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STIMULATION OF DNA SYNTHESIS BY HEATED HUMAN SERUM IN PRIMARY CULTURED NORMAL ADULT RAT HEPATOCYTES

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Summary

In primary culture of normal adult rat hepatocytes, human serum heated at 56°C for 30 min stimulated dose-dependently [^{3}H]thymidine incorporation into trichloroacetic acid insoluble fraction of the cells, most of which was solubilized into hot trichloroacetic acid solution. The solubilized fraction was reduced when hydroxyurea was added to the culture. The heated serum also increased dose-dependently protein synthesis and cell viability determined from morphological findings. These results suggest that human serum has heat-stable factors stimulating DNA synthesis and maintaining cell viability of cultured rat hepatocytes.

Although primary culture of hepatocytes has been used as an experimental model suitable for studying liver function and its pathological mechanism, DNA synthesis or mitosis has not been observed in this system except when fetal or post-hepatectomized regenerating liver was used as a source (1,2), or when hormones were added to the culture medium (3,4).

In this paper we present evidence that in heated human serum there are stimulating factors of DNA synthesis in addition to those which maintain cell viability in primary cultured hepatocytes prepared from normal adult rats, and describe a simple *in vitro* method for studying liver regeneration.

Materials and Methods

Preparation of samples. Pooled human serum was obtained from healthy male adults and stored at -20°C until use. Immediately prior to use, the serum was sterilized by passing it through 0.45μm filter (Millipore Corporation,Bedford,Mass.), and the heated serum was prepared by heating it at 56°C for 30 min.

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Isolation and culture of rat hepatocytes. Hepatocytes were isolated from male Wistar rats (Nihon Rat Co.,Urawa,Japan), weighing 150-200 g, by the method of Berry & Friend (5). 3.0-4.5 x 10^5 viable cells were distributed in 60 x 15 mm plastic Petri dishes (Falcon Plastics,Oxford,Calif.), containing Leibovitz's L-15 medium (GIBCO Laboratories,Grand Island,N.Y.) of final volume adjusted to 3 ml with samples at the concentrations described in each experiment. Viability of the hepatocytes was examined by exclusion of trypan blue.

Assay of [^{3}H]thymidine incorporation into cultured rat hepatocytes and protein synthesis of the cells. After incubation with 1μCi of [^{3}H]thymidine (New England Nuclear,Boston,Mass.)per dish added at the indicated time, the cultured cells were detached from the dish by scratching and collected by centrifugation. These cells were washed 3 times with 4 ml saline. After repeated freezing and thawing, they were suspended in 4 ml of 5 % trichloroacetic acid (TCA), and the supernatant was discarded. This process was repeated 3 times. After solubilizing the TCA insoluble fraction by adding hydroxide of hyamine 10-X (Packard Instrument Company, Inc.,Ill.) and neutralizing pH with acetic acid, the radioactivity in the fraction was measured in a liquid scintillation counter. To the TCA insoluble fraction, 1 ml of 10 % TCA was added and heated at 100°C for 15 min, and the supernatant was used as hot TCA soluble fraction. Hydroxyurea inhibition was performed by adding 25 mM hydroxyurea to the medium at the starting time of the incubation. Protein synthesis was measured by the incorporation of [^{3}H]proline (New England Nuclear,Boston,Mass.)(1μCi per dish) into TCA insoluble fraction following a process similar to that used in [^{3}H]thymidine incorporation. In pulse-labeled experiments, [^{3}H]thymidine of [^{3}H]proline was added to the medium serially at the indicated times, and the incorporation rate was determined by dividing the total radioactivity in TCA insoluble fraction by the incubation period with labeling. All samples were examined in duplicate.

Morphological observation of cultured rat hepatocytes. Morphological study was performed with an inverted microscope, and the grades of cell viability were classified as described in the footnote to Table 3.

