UDC 57.021

REGULATION OF LEUCOGENESIS BY EXTRACELLULAR UBIQUITIN IN RODENTS AFTER CHEMICALLY INDUCED INHIBITION

R. SUJASHVILI, I. IORAMASHVILI, K. APTSIAURI, N. GVINADZE

Iv. BeritashviliCenter of Experimental Biomedicine, Tbilisi, Georgia
E-mail: sujaruss@gmail.com

To study the influence of intraperitoneally injected extracellular ubiquitin on regeneration of leucopoiesis calculation of nuclear cell count in bone marrow (BM) and peripheral blood (PB) smears stained with azure-eosin was performed. In the first, control group of animals inhibition of haematopoiesis achieved by means of 100 mg/kg cyclophosphamide LD_{50} 50–200 mg/kg injection. Bone marrow and peripheral blood samples from the first group of rats had been taken at 24, 48, 72, 96 and 168 h points after injection of cytostatic. Animals of the second, test group were injected by 200 μg/ml ubiquitin 72 h later after cytostatic injection. Our experiments revealed that ubiquitin makes corrections in regeneration of leucopoiesis and leads to normalisation of the process. Ubiquitin regulates stem cell activity, normalizes the release of functional cells into bloodstream, supposedly retains progenitor cells in zones of differentiation and maturation, and restores the nuclear cell ratio in PB and BM. We suppose that obtained results are important for elucidation of new pathways of ubiquitinylation and give us possibilities to find new therapeutics for regeneration of leucopoiesis that is very essential for treatment of radiated bone marrow and chemotherapeutic side effects in cancer patients.

Key words: *extracellular ubiquitin, regeneration, leucopoiesis, bone marrow, peripheral blood, abortive elevation.*

Introduction. Regulated protein metabolism by the ubiquitin-proteasome system (UPS) is essential for the survival of eukaryotic cells. Ubiquitination is involved in regulation of various cellular processes like cell cycle control, signaling pathways, transcription, DNA repair and protein quality control. Aberrations in UPS are implicated in the pathogenesis of cancer and are correlated with a variety of human diseases. UPS plays an essential role in regulation of apoptosis. Suppression of UPS functions by different inhibitors can cause cell death. The exact role of the UPS in the pathology of these diseases however, remains poorly understood [1].

Recently it has been suggested that in vitro microinjected extracellular ubiquitin leads to inhibition of cell cycle. It induces growth suppression and mediates apoptosis of blood cells via accelerated degradation of STAT3 through the UPS [2, 3]. The results of our earlier experiments showed that in vivo injected extracellular ubiquitin inhibits mitotic activity of hepatocytes in healthy rats, but significantly stimulates it in alcoholic liver after partial hepatectomy [4–6]. Another our study showed that extracellular ubiquitin caused evident changes in dynamics of regeneration of leucopoiesis. Mitotic activity in bone marrow of test group animals injected by ubiquitin was decreased by about 53 % as compared with the intact animals of control group [7]. Evidently, changes in bone marrow cells proliferative activity is caused by modification of several proteins after ubiquitin injection, i.e. ubiquitinylation. To date is suggested the new role of extracellular ubiquitin in regulation of cell cycle. It was identified as an agonist of the G-protein-coupled chemokine receptor CXCR4. But CXCR4 has been found in membrane of leukocytes, where it regulates intracellular Ca^{+2} ions flux during mitosis and is involved in regulation of hematopoiesis [8–11]. Ubiquitin plays a significant role in the etiology of acute lymphoblastic leukemia. In ALL bone marrow and cell lines a greater expression of C-terminal truncated version of ubiquitin was observed. Though, it is evident that ubiquitin has a significant role in regulation of hematopoiesis [12].

