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Inhibitory effect of 57% hepatectomized mice serum on the growth of L-cells

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Abstract

Adult mammalian liver cells, which are in cell cycle's G_0 phase, differentiate to accomplish specific functions and do not divide. Only as a result of damage or partial hepatectomy (PH), the cells multiply in a rapid way and cause the organ's regeneration. In one of our previous studies, we observed that sera obtained from 35% partially hepatectomized mice has an inhibitory effect on the growth of the cells after the fourth day. In this study, sera are obtained from 57% hepatectomized mice and added to medium of L-cells. In the different days, viable cell number and labelling index were investigated. According to the results obtained from the experiments it was determined that sera obtained from 57% partially hepatectomized mice has an inhibitory effect on the growth of the L-cells in early days.

Key words: Partial hepatectomy, serum, mouse, L-cells, humoral factor

%57 hepatektomi uygulanmış fare serumunun L-hücrelerinin çoğalmasını baskılayıcı etkisi

Özet

Erişkin memeli karaciğer hücreleri, hücre siklusunun G_0 fazında olup, belli fonksiyonları yapmak üzere farklılaşmışlardır ve bölünmezler. Ancak, yaralanma veya parsiyal hepatektomi (PH) sonucu, hücreler hızlı bir şekilde çoğalarak organın rejenere olmasını sağlarlar. Daha önceki bir çalışmada, %35 PH yapılan farelerden elde edilen serumun, L-hücrelerinin büyümesini dördüncü günden sonra inhibe ettiği tesbit edilmiştir. Bu çalışmada ise, %57 hepatektomi uygulanan farelerden serum elde edilmiş ve bu serum L-hücrelerinin yaşama ortamlarına ilave edilmiştir. Farklı günlerde hücreler toplanarak, canlı hücre sayıları ve işaretlenme indeksleri saptanmıştır. Elde edilen sonuçlara göre, %57 PH uygulanan hayvanlardan elde edilen serumun L-hücrelerinin büyümesi üzerinde ilk günlerde inhibisyon etkisi meydana getirdiği tesbit edilmiştir.

Anahtar sözcükler: Parsiyal hepatektomi, serum, fare, L-hücreleri, humoral faktör

Introduction

Adult mouse liver hepatocytes are differentiated cells which are in cell cycle's G_0 phase under normal conditions and execute rather important functions of the organism. Mitotic index (MI) in liver cells of such characteristics is in such a low level that it is not even worth of attention. But when PH surgery is applied to the livers of adult mice, the diffentiated liver cells gain their dividing characteristics and start to multiply (Higgins and Anderson, 1931; Bucher, 1963; Bucher and Farmer, 1998; Fausto, 2001). This regenerative growth observed in the liver, continue until the liver reaches its dimension to the one prior to the surgery.

Altun (1996) observed that after the performance of PH in a ratio of 35%, the regeneration occured rapidly up to the third day and slowed down during the following days. It was determined that as a consequence of applying PH in a ratio of 57%, regeneration was faster and in a higher ratio than that of the application of 35% (Altun and Özalpan, 1998).

It is asserted that a factor, present in the serum, is effective for the liver's reaching its previous dimension by starting cell multiplication following hepatectomy. Parabiotic rat couples were produced in order to determine this factor named as humoral factor (Bucher et al., 1951; Moolten and Bucher, 1967; Sakai, 1970). It was reported that from the rat couples whose blood circulations were connected to each other, even the liver of the rat which had not undergone PH grew. Besides, Moolten and Bucher (1967) observed that this growth, which occured in the parabiotic pairs, was related to the portion of removed liver.

Stimulative effects of humoral factors on some tumors were also found. Paschkis et al. (1955) who investigated different growths such as PH, unilateral nephrectomy and leg-fracture determined that in rats with PH, some tumors grew better. The investigators did not observe a relation between nephrectomy and leg-fracture and tumors. Consequently, they put forth that humoral factor may cause the rapid growth of the tumors and this factor behaved selectively. Although, Ono et al. (1986) expressed that tumors of the animals, administered X-5563 and Ehrlich ascites tumor cells (EAT) were not affected by PH, in the MH-134 cells the response showed a difference depending on the administration time. In a similar way, Udintsev and Shakhov (1989) indicated that the inhibition of EAT and Pliss' lymphosarcoma in animals with PH was produced by humoral factor.

