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Cell Density-Dependent DNA Fragmentation and Its Suppression by Heparin in Primary Culture of Adult Rat Hepatocytes

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SUMMARY : We show here that the internucleosomal DNA fragmentation, which is a biochemical hallmark of apoptosis, was induced in a cell density-dependent manner in primary culture of adult rat hepatocytes. This DNA fragmentation could be suppressed by a gene expression inhibitor, indicating the active nature of this process. Moreover, the viability changes in high and low cell density cultures showed a tendency corresponding with the incidence of the DNA fragmentation in them. These results suggest that in hepatocytes there may be a cell density-dependent apoptosis mechanism. In this report, we also show that heparin could suppress this DNA fragmentation with high specificity, and the cell death to some extent.

Apoptosis or programmed cell death is an active process of self destruction. It has been known to play an important role in many physiological processes, such as embryonic development, clonal selection in the immune system, and normal cell turnover in adult tissue (1-4). In the liver *in vivo*, it has been reported that apoptosis of hepatocytes occurs during the regression of liver parenchymal hyperplasia (5,6). This notion allowed us to suppose that cell density might regulate the induction of apoptosis in hepatocytes, as well as many other cell functions and gene expressions in hepatocytes (7-9). In this report, we tried to examine this possibility using the primary culture of adult rat hepatocytes. Analysis of the internucleosomal DNA fragmentation (1-4) provided evidences implying the occurrence of cell density-dependent apoptosis and its suppression by heparin in cultured hepatocytes.

EXPERIMENTAL PROCEDURES

Cell preparation and culture — Hepatocytes were prepared from adult Wistar rats (female, 7-9 weeks old) by *in situ* collagenase perfusion method (10) and

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subsequent low speed centrifugation. Cell viability was assessed by the trypan blue exclusion method, and was always higher than 85%. The isolated hepatocytes were resuspended in Williams' medium E supplemented with 10 % fetal bovine serum, and 10^{-7} M dexamethasone, and were seeded into type I collagen-coated 25 cm^2 culture flasks. After 4 h incubation at 37 °C in a 5 % CO_2 atmosphere for cell attachment to flasks, medium was replaced with fresh Williams' medium E supplemented with 10^{-7} M insulin , 10^{-7} M dexamethasone, and 0.7 μg/ml aprotinin. After subsequent culture for 20 or 44 h, cells were harvested by treatment with collagenase solution, which was the same solution as the one used in hepatocytes isolation from rat liver, at 37 °C for 0.5 h. Then dissociated cells were collected by centrifugation at 100 x *g* for 5 min at 4 °C, and stored in -80 °C freezer.

DNA isolation and analysis — Total DNA was isolated from the stored cells by the conventional method (11) using SDS, proteinase K and phenol, and was analyzed by 0.8 % agarose gel electrophoresis (11).

Assessment of viability — Viability of cultured hepatocytes was assessed by MTT assay (12) with slight modifications (13). Hepatocytes culture and MTT reaction were done in 24 wells culture plate coated with type I collagen. After 4 h of MTT reaction, medium was removed and the cells were treated by 100 μl of 0.25 % trypsin. After solubilization of MTT formazan by 1 ml of isopropanol, 100 μl of the each solution was transferred to 96 well microplate and $OD_{595\text{-}655}$ was measured by microplate reader.

RESULTS AND DISCUSSION

To examine whether cell density affects the incidence of apoptosis in hepatocytes, we prepared the primary culture of adult rat hepatocytes under three different cell density conditions, and ascertained whether the internucleosomal DNA fragmentation would occur (Fig. 1). Interestingly, although we used the ordinary medium without adding any special factors, clear DNA fragmentation was observed in high cell density culture at 48 hr (*lane 3*). In lower cell density culture, on the other hand, the DNA fragmentation pattern became weaker (*lane 2*), and when cell density was one-fourth of the high, no apparent DNA fragmentation could be detected (*lane 1*). Similar results were obtained, even if the concentrations of insulin and dexamethasone in the mediums were reduced to one hundredth (10^{-9} M) (data not shown). These results suggest that in hepatocytes there could be a DNA fragmentation-inducing mechanism that works specifically at high cell density. It should be noted that this DNA fragmentation could be significantly suppressed by a gene expression inhibitor, cycloheximide (data not shown), indicating the active nature of this process, as reported in many other apoptosis systems (1-4).