Hematopoiesis involves the maintenance and proliferation of hematopoietic progenitor cells (stem cells) and their differentiation into mature erythrocytes, leukocytes and platelets. Hematopoietic stem cells are undifferentiated cells with capacities for differentiation and self renewal. Under the action of a large number of bone marrow and circulating hematopoietic growth factors, pluripotent hematopoietic stem cells produce progenitor cells and mature hematopoietic cells differentiated throughout a number of cell divisions until blood cells are formed ready to leave the bone marrow and enter the bloodstream [13, 14].

Investigation of regulation of spontaneous regeneration of leucopoiesis by using different bio-

logically active agents, such as ubiquitin studied in present work, is important for further elucidation of mechanisms underlying regeneration disorders in bone marrow after irradiation and chemotherapy, as well as ubiquitilation pathways playing an essential role in leucogenesis.

Materials and methods. To study the influence of intraperitoneally injected extracellular ubiquitin on regeneration of leucopoiesis calculation of nuclear cell count in BM and PB smears stained with azure-eosin was performed. Samples were observed under the light microscope Amplival («Zeiss», Germany). Methods for statistical analysis OriginPro, ImageJ and ANOVA were used for quantitative analysis. All reagents purchased from SIGMA and ABCAM.

Wistar female rats with weight about 120–150 g were used in experiments. In the first, control group of animals inhibition of haematopoiesis was achieved by means of 100 mg/kg cyclophosphamide LD_{50} 50–200 mg/kg injection. Animals were placed separately in individual cages. Bone marrow and peripheral blood samples from the first, control group of rats had been taken at 24, 48, 72, 96, 168 h after injection of cytostatic. The second, test group was injected by 200 μg/ml ubiquitin 72 h later after cytostatic induction. Nucleated cell counts were performed on PB and femora BM smears prepared after decapitation of animals. Femoral marrow content was expelled by syringe containing 0.5 ml of saline. Clots of bone marrow were homogenised by agitation through the syringe. 5,000 cells per sample were counted. Total cell count and calculation of number of nucleic figures per 1,000 cells (‰) was carried out.

Animals were anesthetized by ether before decapitation. Treatment of animals performed in accordance with regulations established by the Institute animal's ethic committee (Protocol N06/13.10.2014).

Results and discussion. We are interested here in the regulation of spontaneous regeneration of leucopoiesis, sub-process of hematopoiesis, by means of in vivo injected extracellular ubiquitin. Like other blood cells, leukocytes are originated from a pool of hematopoietic stem cells. Under an action of growth factors, stem cells differentiate in progenitor cells, which in turn will produce precursor cells after a number of divisions. Due to the number of divisions and the quantity of cells involved in leucopoiesis, issues may arise at different cellular levels and sometimes result in diseases affecting white blood cells [15].

The cytotoxic chemotherapeutic agents used in the treatment of most cancers have effects on hematopoiesis. Ionizing radiation to areas of bone marrow and alkylating agents, when administered at high doses or for long periods of time, may lead to progressive depletion of hematopoietic stem cells. Another long-term effect is the development of myelodysplasia and acute myeloid leukemia due to use of alkylating agents. This often results in decreased reserve capacity and may cause long-term leucopenia. Drugs that are more effective against actively proliferating cells tend to cause earlier and short-lasting leucopenia, primarily because they have little effect on marrow repopulating pluripotent hematopoietic stem cells, but instead they kill hematopoietic progenitors and precursors that are proliferating actively [16–18].

We conducted quantitative analysis of bone marrow (BM) regeneration rate and count of peripheral blood (PB) cells after cytostatic and following ubiquitin injection. Ubiquitin was tested for its effect on BM spontaneous regeneration. To achieve an effect of leucopenia, we used anticancer drug cyclophosphamide. It has an ablility to introduce alkyl radicals into DNA strands forming DNA cross-linkage. Formation of cross-links between and within DNA strands is irreversible and leads to cell apoptosis. Cyclophosphamide is used to treat in different types of cancer. It may cause BM suppression and myelodysplasia – ineffective blood cell production in the BM. Cyclophosphamide interacts with leukocytes at all stages of their maturation and leads to inhibition of leukopoiesis.