Results

Time course of [^{3}H]thymidine or [^{3}H]proline incorporation rate into cultured rat hepatocytes. As shown in Fig. 1A, when the viability of the hepatocytes at plating was 90 %, the [^{3}H]thymidine incorporation rate increased at the incubation times of 3, 9, and 27 hr, and decreased thereafter in serum-free culture. By adding the heated serum, however, a similar and enhanced pattern for the initial 33 hr and a gradual increase thereafter up to 51 hr were observed; it was depressed by adding the non-heated serum. As shown in Fig. 1B, when the viability was 67 %, the incorporation rate

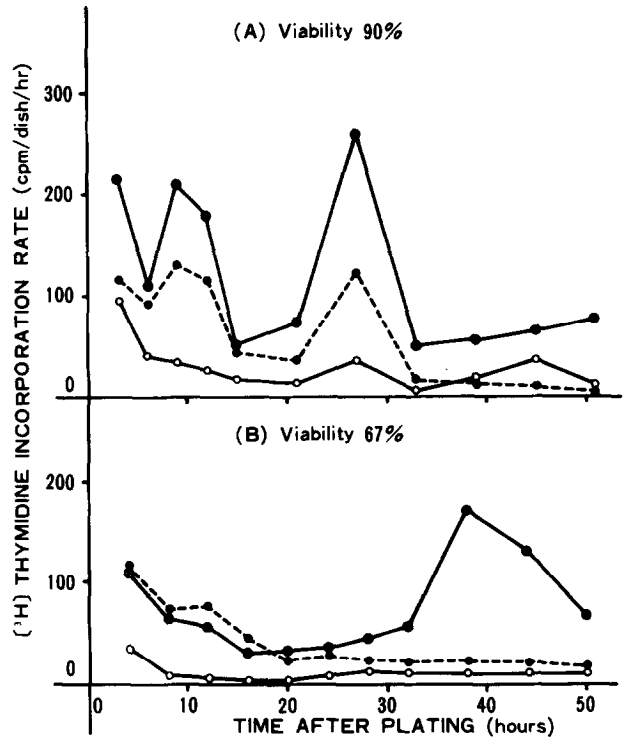

Fig. 1 Time course of [^{3}H]thymidine incorporation rate into primary cultured rat hepatocytes.

Each point indicates the incorporation rate during the period of time between the addition of [^{3}H]thymidine to the medium and the determination of the incorporation. The serum concentration in the medium was 7 %. ●---● , cultured in serum-free medium ; ○—○ , cultured with the non-heated serum ; ◎—◎ , cultured with the heated serum.
(A) Viability of the cells was 90 %, and the number of viable cells plated was 3.8 x 10^5 per dish. [^{3}H]thymidine was added to the medium 3 hr before the determination during the initial 15 hr, and 6hr before the determination during the rest of the incubation period. (B) The viability was 67 %, and the number was 4.3 x 10^5 per dish. [^{3}H]thymidine was added 4 hr before the determination during the initial 32 hr, and 6 hr before the determination during the rest of the period.

decreased gradually in serum-free culture and was almost negligible all through the incubation period in the culture with the non-heated serum. However, when the heated serum was added, although the incorporation rate decreased gradually for the initial 24 hr, it showed a marked increase between 38 and 50 hr. This increase was found to start at 33 hr when the viability was 74 %. The [^{3}H]

Table 1 Effect of heated human serum on [^{3}H]thymidine incorporation into primary cultured rat hepatocytes

Serum Concentration (%)	TCA insoluble fraction (cpm/dish)	Hot TCA soluble fraction (cpm/dish)	Hot TCA soluble fraction reduced by hydroxyurea (cpm/dish)
0	29	16	10
10	264	218	114 (42.3)
20	695	603	293 (42.2)
30	869	776	370 (42.6)

TCA : trichloroacetic acid. Figures in parentheses indicates the ratio of hot TCA soluble fraction reduced by hydroxyurea to TCA insoluble fraction expressed as %. Viability of the cells was 68 %, and the number of viable cells plated was 4.0×10^5 per dish. [^{3}H]thymidine was added to the medium at 24 hr, and the incorporation into TCA insoluble and hot TCA soluble fractions was determined at 48 hr of the incubation.

proline incorporation rate, using the hepatocytes with 67 % viability, decreased gradually from 100 to 80 cpm/dish/hr for the initial 16 hr and became a plateau thereafter up to 50 hr in the culture with the heated serum, while it was undetectable all through the incubation period with addition of the non-heated serum.

Effect of heated human serum on [^{3}H]thymidine incorporation into the cultured rat hepatocytes. [^{3}H]thymidine incorporation into TCA insoluble and hot TCA soluble fractions, and the reduction in that into hot TCA soluble fraction by addition of hydroxyurea, which is assumed to be DNA, increased dose-dependently with the amount of heated human serum added to the medium. The DNA fraction was about 43 % of the incorporation into TCA insoluble fraction (Table 1).