As an approach to explore the trigger factor of the DNA fragmentation in high cell density culture of hepatocytes, we examined the effect of the conditioned mediums from high and low cell density cultures on the DNA fragmentation (Fig. 2). If the cell density-dependent DNA fragmentation is triggered by the soluble factor secreted or released from hepatocytes themselves, then the conditioned medium from high cell density culture may induce DNA fragmentation in cells at low density. So we tried to test this presumption. On the basis of the result of the time course experiments, which revealed that the DNA fragmentation occurs in 4-24 h after cell seeding (data

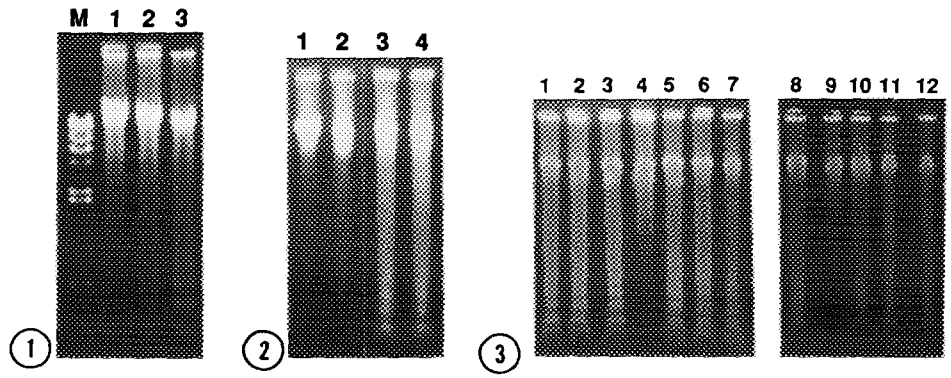

Fig. 1. Cell density-dependent internucleosomal DNA fragmentation in primary culture of adult rat hepatocytes. Isolated hepatocytes were seeded at three different cell densities, 3 x 10^4 cell/cm^2 (*lane 1*), 6 x 10^4 cell/cm^2 (*lane 2*), and 12 x 10^4 cell/cm^2 (*lane 3*), and cultured for 48 h. Then cultured cells were harvested, their DNAs were isolated, and they were analyzed by agarose gel electrophoresis. The amount of DNA applied to each lane was normalized to (DNA derived from 6 x 10^5 cells)/*lane*. *Lane M* is a size marker of λ DNA-*Hind* III.

Fig. 2. Effect of the conditioned mediums of high and low cell density cultures on the cell density-dependent DNA fragmentation. The conditioned mediums at 24 h of both high (12 x 10^4 cell/cm^2) and low (3 x 10^4 cell/cm^2) cell density cultures were prepared. They were added to newly prepared high and low cell density cultures inversely at 4 h of medium replacement instead of fresh mediums. Cells were harvested at 24 h. Other details followed the legend of Fig. 1. *Lane 1*, control of low cell density culture; *lane 2*, low cell density culture with the conditioned medium of the high; *lane 3*, control of high cell density culture; *lane 4*, high cell density culture with the conditioned medium of the low.

Fig. 3. Effect of some factors or substances on the DNA fragmentation. Several flasks of high cell density culture (12 x 10^4 cell/cm^2) were prepared. Each factor or substance was added to a flask at 4 h of medium replacement. Cells were harvested at 24 h. Other details followed the legend of Fig. 1. Two independent experiments were done (*left* and *right panels*). *Lanes 1* and *8*, control; *lane 2*, acidic FGF (20 ng/ml); *lane 3*, EGF (100 ng/ml); *lanes 4* and *9*, heparin (5 μg/ml); *lane 5*, laminin (100 μg/ml); *lane 6*; type IV collagen (50 μg/ml); *lane 7*, type I collagen (200 μg/ml); *lane 10*, heparan sulfate (5 μg/ml); *lane 11*, chondroitin sulfate (5 μg/ml); *lane 12*, keratan sulfate (5 μg/ml). Although data are not shown in this figure, basic FGF, dermatan sulfate and hyaluronic acid were also examined, but they showed no effect.

not shown), we used the conditioned mediums of 4-24 h for the experiments of Fig. 2. In result, we found that the conditioned medium of the high cell density culture showed no effect on the DNA of the low (Fig. 2, *lanes 1* and *2*). Similar result was obtained when we applied the medium that had been used during the initial cell attachment (0-4 h) of the high (data not shown). These results suggest that the soluble factor secreted or released from hepatocytes could not be involved in the induction of the DNA fragmentation. The opposite experiments using the conditioned mediums of the low also demonstrated that the soluble factor suppressing the DNA fragmentation could be absent (Fig. 2, *lanes 3* and *4*, and data not shown). It should be noted that these data simultaneously suggest that changes of the nutritional

condition caused by the difference of the cell density could not be involved in the induction of the DNA fragmentation. Considering the data described above together, we conclude that in hepatocytes there may be a mechanism of DNA fragmentation, probably due to apoptosis, which may be induced by not soluble factors but cell density itself.