By investigating the effects of blood sera, obtained from animals with PH under *in vivo* and *in vitro* conditions more detailed information about humoral factor was attempted to be attained. It was observed that in the *in vivo* studies the area of drawing blood from which serum was obtained, affected the result, and sera obtained from hepatic portal vein or cardiac right atrium had a stimulatory effect on normal and regenerated liver (Adibi et al., 1959; Moya, 1963; Levi and Zeppa, 1972). Besides, it was shown that serum with PH increased tumor growth in the *in vitro* tumors (Ramantanis and Deliconstantinos, 1985; Asaga et al., 1991; de Jong et al., 1995, 1998-1999). In the *in vitro* cultures of some tumors whose growth was observed to increase in the form of a stimulation by serum with PH, it was found that the effect produced changed depending on the time of serum administration or cell concentration (Asaga et al., 1991; de Jong et al., 1995). Sakai and Kountz (1975) determined that serum with PH increased DNA synthesis in lymphocyte and hepatocyte cultures. Chen et al. (1996) in their experiments with cirrhosis and non-cirrhosis rats, with two different PH ratios, observed that serum obtained from these animals increased DNA synthesis of hepatocyte cultures. It is asserted that together with liver dissection, during the first 24 hours a signal protein having a molecular weight of 5,000-10,000 was responsible for the production of these effects and also this protein contained a factor which increased cell growth (Takahashi et al., 1992). Nevertheless, Makowka et al. (1983) informed that liver's cytosole contained stimulatory and inhibitory factors.

In our previous studies, it was observed that serum obtained from mice with 35% PH, inhibited the growth of L-cells *in vitro* following the fourth day of its administration (Altun et al., 2002). Besides, regeneration was in proportion with the portion removed during hepatectomy, in paralel to PH ratio regeneration also increased (Altun and Özalpan, 1998). The aim of this research was to determine mode of effect of the serum obtained from mice with hepatectomy ratio increased to 57%, on L-cells *in vitro*.

Material and methods

Animals and the preparation of sera

The animals used were 2.5 months old male Balb/C strain inbred mice (*Mus musculus*) whose body weights varied between 20-30 g (n=23) The animals were placed in polycarbonate cages and fed with pellet mouse food (Hipodrom Ltd.) and tap-water given ad libitum.

PH operation (57%) was carried out by removing the left lateral and median lobe of the liver of mice under ether anesthesia (Higgins and Anderson, 1931). Three days after the operation, blood was drawn from the carotid artery of hepatectomized mice. The blood

was centrifuged and the serum was separated. After the serum were sterilized, they were used freshly without freezing.

Cells and experimental groups

In the experiments, tumor L-cells obtained from mice subcutaneous connective tissue in 1943 were used (Shannon, 1972). The cells were propagated in 10% Fetal bovine serum and Medium 199.

Cells were removed from the surface of culture bottles by addition of 0.25% trypsin and centrifuged for 3 minutes (1,500 cycle/min). With the addition of Medium-199 on the cell precipitate, the cells became ready for seeding.

Control 1: 10% Fetal bovine serum and 90% Medium-199

Control 2: 10% Fetal bovine serum, 5% normal mouse serum and 85% Medium-199

PH-57: 10% Fetal bovine serum, 5% hepatectomized (57%) mouse serum and 85% Medium-199.

Growth rate

L-cells were seeded on sterile microplates having 24 wells in a concentration of 10^4 cells in each well. Microplate wells were divided into the above mentioned experimental groups and 1.5 ml of medium of each experimental group was added on L-cells in each well. During the experimental period, the microplates were kept in an atmosphere of 5% CO_2 and 95% air at 37°C with pH 7.2 in a dessicator.

The growth rate of L-cells were determined on 2nd, 4th and 6th days. For this process, the cells multiplying in microplate were collected separately with trypsin and by the application of viability test (Phillips, 1973), L-cells count for each group and day were made.

Labelling index

In order to determine the labelling index of L-cells at the end of 2nd, 4th and 6th days, the cells were kept in a medium containing 5 mCi/ml ^{3}H-thymidine (^{3}H-TdR) for 30 minutes and they were then fixed with 1:3 w/w acetic acid:ethanol. By the use of K2 emulsion (Ilford) the autoradiography of the preparations was made. At the end of 10 days of the exposition period, the autoradiograms were developed with D19b developer and stained with Giemsa. The labelling index was determined by counting 900-1,300 cells of each group and day.

Statistical evaluation

The values of growth rate and labelling index obtained in the experiments are given as arithmetic means and standard error of each mean. The significance of the variation was determined by Student-t test.

