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# EFFECTS OF HEPATOCYTE CONDITIONED MEDIUM ON PRIMARY HEPATOCYTE CULTURES

NIKOLAUS BERNHARD BRESGEN AND PETER MARIA ECKL

Institute of Genetics and General Biology, University of Salzburg, Hellbrunnerstraße 34
A-5020 Salzburg, Austria

## Zusammenfassung

Es wurde die Wirkung eines „konditionierten" Mediums (CM) gewonnen 3 Stunden nach Aussaat primärer Hepatocyten - auf primäre Hepatocyten untersucht. Dazu wurden primäre Rattenhepatocyten bis zu 72 Stunden in serumfreiem MEM oder CM, sowohl mit als auch ohne Zusatz von Insulin und EGF kultiviert. Nach einer Inkubation von 3, 12, 24, 36, 48 und 72 Stunden wurden die Zellen fixiert und mit DAPI gefärbt. Verschiedene zeitabhängige Effekte konnten festgestellt werden:
Drei Stunden nach Aussaat war der Prozentsatz nicht ausgebreiteter Zellen bei Kultivierung in CM im Vergleich zur Kultivierung in MEM signifikant erhöht. Die Anzahl angehefteter, toter Zellen nahm bis 24 Stunden nach Aussaat zu und war zu diesem Zeitpunkt in CM-Kulturen signifikant erhöht, die Anzahl angehefteter, lebender Zellen reduziert. 36 Stunden nach Aussaat war die Anzahl lebender Zellen bei Kultivierung in CM signifikant erniedrigt. Nach einer Inkubation von 72 Stunden war die Anzahl lebender Zellen sowohl in CM- als auch in Kontrollkulturen am geringsten, lag jedoch in CM-Kulturen signifikant unterhalb jener der Kontrollkulturen. Der Zusatz von Insulin und EGF führte zu einer Zunahme in der Zahl lebender Zellen und es konnten keine signifikanten Unterschiede zwischen CM- und Kontrollkulturen festgestellt werden. Der deutlichste Effekt des CM war die signifikante Induktion von apoptotischen Zellen 24 und 36 Stunden nach Aussaat.
CM beeinflußt die Hepatocyten scheinbar im Besonderen während der Anpassungsphase an die *in vitro* Umwelt, ein Einfluß, welcher von der Vitalität der Zellen nach der Isolation abhängig zu sein scheint.

**Keywords**: Hepatocyten, Primärkultur, Konditioniertes Medium, Apoptose

**Abstract** 

The effects of a hepatocyte conditioned medium (CM) collected 3 hours after plating was investigated. Primary rat hepatocytes were cultured for 72 hours in serum free MEM-medium or CM, with or without supplementation with insulin and EGF. The cells were fixed after 3, 12, 24, 36, 48 and 72 hours of incubation and stained with DAPI. Several time dependent effects could be observed:
Three hours after plating the percentage of non-spread cells was significantly increased in CM cultures compared to cells cultured in MEM. Up to 24 hours after plating the number of adherent dead cells was increased and significantly higher in CM cultures at this timepoint. The number of adherent viable cells was reduced. 36 hours after plating the number of viable cells was significantly lower when the cells were cultured in CM. After 72 hours of incubation this number was lowest in CM and control cultures, however, significantly lower in CM cultures. Supplementation with insulin and EGF led to an increase of the number of viable cells and no significant differences between CM and control cultures were detectable. The most prominent effect of CM was the induction of apoptotic cells, which became significant 24 and 36 hours after plating.
CM seems to influence hepatocytes especially during the phase of adaptation to the *in vitro* conditions. This influence may depend on the viability of the hepatocytes after isolation.

