) (Fig. 3).

DISCUSSION

The present study demonstrates first that both lymphocyte apoptosis and mobilization of progenitor cells seem to depend strongly on trainings status. While an increase of both lymphocyte apoptosis and progenitor cell mobilization after exercise was found in blood and BM of untrained animals, these processes were not found in trained animals. Second, the application of apoptotic cells or their supernatant into recipients was followed by an increase of hematopoietic progenitors in blood, suggesting a link between apoptosis of lymphocytes and mobilization of progenitor cells.

It has been repeatedly demonstrated that intensive exercise significantly increased both percentage and total numbers of circulating apoptotic lymphocytes (19, 20). Thereby, it has been shown that apoptosis in specific subtypes of lymphocytes (e.g., senescent cells) after subjects exceed a specific duration or intensity of exercise (34). It has been suggested that the expression of several potential mediators is amplified after exceeding a specific cell death-inducing threshold (13, 35–37). This threshold is assumed to be modulated by the athlete’s training status, as indicated by its inverse relationship to lymphocyte apoptosis and progenitor cell mobilization after exercise. While an increase of both lymphocyte apoptosis and progenitor cell mobilization after exercise was found in blood and BM of untrained animals, these processes were not found in trained animals. Second, the application of apoptotic cells or their supernatant into recipients was followed by an increase of hematopoietic progenitors in blood, suggesting a link between apoptosis of lymphocytes and mobilization of progenitor cells.
on the smaller effect of acute exercise on apoptosis in trained mice might be also affected by the higher preexercise percentage of apoptotic lymphocytes.

To investigate potential mechanisms for an increased apoptosis resistance in trained subjects, Avula et al. (2) demonstrated that splenic lymphocytes from trained mice were less sensitive to H_2O_2-induced apoptosis compared with cells from nontrained mice, indicating the upregulation of cellular defense mechanisms by regular exercise training (2). Likewise, it was demonstrated that cells initiate in response to repeated exercise stress the expression of antioxidant enzymes and heat shock proteins, which exhibit protective effects against cellular stressors associated with acute bouts of exercise (6). Especially, the expression of heat-shock proteins has been related to anti-apoptotic properties (7).

Regarding the mobilization of progenitors, it was previously demonstrated that both acute exercise and regular training are stimuli for mobilization of HPCs and EPCs into blood (22, 29). It has been suggested that the mobilization of HPCs indicates a response to exercise stress with an increased demand on undifferentiated hematopoietic cells to increase blood volume, oxygen transport capacity, and immune competence (12). It is further speculated that HPCs have the ability to differentiate and migrate into damaged tissues to improve barrier functions and to stimulate tissue repair or adaptation processes in response to exercise (4, 24). The simultaneous increase of HPC numbers in BM in response to exercise agrees with previous studies. On the one hand, this increase is a result of an increased self-renewal of HPCs to increase the total progenitor cell pool in BM (3). On the other hand, it might be a direct adaptation to an increased hematopoiesis. This assumption is supported by a study of Baker et al. (3) who found that increases of HPC numbers in BM correlated with increases of osteoblasts, which are known to be positive mediators for hematopoiesis (39).

Similar to the finding for EIA, in the present study it was found that untrained mice mobilized both HPCs as well as EPCs in response to acute exercise, while trained animals did not. Therefore, it can be assumed that the sensitivity of exercise-induced progenitor cell mobilization might be inversely related to training status, too. Taken together, the present results suggest that exercise promotes both cell death and cell regeneration, and both processes depend on the subject’s training status. Moreover, the similar regulation of EIA and exercise-induced mobilization of hematopoietic progenitors by training status suggest that both processes might be connected in some way. This could be confirmed by the mobilization of HPCs in response to application of apoptotic lymphocytes, as well as their supernatant. Interestingly, the process was restricted to HPCs, suggesting some kind of specific stimuli. However, the exact nature of the signaling pathway remains unclear. We cannot exclude that apoptotic cells may also stimulate thymopoiesis or homeostatic proliferation of existing T cells to maintain peripheral T-cell numbers and diversity. Nevertheless, these findings suggest a novel role for EIA as a process relevant to regenerative and adaptational responses in the postexercise period.

Based on the results that the supernatant of apoptotic cells provides signaling function, small vesicles like ABs might exert these signaling functions. It was previously shown that, in the course of apoptosis, a series of molecular events culminate. During this process, the release of small membranous particles, termed ABs, from the cell surface occurs (9). Originally, the release of ABs was speculated to be an attempt by the cell to reverse cell death by removing parts of the pro-apoptotic signaling machinery (8). Otherwise, dying cells are known to release “find-me” signals that are sensed by motile phagocytes, which help attract these phagocytes (28).

Indeed, the clearance of apoptotic cells by macrophages has been shown to be more than a neutral method to remove cell waste (26). The ingestion of dying cells and/or their components such as AB can influence the immune response markedly by enhancing or suppressing inflammation (32). In contrast, inefficient efferocytosis is followed by impaired wound healing and tissue repair (38, 39). Endocrine/paracrine signaling via AB to neighboring cells modulates both immune responses and repair mechanisms in endothelial cells (40). This suggestion is supported by early findings of Hristov et al. (9), who demonstrated that ABs from endothelial cells enhance the number and initiate the differentiation of human EPCs in vitro. Similarly, it was proved that administration of ABs promoted incorporation of Sca-1 progenitor cells. In this study, it was found that ABs deliver micro-RNA-126, which affected progenitor cell activity (40). Otherwise, it was shown that progenitor cells can phagocyte ABs, followed by an increase of proliferation (9). A possible active compound within the apoptotic blebs for inducing biological effects could be DNA. Recently, an intercellular DNA transfer by uptake of ABs has been demonstrated in embryonic fibroblasts, monocytes, and endothelial cells (9).

From an exercise-immunological point of view, EIA was often viewed as detrimental for immunity. Present data might indicate a novel role of EIA representing the initial point for inducing regenerative processes after exercise-induced cell damage and increasing cell turnover. In this sense, Simpson (33) argued that lymphocyte apoptosis might be a mechanism to remove senescent, activated, or potentially autoreactive lymphocytes. However, we did not perform a more detailed phenotypic analysis of the apoptotic T cells. Given that senescent cells die by apoptosis, exercise might create “vacant space” for newly functional lymphocytes to occupy and expand the naive T-cell repertoire (33). This assumption is supported by our results, because mobilized HPCs are suggested to differentiate in different cells of the hemopoietic lineage, including lymphocytes (10).

In summary, our data give evidence for a fine tuning of mobilized dying and regenerative cells during acute and chronic exercise, in which apoptotic lymphocytes and their components exert signaling functions relevant to HPCs. The underlying molecular mechanisms may involve novel cell-to-cell signaling mechanisms such as circulating micro-RNAs but are hypothetical at the moment. Whether an increased cell turnover is a general mechanism of exercise-induced adaptation remains to be determined.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: F.C.M. conception and design of research; F.C.M. and K.K. analyzed data; F.C.M. and K.K. interpreted results of experiments; F.C.M. drafted manuscript; F.C.M. and K.K. edited and revised manuscript; F.C.M. and K.K. approved final version of manuscript; K.K. performed experiments; K.K. prepared figures.
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