Protein synthesis and morphological observation of rat hepatocytes cultured with human serum. [^{3}H]proline incorporation into

Table 2 Effect of human serum on protein synthesis of primary cultured rat hepatocytes

Serum concentration (%)		[^{3}H]proline incorporation (cpm/dish)
	0	146
Heated serum	5	182
	10	194
	20	231
	30	328
	40	547
Non-heated serum	5	35
	10	11
	20	16
	30	13
	40	7

Viability of the cells was 74 %, and the number of viable cells plated was 3.0×10^5 per dish. [^{3}H]proline was added to the medium at 24 hr, and the incorporation into TCA insoluble fraction was determined at 48 hr of the incubation.

TCA insoluble fraction increased together with the amount of the heated serum added to the medium. However, it was depressed markedly when the non-heated serum was used (Table 2). The grades of cell viability from the morphological findings in the culture with the serum corresponded well with the observations in the protein synthesis (Table 3).

Discussion

In primary culture of hepatocytes isolated from intact adult rat, it has been found that human serum has a stimulating effect on DNA synthesis when it is added to the incubation medium after heat treatment. This is based on the fact that the heated serum increased dose-dependently [^{3}H]thymidine incorporation into TCA

Table 3 Morphological findings of rat hepatocytes cultured with heated human serum

Serum concentration (%)	Adhesion	Elongation	Aggregation
0	1	1	1
5	1 - 2	1 - 2	1
10	2	2	2
20	2 - 3	3	2
30	3	3	3
40	4	4	4

Viability of the cells was 74 %, and the number of viable cells plated was 3.0×10^5 per dish. The incubation time was 27 hr.
Adhesion : 1 indicates that about 10 % and 4 indicates that 90 % of the cells adhere to the plate. Elongation : 1 indicates that less than 10 % and indicates that 4 more than 70 % of the cells attached to the plate form a spread-out and elongated shape. Aggregation : 1 indicates that less than 10 % and 4 indicates that more than 70 % of the cells attached to the plate aggregate into trabecular structures.

insoluble fraction of the cells, most of which was solubilized into hot TCA solution and reduced when the culture was performed by addition of hydroxyurea. In order to reveal the effect constantly, using the hepatocytes with viability in the range of 67 to 90 %, it was found essential to examine [^{3}H]thymidine incorporation at an appropriate time after plating, because an increase in the incorporation was observed repeatedly up to 27 hr in the case of 90 % viability, but it appeared only after 32 hr in the case of 67 % viability. Therefore, we added [^{3}H]thymidine at 24 hr and determined the incorporation at 48 hr of incubation. In addition, it has been also found that heated human serum has a favorable effect on maintenance of cell viability, because [^{3}H] proline incorporation and the grades of cell viability from the morphological findings increased dose-dependently with the amount of serum added to the medium.

It can be postulated that platelet-derived growth factor was contaminated in serum (6). However, its effect can be neglected, because there was no significant difference when plasma was used instead of serum.

It is hard to explain why DNA synthesis was stimulated periodically during the incubation. However, it might correspond to the observations in in vivo experiments that the percentages of mitotic labeling with [^{3}H]thymidine in the hepatocytes of the growing rat showed a pattern of waxing and waning (7), that the hepatocytes in various parts of the post-hepatectomized regenerating liver differed in their DNA synthesis activity and that even in the zone of optimal conditions for proliferation, only 30 % of the cells work simultaneously for DNA synthesis (8).

The mechanism by which heated human serum stimulates the DNA synthesis and maintains cell viability is an interesting problem. It is possible to assume that in human serum there are heat-stable stimulating factors and heat-labile inhibitors to these stimulators which are associated with DNA synthesis and cell maintenance of the cultured rat hepatocytes, and also heat-labile toxic substances to cell viability, because the non-heated serum depressed markedly the protein synthesis as well as the viability determined from the morphological findings. Complements are unlikely to be these substances, because when complements of guinea pig were added to the incubation medium with the heated serum, there was a slight decrease in the effect of the serum on DNA synthesis irrespective of the amount of the complements added.

Of the sera of monkey (macaca irrus), horse, pig, fetal and newborne calves, rabbit, dog, rat, guinea pig, and chicken tested, the sera of monkey and horse had a similar effect to human serum. The difference among species is quite puzzling.

In the present study it is not clear how heated human serum stimulates DNA synthesis and whether or not mitosis or cell division actually occurs. Further studies concerning these problems are now in progress. In addition, using this culture system, alterations in these stimulating factors are being examined in the serum of patients with various types of liver diseases.

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