In vivo Introduced Extracellular Ubiquitin Regulates Intracellular processes

Rusudan Sujashvili, Ekaterine Bakuradze, Irina Modebadze, Davit Dekanoidze

Abstract—Extracellular ubiquitin in vivo effect on regenerative liver cells and liver histoarchitectonics has been studied. Experiments were performed on mature female white rats. Partial hepatectomy was made using the modified method of Higgins and Anderson. Standard histopathological assessment of liver tissue was used. Proliferative activity of hepatocytes was analyzed by colchicine mitotic index and immunohistochemical staining on ki67. We have found that regardless of number of injections and dose of extracellular ubiquitin liver histology has not been changed, so at tissue level no effect was observed. In vivo double injection of ubiquitin significantly decreases the mitotic activity at 32 hour point after partial hepatectomy. Thus, we can conclude that in vivo injected extracellular ubiquitin inhibits proliferative activity of hepatocytes in partially hepatectomyzed rats.

Keywords—Liver, regeneration, proliferation, ubiquitin.

I. INTRODUCTION

TODAY scientists consider that the use of intracellular mechanisms can be efficacious in treatment of human diseases and so it has set the stage for attempts to selectively inhibit the activities of disease-specific components of the ubiquitin-proteasome system (UPS), since there are many components of the UPS that might be targeted for inhibition in the context of particular disease.

Targeted therapy, which first became available in the late 1990s, is currently a very active research area. This constitutes the use of agents specific for the deregulated proteins of injured cells. In considering the UPS for drug development it is important to know what the disease to be targeted is and what the nature of the alteration in the pathway is that we are trying to modify with molecule therapeutics? Also, it is important to consider how particular classes of proteins within the UPS can be targeted [1],[2].

From this point of view it is evident that all components of ubiquitin-proteasome pathway must be properly investigated to the effect of gaining and understanding of their functional and structural peculiarities, as well as possibilities of implication in new therapy of different human diseases [3].

In different studies have been suggested, that injected proteins can be used as authentic tests of the behavior of endogenous proteins [4],[5]. Moreover, it has been shown, that the extracellular ubiquitin incorporates into hematopoietic cells and mediates their growth suppression and apoptosis

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through proteasome-dependent degradation of selective cellular proteins such as STAT3 (transcriptional activator) [5]. At that if it is remembered that STAT3 is involved in a liver regeneration [6],[7], perhaps it points to the events of a similar nature in hepatocytes. Referring to these data and taking into account accumulated information about UPS and liver diseases, we supposed that it was not unlikely to make some definite changes in morphological structure of the liver and in the course of hepatocyte proliferation after in vivo transduction of extracellular ubiquitin. Limited number of studies has been directed to the investigation of behavior of extracellular ubiquitin. Different authors examined effect of extracellular ubiquitin by using the microinjected cultured cells, but there is no information about in vivo experiments.

The goal of our study was to investigate the effect of extracellular ubiquitin on liver histoarchitectonics and regenerative cells in vivo.

II. MATERIALS AND METHODS

Experiments were carried out on female white rats weighing 140 gr. Ubiquitin and other reagents purchased from Sigma. Partial hepatectomy was performed using the modified method of Higgins and Anderson [8]. Light microscope "Zeiss" has been used for observation of samples. Statistical analyses were performed using Student's test.

A. Animal Groups

Animals were divided into four groups: I – control group (intact animals), II - different doses of ubiquitin (200μg-500μg) were intraperitoneally injected into the animals once per day during three days and test material has been taken 24 hours later after the first, second and third injections, III - partially hepatectomized animals, IV - partially hepatectomized animals with double intraperitoneal injections of ubiquitin (200 μg/ml) at once and 12 hours later after partial hepatectomy. Material from III and IV groups has been taken at 29th, 32nd, 36th, 48th hours after partial hepatectomy. Animals were anesthetized by ether before decapitation.

B. Histological Analyses

For histopathological study, tissue of animals from each group after separation was fixed in 4% paraformaldehyde solution prepared in 0.1M phosphate buffered saline pH7.4. Then the samples were embedded in paraffin, sectioned using a microtome and stained with H&E.

C. Proliferative Activity Analyses

1mg/kg of colchicine was introduced into the animals of intact and test groups for proliferative activity test by determination of colchicine mitotic index. For estimation of

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proliferative activity, 5000 cells per sample were counted and mitotic index was determined by number of mitotic figures per 1000 cells (‰).

D.Immunohistochemical Analyses

On dewaxed and rehydrated slides, we performed endogenous peroxidase blocking with 3% hydrogen peroxide for 10 minutes. Heat induced epitope retrieval in citrate buffer pH6 was used for 15 minutes to unmask Ki67 epitope. Incubation with ki67 primary antibody, clone SP6 from Abcam, for two hour (dilution 1:50) proceeded with the application of biotinized secondary antibodies (Abcam). Extravidin peroxidase was added to slices and visualization was performed with 3,3'-diaminobenzidine as chromogen. We have counted 5000 cells per sample and ki67 positive cells were determined per 1000 cells (‰).