**Keywords**: hepatocytes, primary culture, conditioned medium, apoptosis

## 1 Introduction

When isolated rat hepatocytes are put into primary culture the amount of plated cells/dish sometimes is too low and cell suspension has to be added. If this is done without a medium exchange (see materials and methods) the cells added exhibit a lower viability compared to an addition of cells after a medium change or when no addition of cells is necessary. After 24 hours in culture the number of dead cells in the medium, as well as the amount of cells which are not well spread appears to be increased. During further cultivation an increased fraction of cells exhibits a less viable morphology. Evidence for this assumption comes especially from the analysis of cell spreading. Usually cell spreading can be observed three hours after plating, and 12 hours later almost all the cells are well spread with the typical polyhedral shape. When incubating primary hepatocytes in conditioned medium an increased number of rounded cells can be observed after 12 hours, indicating a delay in cell spreading.
Therefore, the aim of this study was to analyse the behaviour of parenchymal rat hepatocytes cultured in conditioned medium (CM) collected from primary hepatocyte cultures three hours after plating, and the influence of the CM on primary hepatocytes within 72 hours. The endpoints determined were the numbers

of adherent viable and dead cells and the number of apoptotic cells. The investigation was carried out with serum-free MEM-medium with and without the addition of insulin alone, and a combination of insulin and EGF.
In a first series of experiments (described as protocol A) primary cultures (see materials and methods) were „crude cell suspensions" which means that the cells which died in the course of hepatocyte isolation were not removed. In order to reduce the influence of dead cells a second series of experiments utilizing percoll enriched cell suspensions of viable hepatocytes was carried out.

## 2 Materials and Methods

### 2.1 Materials

Female Fischer 344 rats were obtained from HARLAN CPB, Zeist, The Netherlands and were maintained in a temperature and humidity controlled room with a 12h light - dark cycle. Food was provided *ad libitum*. MEM with Earle's salts and non- essential amino acids was obtained from Grand Island Biological Company through Heilmittelwerke Wien, Vienna, Austria. EGF, collagenase, insulin and other cell culture chemicals were obtained from Sigma Chemical Company through Biotrade, Vienna, Austria. Percoll was obtained from Pharmacia LKB and the *in situ cell death detection kit* from Boehringer Mannheim.

### 2.2 Cell isolation and primary culture

Parenchymal rat hepatocytes were isolated from female Fischer 344 rats by the two step *in-situ* perfusion technique described by MICHALOPOULOS et al. (1). After washing twice in calcium free buffer, a cell suspension was prepared by diluting the cell pellet with MEM containing 1.8 mM $Ca^{2+}$ (1:50). For protocol A the cells were plated on collagen coated petri dishes (60 mm ) at a density of 500.000 cells/dish. For protocol B a further cleaning step was carried out to remove dead cells (from the cell suspension) by centrifugation in a Percoll gradient at 400g for 10 minutes. After washing twice in MEM with 1.8 mM $Ca^{2+}$ the cells were plated on collagen coated glass coverslips in 24-well multi well plates at a density of 50.000 cells/well. After plating, the cells were incubated under standard conditions (37°C, 5% $CO_2$ , 95% rel. humidity) in serum free MEM/1.8 mM $Ca^{2+}$ supplemented with nonessential amino acids, pyruvate (1 mM), aspartate (0.2 mM), serine (0.2 mM), penicillin (100 U), and streptomycin (100µg/ml) or serum free conditioned medium for 3 hours. Thereafter the medium was changed and the plates were incubated for a further 21 hours. 24 hours after plating, the cultures were supplemented with insulin ($10^{-7}$ M) or insulin/ EGF (40 ng/ml) or remained unsupplemented. The medium was changed at this timepoint solely when the cells were cultured following protocol A.

### 2.3 Preparation of Conditioned Medium (CM)

Parenchymal rat hepatocytes were isolated and cultured as described above. Three hours after plating the medium was collected and centrifuged (1500 rpm / 10 minutes) to remove dead cells and cell debris. The supernatant (CM) was stored at 4°C until further use. For protocol B CM was collected from cultures of percoll enriched viable hepatocytes plated on 60 mm petri-dishes at a density of 500.000 cells/plate, whereas CM in protocol A was collected from cultures where no percoll cleaning step was carried out.

### 2.4 Fixation

Cells cultured in plates (protocol A) were fixed in methanol/glacial acidic acid (3:1) for 15 minutes, followed by a washing step with distilled water and air dried. To fix the cells grown on coverslips these were removed from the plates and transfered into wells of another plate containing PBS (calcium free) for 1 minute. After fixation in methanol/glacial acidic acid (3:1) for 15 minutes, the cells were rinsed with distilled water and air dried.