Results

The multiplication rate of L-cells propagated in medium to which 5% serum obtained from mice on the third day following 57% PH ratio performance is shown in Figure 1. As seen in the Figure, two days after initially seeding 10^4 cells, 34,545 cells were counted and in the forth day, the cell number reached 46,153. After the fourth day, the multiplication rate

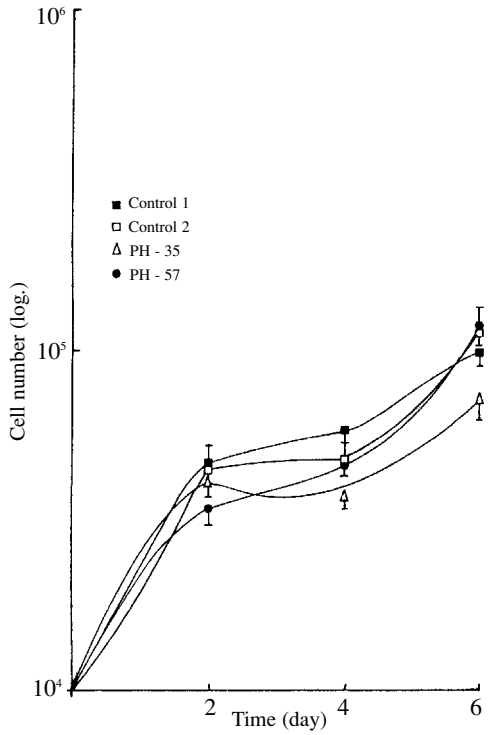

Figure 1: The growth rate in the L-cells.

increased a little and on the sixth day the cell number was found to be 110,750.

When the cells which were propagated in a medium containing normal and with PH serum were examined under the microscope, a layer similar to an oily one at the surface of the medium was observed. Besides, a large number of vacuoles in the cells cytoplasm were observed (Figure 2).

The values of labelling index of L-cells propagated in serum with 57% PH are given in Table 1. From these values, it was determined that the labelling index started with a considerable low level of 11.5%, increased rapidly and after reaching 44.1% on the fourth day, showed a little reduction later on. The labelling index of L-cells on the sixth day was found as 28.5%.

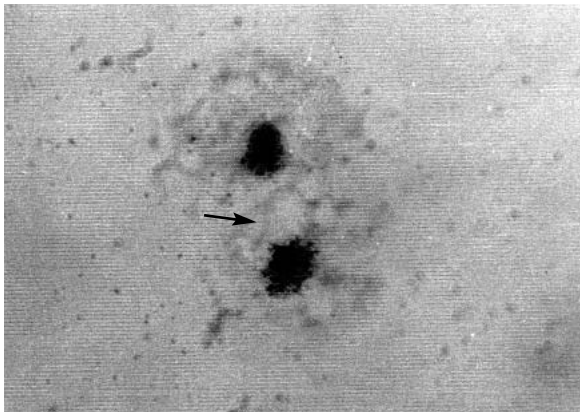

Figure 2: L-cells in the PH-57 group (➔ vacuole).

Discussion

With the addition of serum, obtained from the carotid arteries of mice with 57% PH, the multiplication rate and labelling index of L-cells were examined. According to the results obtained, it was determined that L-cells exhibited a slow multiplication during the first days and after the fourth day, the multiplication rate increased. Even though, this characteristic was also observed in the values of labelling index, little reduction in the synthesis rate could be seen on the sixth day. These results were compared with those of the ones obtained with our previous studies with normal and with 35% PH serum (Figure 1, Table 1), (Altun et al., 2002). When Figure 1 is examined it can be seen that on the second day in comparison with Control 1, Control 2 and PH-35 groups, in the PH-57 group a lower level of multiplication rate occured. Contrarily, it was observed that with the increase of PH-57 group's multiplication rate in the following days, on the sixth day a higher cell number was reached in proportion to other groups. In respect to control groups during the first days, this decrease observed in L-cells group, propagated in serum with 57% PH, showed a significant difference ($p<0.05$) only in Control 1 group.

Table 1: The change in labelling index of L-cells, depending on days (±SE).

	Time (day)		
Groups	2	4	6
Control 1	35.4±7.6	41.3±5.0	42.7±0.7
Control 2	37.2±8.2	32.7±5.3	31.2±4.8
PH-35	14.4±2.1	16.8±9.7	14.4±0.8
PH-57	11.5±3.0	44.1±9.4	28.5±6.8

As in our previous study (Altun et al., 2002), an oily layer was present on medium's surface and vacuoles were observed in cell cytoplasms. It is known that when the environmental conditions of the cells propagated *in vitro* are not favourable, vacuoles start to form in the cytoplasm and the culture starts to degenerate (Freshney, 2000). Besides, the importance of serum's amount in the medium for the attachment of cells to the surface was reported (Bliokh et al., 1982). Consequently, as Altun et al. (2002) had disclosed, L-cells were also affected unfavourably by serum with 57% PH added to the medium.

Sakai and Kount (1975) determined that as a result of administrating serum with PH, obtained from rats, to fetal liver cells and lymphocytes an increase occured in amount of DNA and in the rate of mitosis *in vitro*. Asaga et al. (1991) observed that serum, obtained after 1-4 days from PH, stimulated the growth of both AH130 cells *in vitro* and Walker 256 cells *in vivo*, in the case of tumor transplantation 7 days after PH, the growth of tumor was smaller.