III. RESULTS

Typical structure of liver tissue has not been changed in test groups as compared with liver of intact rats. Classical lobules with portal triads at the vertices and a central vein in the middle are well visualized. Diameter of sinusoidal capillaries has not been changed. No pathological deviations were mentioned in cytoplasm of hepatocytes. Regardless the number of injections and dose of extracellular ubiquitin histology of liver remains the same as in intact groups (Fig. 1).
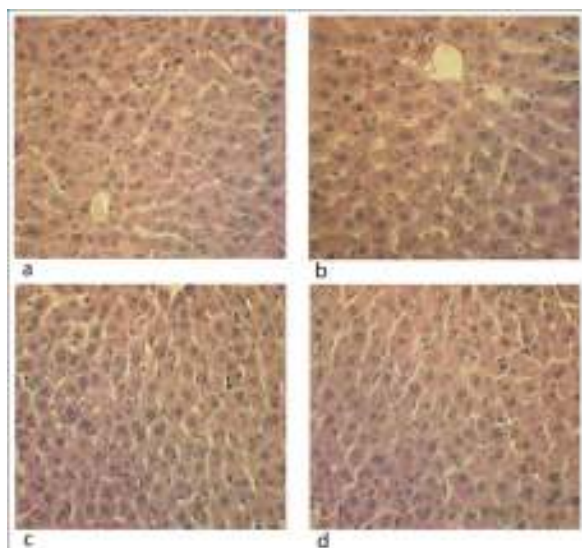

Fig. 1 Liver histology after injection of extracellular ubiquitin. a - control group, b, c, d - liver tissue after the first second and third injections of extracellular ubiquitin respectively (magnification 20x10)

Extracellular ubiquitin decreases proliferative activity of hematopoietic cells due to accelerative degradation of transcriptional factors regulating cell cycle [9]. Transcriptional activators are essential for early response genes activation which reaches the peak at 6 hour after partial hepatectomy. The peak of these genes is connected with mitotic activity of hepatocytes. The first mitosis usually occurs only at 24-26 hours after surgery and it reaches maximum value at 32 hours

after partial hepatectomy [8],[10]. These data seems to be in correlation with our results as we have found that in vivo double injection of ubiquitin significantly decreases the mitotic activity in the IV test group as compared with the mitotic activity of hepatectomyzed liver cells of the III group at 32 hours after partial hepatectomy. Particularly colchicine mitotic index of hepatocytes in hepatectomyzed rats (III group) equals to 4±1.3, mitotic index is 0.7±0.3‰ after the double injection of 200μg ubiquitin in the test group at 32 hour point after partial hepatectomy (Fig.2).
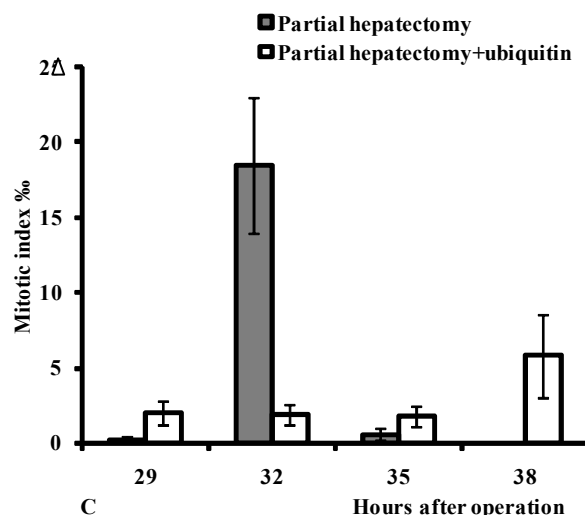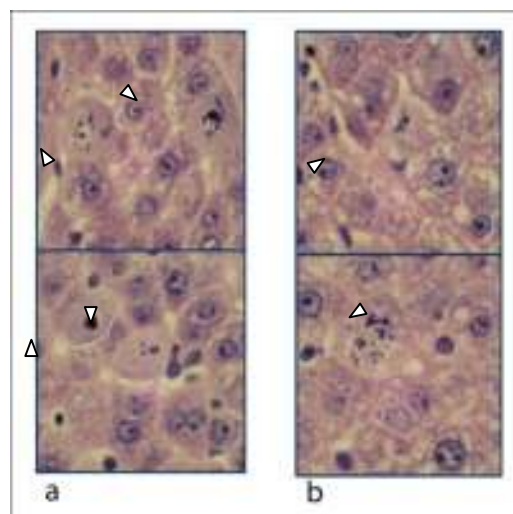

Fig. 2 Influence of extracellular ubiquitin on changes of colchicine mitotic index after partial hepatectomy. a - Mitotic figures ( ) in hepatectomized liver at 32 hours after PH; b - Mitotic figures in hepatectomized liver after the injection of ubiquitin at 32 hours after PH (magnification 90x10); c - Quantitative analysis of mitotic index