### 2.5 Staining and microscopical analysis

The fixed cells were briefly rinsed with Mc Ilvaine buffer (0.2M $Na_2HPO_4$ / 0.1 M citric acid buffer, pH=7) and the cells stained with DAPI in Mc Ilvaine (0.2µg DAPI/ml) for 40 minutes in the dark. After washing twice with distilled water, the cells were air dried. For microscopical analysis the stained cells were mounted in glycerol and analysed under the fluorescence microscope (REICHERT UNIVAR) using epifluorescence combined with phase contrast. 4000 cells were analysed for each timepoint and treatment group per experiment.

### 2.6 TUNEL Assay

Cells grown on coverslips were transferred to PBS as described above and fixed in 4% paraformaldehyde (in PBS, pH=7.4) for 30 minutes at room temperature. The staining of apoptotic cells was carried out according to the protocol for adherent cells of the *In Situ Cell Death Detection Kit* from BOEHRINGER MANNHEIM.

## 3 Results

Microscopical analysis allowed the quantification of three different morphological criteria of adherent cells. Viable cells with nuclei of normal size and shape, dead

cells with small pycnotic nuclei and highly condensed chromatin and cells in which the nuclei are disintegrated into „bubble-like" chromatin fragments. The latter group proved to represent apoptotic cells as demonstrated by positive staining in the TUNEL assay (see materials and methods).
The results of cultures following protocol A indicate a significant influence by dead cells, which have not been removed before plating, mainly reflected by increased variances, thus reducing the degree of significance of the observed effects. Additionally, CM used in the first series of experiments was collected from different cultures for each experiment, leading to large differences between single experiments. In order to address these effects more clearly the results presented here are those obtained from two experiments following protocol B (removal of dead cells prior to plating). In these experiments CM was collected from one primary culture. The main effects, however, are comparable between the two experimental series.
One variable which may be responsible for some differences between the results of the two experiments of series 2 (protocol B) is the viability of hepatocytes after isolation. The viability after perfusion was 92% in experiment 1 and 80 % in experiment 2. After the Percoll cleaning step, the viability was raised to 95% in both experiments.

### 3.1 „Fate" of the viable cells

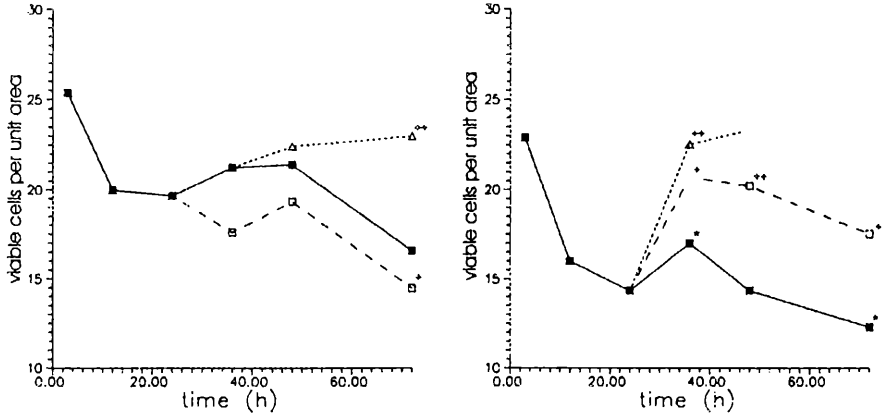


Fig. 1: Time course of adherent viable cells per unit area
■ without Insulin / EGF, □ with insulin, Δ with Insulin / EGF
Data points represent the mean values of two experiments
Significant compared to the control (* $p<0.05$)
Significant compared to cultures without insulin and EGF (+ $p<0.05$; ++ $p<0.005$)

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### 3.1 „Fate" of the viable cells