With the suggestion of tumoral factor's responsibility of initiating the growth after PH, studies were performed with blood sera from different area in order to investigate this factor's secretion site to the circulation system. Adibi et al. (1959) who infected serum obtained from hepatic vein and left ventricules of rats with 67% to rats again with PH, after 24 hours examined mitotic indices of

hepatocytes. While liver's mitotic indices in the peripheral blood serum obtained from left ventricule was 9.19, it was established that with the hepatic vein serum, mitotic indices reached 18.78. In respect with the results they obtained, they asserted that the factors which stimulated and inhibited growth were in balance under normal conditions, with the decrease of growth inhibitory concentration by PH the balance change towards the stimulating factor and this factor was released by the liver. Moya (1963) who applied PH in a ratio of 2/3 to rats from the sera obtained from their hepatic and portal veins, and from carotide artery observed that artery serum produce inhibition in normal rats and on the other one produced a much lesser inhibition rate in comparison to the artery serum. Moya (1963) disclosed this situation as the decrease in the inhibitory substance's amount while passing into the tissues. Moya (1963), who added normal and PH serum to AH130 cells *in vitro* observed that while a decrease occured in cell number of the cultures on the 24th hour and sixth day, serum with PH produced an effect similar to the normal culture medium. 24th hour following 70% PH serum obtained from portal vein was also found to increase liver DNA synthesis amount in normal rats (Levi and Zeppa, 1972). Colorectal liver tumors were examined by de Jong et al. (1995, 1998-1999). When these investigators added portal and systematic sera to CC 531 cells 24 hours and 14 days after PH, they observed that an effect in the form of stimulation when the cell amount was small and of inhibition when it was large occurred (de Jong et al., 1995). de Jong et al. (1998-1999) indicated that portal serum taken on the third day after 70% PH application increased the multiplication of CC 531 cells in a rate of 25-40%. In contrast to this, when the same investigators added systematic serum, taken on the 14th day, to the same cells they observed an increase in the cell multiplication. Chen et al. (1995) applied PH in ratios of 33% and 70% to cirrhosis and non-cirrhosis rats and found that the serum which they obtained from the animals on the second and 48th hours did not affect the mitosis of hepatocyte cultures, but produced an increase in DNA synthesis.

Takahashi et al. (1992) asserted that in the portal serum, obtained from rats with 70% PH after 24 hours, a protein with a molecular weight of 5,000-10,000 contained a factor which stimulated cell growth.

The effects of hepatectomy ratios on parabiotic pairs produced by connecting the blood circulations of two rats one of which had PH, was investigated by Moolten and Bucher (1967). The investigators who examined three different ratios PH, 34%, 68% and 85%, observed that a significant increase in normal rat liver DNA did not occur only in the group to which 35% PH was performed. It was determines that following PH performance to cirrhosis and non-cirrhosis patients most of which had hepatocellular carcinoma, human hepatocyte growth factor (hHGF) was present in their peritoneal fluid and blood serum and this factor which was in relation with removed liver is portion increased DNA synthesis in rat hepatocyte cultures *in vitro* (Miyata et al., 1996a, 1996b). Higaki et al. (1999) reported that in patient with PH, the growth factor was in a higher level in portal serum than that of peripheral serum. Furthermore, in the human liver regeneration after PH, it has been determined that the activity of liver regeneration, mainly referring to proliferation of hepatocytes, is affected by a number of factors (both stimulatory and inhibitory) released from local or from other parts of the body (Wu et al., 1998).

By inoculating tumor at the same time to animals with PH, whether PH had an effect on tumor growth or not was investigated. While an increase was observed in hepatoma and Walker 256 tumors, inoculated at the same time with PH, any change in Jensen sarcoma's and Murphy lymphosarcoma's growth was not observed (Paschkis et al., 1955). Ono et al. (1986) who reported that X-5563 and EAT tumors were not affected by PH, determined that an inhibition occured in tumor growth depending on transplantation time. In similar way, it was determined that the growth of EAT cells, inoculated at the same time with 35% PH was inhibited on the 10th day (Altun, 1996). This different behaviour of PH on tumors was expressed by Paschkis et al. (1955) as a selective behaviour of humoral factor which started liver regeneration. Udintsev and Shakhov (1989) also confirmed that the inhibition which occured in the growth of EAT and Pliss' sarcoma of animals with PH was formed by the humoral factor.

A study showing the effect of serum, obtained from mice with a lower PH ratio, on L-cells is present in the literature. The other studies show differences in the viewpoint of both cell type and serum. When all these investigations are taken into consideration with respect to L-cells, besides the area where serum was obtained and the selective effect of humoral factor on the tumors, the growth of tumors was also affected by

PH ratio and this observed inhibition may be produced by the effect of one or more than one of these factors.

In conclusion, it was determined that even though mouse serum with 57% hepatectomy inhibited the growth of L-cells on the second day, this effect was alleviated during the following days.

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