Next, we tried to search other factors that have the ability to regulate this DNA fragmentation. Several kinds of factors or substances were examined for their activity suppressing the DNA fragmentation (Fig. 3). Among the ones we tested, only heparin showed a significant suppressing activity (*lanes 4* and *9*). Interestingly, other glycosaminoglycans including heparan sulfate did not show any activity in spite of their partial similarity in structure to heparin (*lanes 10-12*). These results suggest that heparin has the ability to suppress the DNA fragmentation through a specific mechanism.

We, next, examined the relationship between the incidence of the DNA fragmentation and the viability change in cultured hepatocytes by MTT assay (12,13). In correspondence with the data of the DNA fragmentation (Fig.1), the extents of cell death at 1 or 2 day in high cell density culture were higher than the ones in low cell density culture (Fig. 4). Moreover, addition of heparin, which resulted in the suppression of the DNA fragmentation (Fig. 3), also resulted in some increase in viability at high cell density (Fig. 4). These results suggest the close correlation between the DNA fragmentation and the cell death process.

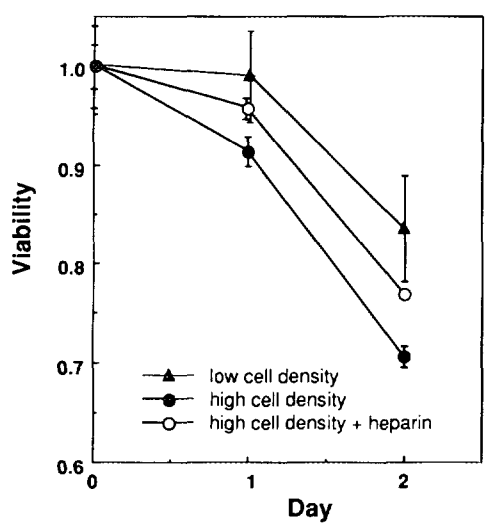

Fig. 4. Effect of cell density and heparin on the viability of the cultured hepatocytes. The viability of cultured hepatocytes was assessed by MTT assay (12,13). The values of viability on the graph are the relative values to each OD at 0 day by MTT assay as 1. Each value represents the mean ± SD of 4 replicates.

As described above, we showed here two novel phenomena in cultured hepatocytes; one is the cell density-dependent DNA fragmentation, the other is the effect of heparin to suppress this DNA fragmentation. We think this DNA fragmentation is most likely to result from the induction of apoptosis, judging from its fragmentation pattern (Fig. 1), its dependency on gene expression, and its correlation with the decrease in viability (Fig. 4). We could little observe the morphological change, which is another characteristic of apoptosis (1-4), as far as we performed preliminary investigation by phase contrast microscopy and fluorescence microscopy using Hoechst 33342 (data not shown). We think, however, this doesn't necessarily deny the possibility of the apoptosis, because recently the variety of apoptosis mechanisms is being suggested (4,14), and the type of apoptosis that show no apparent morphological changes has been also reported in hepatoma (15) and *in vivo* cell turnover (16). Further experiments including more detailed morphological study are now in progress to ascertain whether the phenomena shown here are truely due to a type of apoptosis.

Considering the results in Figs. 1 and 2, it seems certain that cell density itself plays a important role to determine the incidence of the DNA fragmentation in hepatocytes. In general, most of the known stimuli that regulate apoptosis are classified to the soluble factor group, such as growth factor, hormone, or cytokine (3,4). Therefore, if the phenomena shown here are due to apoptosis, the cell density-dependency shown here is considered to belong to a unique class of the stimuli.

The effect of heparin to suppress the DNA fragmentation (Fig. 3) is intriguing, because, as far as we know, there is no other example for this type of activity of heparin (17). Its low working concentration (5 μg/ml) and its high specificity (Fig. 3 *right panel*) allow us to suppose the occurrence of a specific physiological process.

In any case, it is certain that the phenomena we showed, that is, the cell density-dependent DNA fragmentation and its suppression by heparin, are novel and interesting ones. The elucidation of their physiological significance and detailed mechanisms is a future subject to be resolved.

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