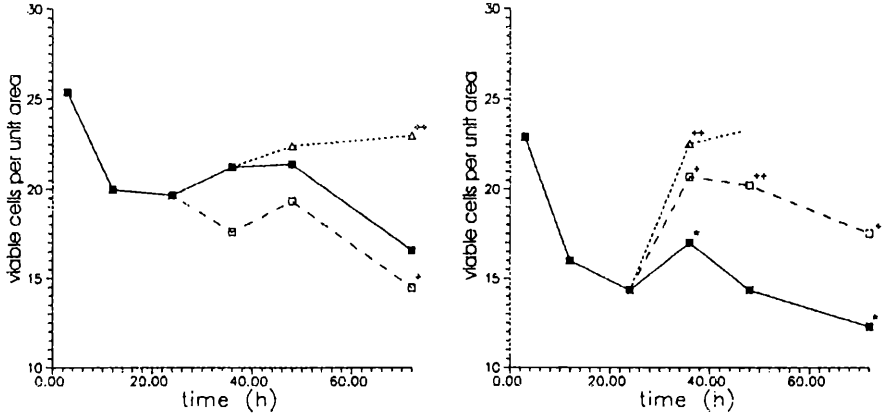


Fig. 1: Time course of adherent viable cells per unit area

■ without Insulin / EGF, □ with insulin, Δ with Insulin / EGF

Data points represent the mean values of two experiments

Significant compared to the control (* $p<0.05$)

Significant compared to cultures without insulin and EGF (+ $p<0.05$; ++ $p<0.005$)

The time course of viable cells per unit area is shown in Fig.1. In cultures without insulin and EGF the cell number is rapidly decreasing within the first 24 hours of culture, followed by an increase during the next 12 hours. Compared to the control cultures (cells cultured in MEM), the number of viable cells in CM cultures is significantly ($p \leq 0.05$) reduced 36 hours after plating. 72 hours after plating the number of attached viable cells was lowest, however, significantly ($p \leq 0.05$) lower in CM cultures.

Following the addition of insulin and EGF, the number of cells is increasing in control cultures as well as in CM cultures (where this increase is more pronounced). 72 hours after plating the number of viable cells is significantly higher ($p \leq 0.005$) when insulin and EGF was added to the cultures. The addition of insulin alone, 24 hours after plating, leads to an increasing cell number only in CM cultures. On the contrary, the number of cells is decreased in control cultures after addition of insulin and 48 hours after supplementation the cell number is even significantly lower ($p \leq 0.05$) compared to control cultures without supplementation.

Regardless of these differences, there is no significant difference in the number of viable cells between CM cultures and the control at any time point after supplementation with insulin or insulin and EGF.

The number of adherent viable cells/mm$^2$ at four different timepoints is listed in Table I for each experiment. In contrast to the mean values (Fig.1) the number of viable cells in CM cultures compared to the controls is significantly lower 24 hours (experiment 1; $p \leq 0.005$) and 12 hours (experiment 2; $p \leq 0.05$) after plating. Corresponding to the mean values, a significantly lower ($p \leq 0.05$) cell number is observed in CM cultures 72 hours after plating. Consistent with the mean values, the number of viable cells/mm$^2$ is significantly increased at this timepoint after insulin supplementation in CM cultures of experiment 2 ($p \leq 0.005$), but not in experiment 1.

Table I: Number of adherent viable cells/mm$^2$ 3, 12, 24 and 72 hours after plating

| | MEM | | CM | | MEM | CM |
|---|---|---|---|---|---|---|
| time (h) | 1 | 2 | 1 | 2 | Serie 1 | Serie 1 |
| | | | | | | |
| 3 | 92.13 ± 9.87 | 87.33 ± 11.94 | 93.28 ± 11.77 | 68.61 ± 16.02 | 188.5 ± 62.3 | 170.5 ± 23.9 |
| 12 | 80.73 ± 10.25 | 60.56 ± 5.56 | 66.62 ± 12.19 | 41.72 ± 14.54 * | 174.1 ± 42.5 | 177.0 ± 49.7 |
| 24 | 81.32 ± 6.06 | 57.69 ± 5.92 | 59.58 ± 7.28** | 60.70 ± 3.99 | 91.4 ± 34.2 | 126.2 ± 30.7 |
| 72 | 60.46 ± 7.71 | 56.77 ± 4.50 | 43.18 ± 4.72 * | 43.63 ± 4.48 * | n.d. | n.d. |
| | | | | | | |
| 72/I | 49.72 ± 1.96 | 52.76 ± 3.18 | 49.06 ± 7.02 | 74.73 ± 5.50 ** | n.d. | n.d. |
| 72/I,EGF | 89.37 ± 6.32 | 73.17 ± 13.61 | 78.40 ± 8.33 | 80.80 ± 4.94 | n.d. | n.d. |

I + Insulin; I,EGF + Insulin and EGF n.d. not determined

* Significant under the assumption of equal variances ( * P≤ 0.05; ** P≤ 0.005 )