After injection of cytostatic, we can see clearly marked cytopenia in peripheral blood. Changes in total cell count mean of PB and BM cells have a wavelike character with shifted crests and troughs. Intensification of proliferative activity of PB cells is observed at 48, 72, 168 h and at 72, 168 h points in BM, whilst deficit of cells is mentioned at 24, 96 h in PB and 24, 48, 96 h points in BM (Fig. 1, *a*, *b*).

Nuclear cell and total cell count relation (per mille) is given in Fig. 2, *a*, *b*. Here we can see that per mille of nuclear cells in PB increases at 24 h point and then gradually decreases up to 96 h point (during 2–4 days). At 168 h point is mentioned

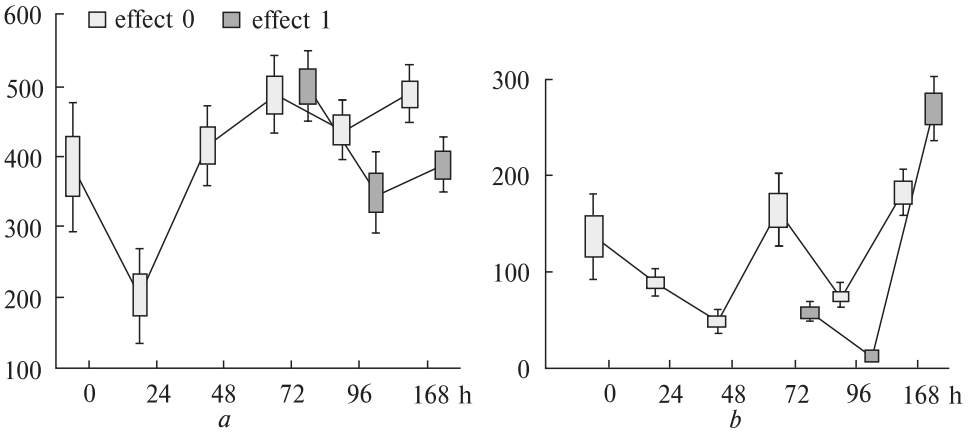

Fig. 1. PB (*a*) and BM (*b*) total cell mean count; effect 0 – control group of rats intraperitoneally injected by 100 mg/kg LD_{50} 50–160 mg/kg cyclophosphamide; effect 1 – test group of rats intraperitoneally injected by 200 μg/ml ubiquitin

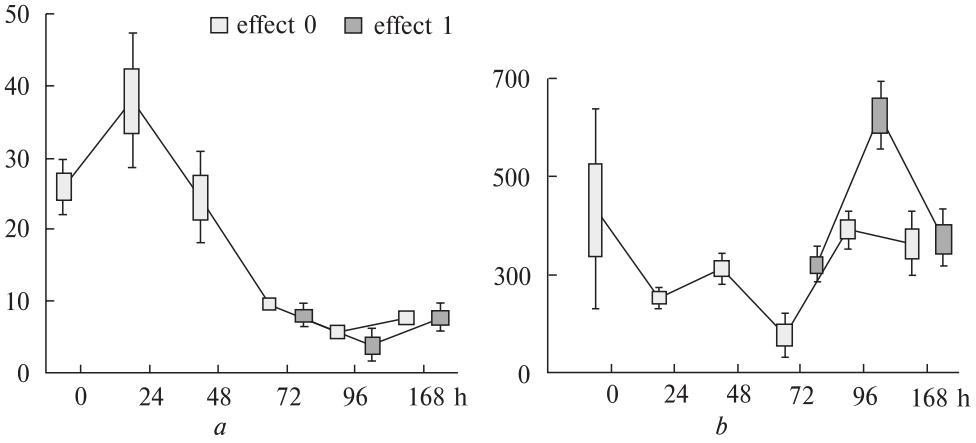

Fig. 2. PB nuclear (*a*) and BM nucleic (*b*) cell count ‰; effect 0 – control group of rats intraperitoneally injected by 100 mg/kg LD_{50} 50–160 mg/kg cyclophosphamide; effect 1 – test group of rats intraperitoneally injected by 200 μg/ml ubiquitin

rise of per mille by 33 % as compared with 96 h point value.