Proliferative activity of hepatocytes was also determined by immunohistochemical assay. Treatment with ubiquitin decreases the quantity of ki67 positive cells in hepatectomized liver. The number of ki67 positive cells is lower in the IV group compared with the III group at 29 hours after the partial

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hepatectomy. Number of these cells goes down within the 7 hours (at 35-hour point after PH). At 38 hours after the operation amount of ki67 positive cells seems to be increased (Fig. 3).
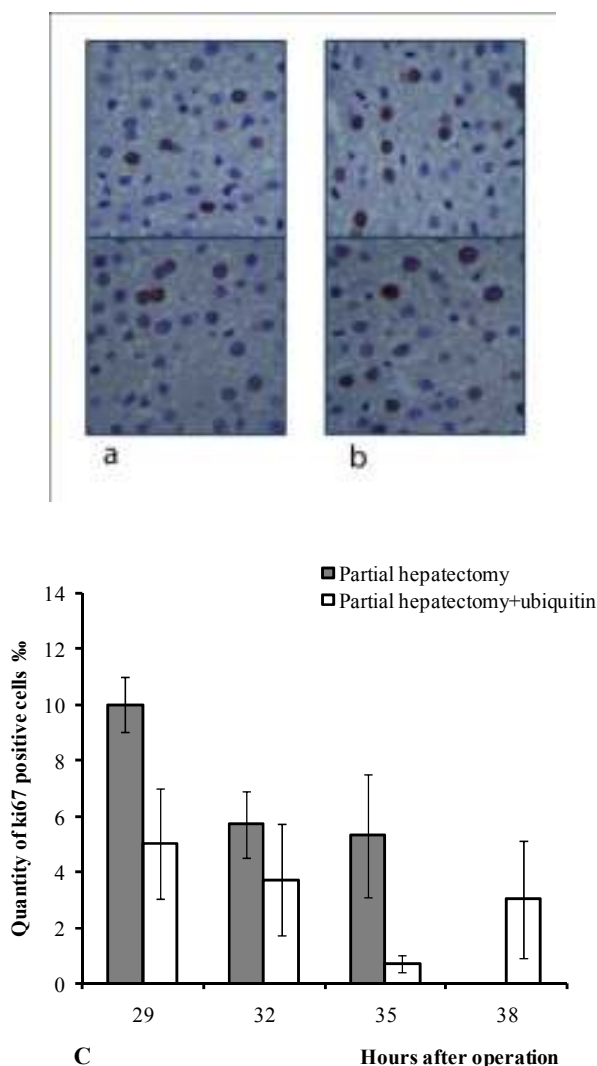

Fig. 3 Influence of extracellular ubiquitin on Ki67 positive cells in the liver after partial hepatectomy. a - immunohistochemicaly stained hepatectomized liver tissue after PH; b - immunohistochemicaly stained hepatectomized liver after the injection of ubiquitin (magnification 60x10); c – quantitative analysis of ki67 positive cells

UPS digests cell proteins not indiscriminately; it participates in the regulated breakdown of selective proteins. Extracellular ubiquitin suppress IgG production in lipopolysaccharide-stimulated splenocytes, inhibits platelet activities and has an inhibitory effect on the growth of normal hematopoietic progenitor cells [4], [11] – [13]. Other studies declare that the treatment with ubiquitin leads to degradation of various signaling molecules activated by IL-6. Exogenous addition of ubiquitin leads to apoptosis of various types of hematopoietic cells, resulting in the suppression of cell growth

[5]. It is not improbable degradation of transcriptional activators that are crucial for activation of early response genes involved in regenerative processes after ubiquitin in vivo treatment, but ubiquitin can decline proliferation affecting different mechanisms of cell cycle regulatory systems in eukaryotic cells. Thus, our results show that proliferative activity is reduced in hepatectomized liver after in vivo introduction of ubiquitin, but it is unclear how does ubiquitin overcomes the barriers before it becomes involved in intracellular processes and which pathways are exactly subjected to ubiquitilation in this particular case.

IV. CONCLUSION

Our results suggest that extracellular ubiquitin may play a role in the control of liver regeneration. On the bases of our results, we can conclude that in vivo injected extracellular ubiquitin inhibits proliferative activity of hepatocytes in partially hepatectomyzed rats. Modifications at tissue level had not been mentioned. Further studies will be necessary to understand the precise mechanisms by which the in vivo introduced extracellular ubiquitin regulates regeneration of normal and pathological liver tissue. Furthermore, here arises a question about extracellular ubiquitin ability to interact with intracellular proteins. It is incomprehensible yet and it generates interest to elucidate how ubiquitin enters the cell plasma membrane or alternatively regulates intracellular processes via modification of membrane proteins. Anyway, obtained results appear to be interesting and create a background for further investigations at molecular level.

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