### 3.2 Cell adhesion and cell spreading

During the first 24 hours it is possible to differentiate between rounded and spread viable cells. At later timepoints, the number of rounded viable cells is very low (< 0.2 cells/ unit area). By calculating the percentage of the number of rounded viable cells a measure for cell spreading can be obtained. A statistically significant difference between control- and CM cultures of this percentage was obtained 3 hours after incubation for the mean values ( 5.45 ± 2.06 for control cultures and 7.97 ± 2.62 for CM cultures; $p \leq 0.05$), but not in the single experiments (Table II). After 12 hours, the percentage of rounded viable cells is significantly increased in CM cultures of experiment 2 ($p \leq 0.005$).

Table II: Percentage of rounded viable cells 3 and 12 hours after plating

| | MEM | | CM | | MEM | CM |
|---|---|---|---|---|---|---|
| time (h) | 1 | 2 | 1 | 2 | Serie 1 | Serie1 |
| | | | | | | |
| 3 | 5.19 ± 3.09 | 5.69 ± 0.34 | 7.83 ± 3.97 | 8.06 ± 0.53 | 28.92 ± 10.31 | 26.14 ± 12.81 |
| 12 | 0.62 ± 0.32 | 0.06 ± 0.12 | 1.01 ± 0.92 | 1.42 ± 1.78 ** | 5.73 ± 3.24 | 9.84 ± 7.3 |
| | | | | | | |
| Δ% | 4.57 | 5.63 | 6.82 | 6.64 | 23.17 | 16.3 |

* Significant under the assumption of equal variances ( * $P \leq 0.05$; ** $P \leq 0.005$ )

Table II allows a comparison of cell spreading between experimental series 1 and 2. After 3 and 12 hours of incubation the percentage of non spread viable cells is remarkably higher in series 1 than in series 2. The percentage in series 1 after 12 hours is comparable to that of series 2 after 3 hours. However, the reduction in the percentage of rounded viable cells between 3 and 12 hours is much higher in the first experimental series.

### 3.3 Dead cells

Fig. 2 shows the time course of the number of adherent dead cells. In CM cultures a significantly higher ($p \leq 0.005$) number of adherent dead cells is obtained after 24 hours of incubation, whereas in control cultures a comparable level is obtained 12 hours later. With longer times in culture this number of adherent dead cells plateaus. In control cultures the number of dead cells is slightly decreasing after 48 hours of incubation, but stays high in CM cultures, although there is no significant difference between CM and control cultures after 72 hours. The addition of insulin did not change this effect. The same effect was observed when the medium was supplemented with insulin and EGF

The time course of viable cells per unit area is shown in Fig.1. In cultures without insulin and EGF the cell number is rapidly decreasing within the first 24 hours of culture, followed by an increase during the next 12 hours. Compared to the control cultures (cells cultured in MEM), the number of viable cells in CM cultures is significantly ($p \leq 0.05$) reduced 36 hours after plating. 72 hours after plating the number of attached viable cells was lowest, however, significantly ($p \leq 0.05$) lower in CM cultures.

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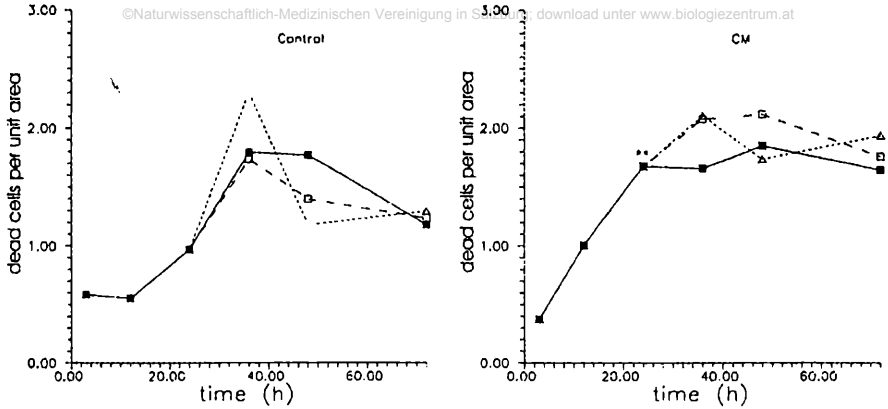