In BM samples the changes of nuclear cell and total cell count relation have again wave-like character. Lowest values are mentioned at 24 and 72 h points and smooth decrease at 168 h point, where it is approximately equal to cell count of control group animals. Peaks are observed at 48 and 96 h points.

Three days later after cytostatic induction, animals were injected intraperitoneally by 200 μg/ml ubiquitin. The most noticeable cytological effect in BM induced by ubiquitin is a significant increase of per mille at 96 h, whilst total mean is strictly decreased.

The nucleated cell count of the BM actually increased approximately – 4 times through 24 h after ubiquitin injection, then strictly decreases during next 24 h and reaches the norm value. Total cell count mean dramatically declines at 96 h in BM that is followed by impressive jump through the next 24 h and at 168 h point is increased about 22 times (Fig. 1, *b* and 2, *b*).

In PB ubiquitin declines the mean of total cell count by 20 % as compared with cytostatic effect at 96 h point. Through the next 24 h number of cells increases and becomes equal to norm. In cytostatic injected animals, without ubiquitin at the same hour point (168 h) mean count of total cells is above the norm by 20 % (Fig. 1, *a*). Nuclear cell ratio in the

Table 1. **Spontaneous regeneration of leucopoiesis after cyclophosphamide injection. Nuclear cell ratio and total cell mean count**

Hours after cyclophosphamide injection	‰ nuclear cells		Total cell mean		Standard Error (P < 0.05)	
	PB	BM	PB	BM	PB	BM
Intact	25	328	386	137	9,75 ± 1,0	44,8 ± 2,5
24	34	252	202	89	6,80 ± 0,7	22,3 ± 1,5
48	24	303	416	48	10,20 ± 1,3	14,6 ± 1,5
72	10	161	489	164	4,75 ± 0,3	26,5 ± 2,0
96	6	389	438	75	2,50 ± 0,2	29,2 ± 2,6
168	8	351	490	183	3,80 ± 0,2	64,0 ± 3,4

Table 2. **Changes in nuclear cell ratio and total cell mean count after injection of extracellular ubiquitin**

Hours after cyclophosphamide+ +ubiquitin injection	‰ nuclear cells		Total cell mean		Standard Error (P < 0.05)	
	PB	BM	PB	BM	PB	BM
72 (6)	8	322	500	58	4,0 ± 0.4	18.75 ± 1.9
96 (24)	4	624	348	12	1.5 ± 0.4	7.75 ± 1.2
168 (96)	8	358	390	270	3.1 ± 0.4	96,9 ± 1,0

PB decreases by about 30 % at 96 h. During the next 24 h, per mille of nuclear cells is increased two times that becomes equal to the ratio of 168 h point of cytostatic injected animals.

Abortive elevation that is observed at 72 h point in PB and BM is accompanied by decline of nuclear cell ratio in group of animals injected by cyclophosphamide. Increase of total cell mean count in bone marrow while abortive elevation might be explained by activation of stem cells proliferation or retention of progenitor cells in zones of differentiation and maturation. Introduction of ubiquitin deepens subsequent depletion at 96 h point in PB. The ratio of nucleated cells is declined in PB and sharply increased in BM. It is evident that ubiquitin strictly diminishes number of precursor cells in BM, as well as functional cells in PB. The sharp depletion at 96 h point in BM is followed by the next boost of elevation of total cell mean count at 168 h point in BM, normalization of total cell mean count in PB and nucleated cell ratio in BM. It is clear that in vivo injected ubiquitin regulates stem cell activity, normalizes the release of functional cells into bloodstream, supposedly retains progenitor cells in zones of differentiation and maturation, and restores the nuclear cell ratio in PB and BM. So, there is clearly expressed effect of ubiquitin on bone marrow cells through the spontaneous regeneration of leucopoiesis. The results of the ubiquitin effect on leucopoiesis spontaneous regeneration and PB are presented in Tables 1 and 2.