Fig. 2: Time course of adherent dead cells per unit area
■ without Insulin / EGF, □ with insulin, Δ with Insulin / EGF
Data points represent the mean values of two experiments
Significant compared to the control (** $p<0.05$)

Table III gives the numbers of adherent dead cells as percentage of all adherent cells. In contrast to the significantly ($p\leq0.005$) increased mean value for adherent dead cells per unit area 24 hours after plating, the percentage of adherent dead cells for both single experiments is significantly higher in CM cultures after 12 hours ($p\leq0.05$) of incubation. Without addition of insulin or EGF the percentage of adherent dead cells in CM cultures is significantly increased after 48 hours in experiment 1 ($p\leq0.05$) and after 72 hours in experiment 2 ($p\leq0.05$). No significant differences in the percentages could be observed after addition of insulin and EGF.

Table III: Percentage of adherent dead cells 3, 12, 24, 48 and 72 hours after plating

| | MEM | | CM | | MEM | CM |
|---|---|---|---|---|---|---|
| time (h) | 1 | 2 | 1 | 2 | Serie 1 | Serie 1 |
| | | | | | | |
| 3 | 2.81 ± 0.93 | 1.64 ± 0.64 | 2.08 ± 0.82 | 1.03 ± 0.31 | 7.49 ± 2.46 | 6.91 ± 1.76 |
| 12 | 3.63 ± 0.44 | 1.39 ± 0.65 | 6.06 ± 1.92 * | 5.18 ± 1.05 ** | 8.52 ± 1.33 | 10.31 ± 3.46 |
| 24 | 4.17 ± 1.11 | 5.39 ±1.98 | 8.76 ± 3.28 | 13.56 ± 6.61 | 8.34 ± 5.18 | 13.30 ± 9.57 |
| 48 | 6.56 ± 2.49 | 8.84 ± 4.76 | 11.7 ± 1.41 * | 10.63 ± 2.43 | 3.02 ± 1.59 | 4.43 ± 2.56 |
| 72 | 5.51 ± 2.27 | 7.80 ± 1.59 | 4.09 ± 1.19 | 17.15 ± 6.39 * | n.d. | n.d. |
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| 72/I | 7.21 ± 2.02 | 8.27 ± 2.75 | 5.46 ± 1.60 | 11.21 ± 0.41 | n.d. | n.d. |

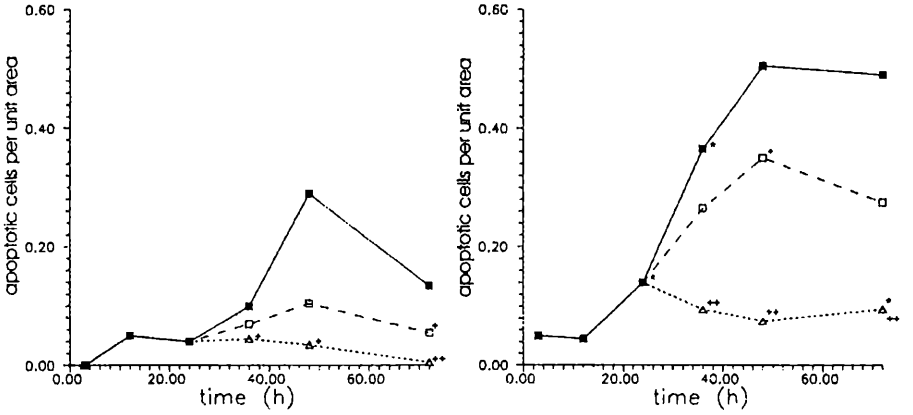
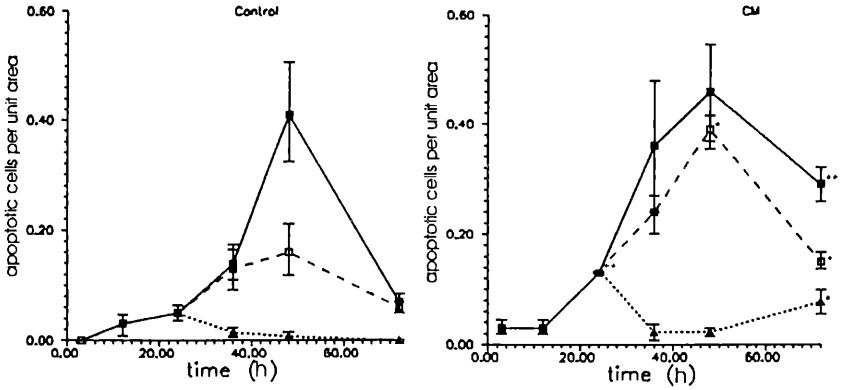