Conclusions. Our experiments revealed that the regeneration of leucopoietic cells is influenced by intraperitoneally injected extracellular ubiquitin. Ubiquitin makes corrections in regeneration and leads to normalisation of the process. It also is evident that when total cell count sharply decreases, portion of nucleic cells in PB elevates significantly. The period of maximal activity (3–4 days) is mostly affected by ubiquitin. It is clear that in vivo injected ubiquitin regulates stem cell activity, normalizes the release of functional cells into bloodstream, supposedly retains progenitor cells in zones of differentiation and maturation, and restores the nuclear cells ratio in PB and BM. So, there is clearly expressed effect of ubiquitin on bone marrow cells through the spontaneous regeneration of leucopoiesis. Observation of extracellular ubiquitin effect on spontaneous regeneration of leucopoiesis after chemical inhibition may elucidate new possible pathways of ubiquitilation, as well as mechanisms of regulation of leukogenesis after chemically induced leucopenia. That is very essential for treatment of radiated bone marrow or chemotherapeutic side effects in cancer patients.

РЕГУЛЯЦИЯ ЛЕЙКОГЕНЕЗА ВНЕКЛЕТОЧНЫМ УБИКВИТИНОМ У ГРЫЗУНОВ ПОСЛЕ ХИМИЧЕСКОГО ИНГИБИРОВАНИЯ

Р.Ш. Суджашвили, И.В. Иорамашвили, К.Н. Апциаури, Н.Н. Гвинадзе

Для изучения воздействия внеклеточно введенного убиквитина на регенерацию лейкопоэза применен метод подсчета количества ядерных клеток в окрашенных азур-эозином образцах костного мозга и периферической крови грызунов. В контрольной группе животных ингибирование гематопоэза вызывалось внутрибрюшинной инъекцией 100 мг/кг циклофосфамида LD_{50} 50–200 мг/кг. Образцы костного мозга и периферической крови первой группы животных получены через 24, 48, 72, 96, 168 ч после введения цитостатика. Животным опытной группы через 72 ч после инъекции цитостатика введен убиквитин 200 мкг/мл. Выявлено, что интраперитонеально введенный внеклеточный убиквитин влияет на процесс регенерации клеток лейкопоэза, корректирует регенерацию и ведет к нормализации процесса. Наши результаты предполагают возможность выявления нового метаболического механизма убиквитилирования и терапевтических путей для лечения облученного костного мозга и побочных эффектов химиотерапии.