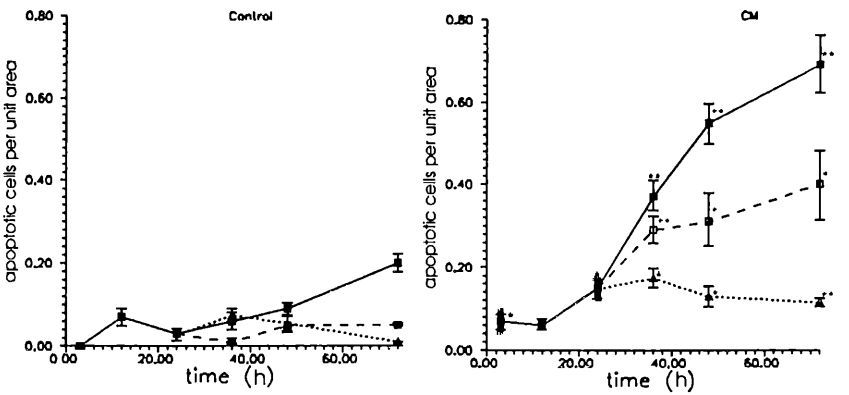

Fig. 3: Time course of apototic cells per unit area
■ without insulin / EGF, □ with insulin, Δ with Insulin / EGF
Data points represent the mean values of two experiments
Significant compared to the control (* $p<0.05$)
Significant compared to cultures without insulin and EGF (+ $p<0.05$; ++ $p<0.005$)

The most prominent effect obtained with cultivation in CM was an increase in the number of apoptotic cells. As demonstrated in Fig. 3 there is a remarkable increase in the number of apoptotic cells, which starts 12 hours after plating and is significant after 24 and 36 hours ($p \leq 0.05$). The number of cells with apoptotic nuclei is further increasing up to 48 hours, followed by a plateau. After the addition of insulin a less pronounced increase of apoptotic cells per unit area in CM cultures was observed. 48 hours after the addition of insulin and EGF the number of apoptotic cells is significantly increased ($p \leq 0.05$) in CM cultures compared to the control cultures.
As demonstrated in Fig. 4 the results of the two experiments are very different at later time points (≥ 24 hours after plating). After 24 hours of incubation a significant increase ($p \leq 0.05$) of the number of apoptotic cells can be observed in CM cultures of experiment 2 at any timepoint and treatment, while the values for the control cultures stayed low. In contrast, the values for non supplemented control cultures are increased 36 and 48 hours after plating in experiment 1. Furthermore, CM cultures without supplements or supplemented with insulin alone led to a decrease of the number of apoptotic cells after 48 hours. After 72 hours a significant increase ($p \leq 0.05$) of apototic cells was induced in every treatment group. The curves for non supplemented and insulin supplemented CM cultures gave a

similar shape, but differ between the two experiments. In both experiments the values for non supplemented cultures are highest. Supplementation of CM cultures with insulin and EGF resulted in a decrease of apototic cells up to 48 hours followed by a signifcant increase ($p \leq 0.05$) at 72 hours in experiment 1, whereas the significant number of apoptotic cells remains constant after treatment with insulin and EGF in experiment 2, the level being significantly higher ($p \leq 0.005$) compared to the control at any time point.




(A) Time course of apoptotic cells per unit area in experiment 1




(B) Time course of apoptotic cells per unit area in experiment 2

Fig. 4: Time course of apoptotic cells per unit area
■ without Insulin / EGF , □ with Insulin , △ with Insulin / EGF
Data points represent the mean values of the single experiments
Significant compared to the control ( * $p<0.05$;** $p<0.005$)

In the first series of experiments, the number of apoptotic cells is significantly increased in CM cultures at 24 and 36 ($p \leq 0.05$) hours.