REFERENCES

1. Ciechanover, A., The ubiquitin-proteasome pathway: on protein death and cell life, *EMBO J.*, 1998, vol. 17, no. 24, pp. 7151–7160.
2. Daino, H., Matsumura, I., Takada, K., Odajima, J., Tanaka, H., Ueda, S., Shibayama, H., Ikeda, H., Hibi, M., Machii, T., Hirano, T., and Kanakura, Y., Induction of apoptosis by extracellular ubiquitin in human haematopoietic cells: possible involvement of STAT3 degradation by proteasome pathway in interleukin 6-dependent hematopoietic cells, *Blood*, 2000, vol. 95, no. 8, pp. 2577–2585.
3. Daino, H., Shibayama, H., Machii, T., and Kitani, T., Extracellular ubiquitin regulates the growth of human hematopoietic cells, *Biochem. Biophys. Res. Commun.*, 1996, vol. 223, no. 7, pp. 226–228.
4. Sujashvili R., Bukuradze, E., Modebadze, I., and Dekanoidze, D., Effect of extracellular ubiquitin on modeled alcoholic liver histoarchitectonics and proliferative activity of hepatocytes, *EMBO, Molecular Insights for Innovative Therapies*, Conference, Heidelberg, Germany, 2011.
5. Sujashvili, R., Bukuradze, E., Modebadze, I., and Dekanoidze, D., In vivo introduced extracellular ubiquitin regulates intracellular processes, *Int. J. Biol. Life Sci.*, 2012, vol. 61, pp. 326–329.
6. Sujashvili, R., Bukuradze, E., Modebadze, I., and Dekanoidze, D. Ubiquitin in combination with alcohol stimulates proliferative activity of hepatocytes, *Georgian Med. News*, 2013, no.10 (223), pp. 86–90.
7. Sujashvili, R., Ioramashvili, I., Gvinadze, N., and Aptsiauri, K., Inhibition of proliferative activity of bone marrow cells by extracellular ubiquitin, *Proc. Georgian NAS, Biomed. Ser.*, 2014, vol. 40, no. 5–6, pp. 265–270.
8. Saini, V., Romero, J., Marchese, A., and Majetschak, M., Ubiquitin receptor binding and signaling in primary human leukocytes, *Commun. Integr. Biol.*, 2010, vol. 3, no. 6, pp. 608–610.
9. Saini, V., Marchese, A., and Majetschak M., CXC chemokin receptor 4 is a cell surface receptor for extracellular ubiquitin, *J. Biol. Chem.*, 2010, vol. 285, no. 20, pp. 15566–15576.
10. Deng, X., Xu, J., *He, G., Tao, Y., Yang, T., Peng, X., and Cao, Y.,* Posttranslational modifications of CXCR4: implication in cancer metastasis, *Recept. Clin. Investig.*, 2014, vol. 1, no. 1, pp. 13–19.
11. Chernock, R.D., Cherla, R.P., and Ganju, R.K., SHP2 and cbl participate in α-chemokine receptor CXCR4-mediated signaling pathway, *Blood*, 2001, vol. 97, no. 3, pp. 608–615.
12. Hegedus, C.M., Gunn, L., Skibola, C.F., Zhang, L., Shiao, R., Fu, S., Dalmasso, E.A., Metayer, C., Dahl, G.V., Buffler, P.A., and Smith, M.T., Proteomic analysis of childhood leukemia, *Leukemia*, 2005, vol. 19, no. 10, pp. 1713–1718.
13. Eastmond, D.A., *Chemical and radiation leukemogenesis in humans and rodents and the value of rodent models for assessing risks of lymphohematopoietic cancers.* U.S. Environmental Protection Agency, Office of Research and Development, National Center for Environmental Assessment, Washington Office, Washington, DC, EPA/600/R-97/090, 1997, pp. 1–134.
14. Ponchin, G., Beaulieu, A., Humblet, C., Thiry, A., Oda, K., Boniver, J., and Defresne, M.-P., Characterization of spontaneous bone marrow recovery after sublethal total body irradiation: Importance of the osteoblastic/adipocytic balance, *PLoS One*, 2012, vol. 7, no. 2, pp. 1–13.
15. Shizuru, J.A., Negrin, R.S., and Weissman, I.L., Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematopoietic system, *Annu. Rev. Med.*, 2005, vol. 56, pp. 509–538.
16. Hollingsworth, J.W., Inhibition by cytotoxic drugs of bone marrow regeneration after irradiation and marrow transplantation, *Cancer Res. (AACR)*, 1959, vol. 19, pp. 165–169.
17. Orrenius, S., Nicotera, P., and Zhivotovsky, B., Cell death mechanisms and their implication in toxicology, *Toxicol. Sci.*, 2011, vol. 119, no. 1, pp. 3–19.
18. Kondo, T., Radiation induced cell death and its mechanisms, *Radiat. Emerg. Med.*, 2013, vol. 2, no. 1, pp. 1–4.

Received 10.08.15