## 4 Discussion

To discuss the effects of the CM in primary hepatocyte cultures it appears advantageous to divide the time course of cultivation into three successive phases. After plating the cells attach to the collagen matrix and start to spread. With respect to the percentage of rounded viable cells, nearly 95% of the cells are well spread after 12 hours of incubation marking the end of phase I. The first detectable effect of CM occurs at this phase, as the percentage of non spread cells is higher in CM cultures after 3 hours of incubation.

The phase which follows could be described as a period of adaptation to the minimal environmental conditions in serum free culture. This phase seems to be of special interest, as the most prominent effects of CM take place during this phase. The adaptation process might be mainly influenced by the ability to recover from the stress of cell isolation during phase I. If this is not possible up to a sufficient degree or in a sufficient period of time, the ability to adapt could be reduced. In the second experiment of series 2, the viability after perfusion was approximately 80% and in control cultures a significantly reduced number of viable cells per unit area at 12 and 24 hours of incubation could be observed compared to the control cultures of experiment 1 (data not shown), where the percentage of viable cells after perfusion was over 90%. Increasing stress caused by cell isolation may lead to an increase in the amount of sublethally damaged cells which are not stained with Trypan Blue, thus considered to be viable cells. A portion of these cells will attach during the first three hours of incubation, but detach within the next 12 - 24 hours due to insufficient repair of the damage. Besides these cells, there might be a fraction of stressed cells, which stay adhered, but cannot adapt to the in-vitro situation as readily as less stressed cells. Reduced numbers of viable cells after 12 and 24 hours as demonstrated in experiment 2 may reflect this situation.

The phase following adaptation is characterized by a time depending increase in degenerative events most obviously represented by a further decrease of the number of adherent viable cells combined with a continously increased number of necrotic and apoptotic cells. As can be seen in Figures 1 - 3, phase III starts in the time interval of 24 and 36 hours. At 72 hours the low values for viable cells in combination with increased numbers of adherent dead and apoptotic cells are characteristic for the degenerating culture. If proliferation stimulating factors like EGF are added during phase II (24 hours after plating) degeneration in phase III is reduced and dedifferentiation pathways may be induced.

The results indicate, that CM leads to an enhanced induction of necrotic and apoptotic events during phase II in a viability dependent manner resulting in more pronounced degenerative events during phase III. For the mean values of both

experiments shown in Figures 1-3 this enhancement is significant 24 hours after plating, however, these values represent the CM effects independent of the different viabilities after isolation. In the case of apoptosis induction the viability dependence becomes most obvious (Figure 4). At a lower viability, a steady significant increase above the control level till the end of incubation is induced by CM. If the viability is high apoptotic events peak after 48 hours of incubation in control cultures as well as in CM cultures followed by a decrease. After 72 hours the number of apoptotic cells is again significantly higher in CM cultures. The different effects seem to be related to cell density in phase II, which is higher in cultures with an increased viability. In control cultures the same factors as present in CM must be secreted during the first three hours of incubation. Under the assumption, that the secretion does not cease before the first medium change, factors which enhance induction of apoptotic events are further produced and the medium of the control cultures becomes conditioned too. With respect to increased numbers of viable cells and the interrelating effects of a higher viability, such an „internal conditioning" could explain an increase in the rate of apoptoses in control cultures, being pronounced further by cultivation in CM as a result of higher concentrations of the same factors. Additional evidence for this assumption comes from the observation, that we could never see such a peak in control cultures in the experiments of series I, where a medium change was carried out 24 hours after plating.

The effects caused by the CM factors are slightly reduced when insulin is added, but remain noticeable, especially in the case of apoptotic cells. When insulin and EGF are added, the effects change more clearly. As proliferation is induced, the number of viable cells is increasing during phase III. Although the number of apoptotic cells in CM cultures is increased above the control level, this increase is much less pronounced. An interaction between proliferation stimulating factors and „degeneration promoting factors" of the CM thus cannot be excluded. At the moment it is unknown if for example factors like TGF-β or TNF, or some yet unknown factors are secreted by parenchymal hepatocytes during phase I, mediating the effects of CM in phase II and III.

In the experiments presented here, no direct interactions between those cells which were added after three hours and the still adherent cells have been investigated. To address this question, as well as the biochemical identification of the CM components will be part of future work.

## 5 Literature

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