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Precision Cut Tissue Slices of the Liver as Morphological Tool for Investigation of Apoptosis

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Abstract. *Background: Apoptosis, programmed cell death, is involved in a broad range of pathological processes. Dysregulation of apoptosis plays a key role in the pathogenesis of hepatitis, toxic liver disease and also liver tumor development. For the study of apoptosis in liver diseases, different in vivo models and different in vitro approaches have been developed. They include cell culture models based on hepatocellular carcinoma cell lines or isolated primary hepatocytes. Materials and Methods: We have established precision cut tissue slices (PCTS) of the liver as a morphological tool for the study of apoptosis. From porcine livers, PCTS were prepared and incubated in a static system with different types and amounts of media. Viability, morphology, spontaneous apoptosis and proliferation were investigated. Apoptosis was induced with actinomycin D and tumor necrosis factor (TNF) alpha. Results: Morphology and viability was well preserved for at least 24 h. After 48 h, deterioration with single and group cell autolysis was seen. There was a low rate of spontaneous apoptosis and proliferation. Using a combination of TNF alpha and actinomycin D, a significant amount of apoptosis occurred. Conclusion: PCTS can be used to directly analyse apoptosis at the tissue level in a qualitative and quantitative manner.*

Inappropriate induction of apoptosis is involved in a broad range of pathological processes. In the liver, it is part of the pathogenesis of different diseases such as viral hepatitis, drug-induced injury, alcoholic liver disease, hepatocellular carcinoma (HCC) and biliary diseases (28, 31). For the study of apoptosis in the liver, different *in vivo* models such as knock-out mice (10) and rat models (14) as well as *in vitro* approaches, including HCC cell lines and isolated primary hepatocytes, have been developed (17, 32).

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Current *in vivo* models, for example in pigs, rats, rabbits and mice, are difficult to standardize and do not represent all human pathomechanisms, especially with regard to immunologically-induced apoptosis. In addition, modern ethical requests demand reduction of the use of animals for scientific purposes. In isolated liver *in vitro* models, the preservation of the three-dimensional organ structure, the analysis of hemodynamic parameters and the possibility of bile collection are the major advantages (12). The complexity and the cost of the set-up, the short-term viability and the high number of animals used are drawbacks of these models, that have prevented their more widespread use (11).

In contrast, cell lines are well characterized, easy to handle and are also available of human origin. With regard to apoptosis, however, the interpretation of the results requires special attention. These cells are *per se* immortalized, most often due to their origin as tumor cells. Compared with the *in vivo* situation, they may react to apoptotic stimuli in a different way (7). When isolated primary liver cells are used, the preparation requires the use of proteolytic enzymes that extensively modify their reaction to apoptotic stimuli (32). It is highly likely that very fragile cells, especially cells sensitive to apoptosis, will be destroyed by these procedures. The cell-to-cell and cell-to-matrix interaction is destroyed. The lobular architecture and, thus, heterogeneous composition is lost. Other cell types apart from parenchymal cells are often washed away. In addition, dedifferentiation may occur (18-20).

Precision cut tissue slices (PCTS) have been used in pharmacological and toxicological research for metabolic studies (1, 2, 21, 22, 26). Although liver slices were first prepared by Otto Warburg in 1923, only recent developments of new tissue slicer models have overcome previous methodical problems such as tissue trauma and poor oxygen and nutrients supply. In contrast to other *in vitro* models, the preparation of PCTS does not require the use of proteolytic enzymes. Thus, cell-to-cell and cell-to-matrix interactions are preserved. Damage of cells sensitive to apoptotic stimuli can be prevented. PCTS retain the original architecture, the cell-to-cell and the cell-to-matrix

interaction. Compared to *in vivo* models, PCTS allow the direct and rapid analysis of tissue under standardized and life-like conditions in a reproducible manner.

We have adapted the method of PCTSs of the liver for the study of apoptosis. Our data demonstrate that PCTSs of the liver allow the maintenance of liver tissues under life-like conditions without significant deterioration for up to 24 h. Induction and execution of apoptosis was studied during this time span. Thus, PCTSs allow novel approaches to the study of apoptosis.

Materials and Methods

Liver tissues. In total, 4 livers of white German landrace pigs had been taken from an abattoir with approval of the veterinarian institution. These animals were electrically stunned according to the slaughtering protocol. The livers were removed, placed on ice and transferred to the laboratory within 20 min.

Preparation of PCTS. Cylindrical tissue cores (0.8 cm in diameter) were cut out of the liver with a sharpened stainless steel tube (Vitron, Tucson, AZ, USA) by slowly turning and advancing the tube into the liver. The cores were then loaded in the Brendel/Vitron tissue slicer (Vitron) filled with ice-cold oxygenated (95% O_2 and 5% CO_2) physiological Ringer's solution and slices were cut (250-300 μm thick). Afterwards, slices were taken from the collecting chamber and transferred to the incubation system.

Incubation. The slices were incubated in a static incubation system using 24-well plastic tissue culture plates (VWR, Darmstadt, Germany) on a rocker platform (Heidolph, Schwabach, Germany) oscillating at approx. 100 rpm in a cell culture incubator at 37˚C with saturated humidity and 5% CO_2. Incubation was tested with the following different media: (i) RPMI medium supplemented with serum [40 ml of RPMI medium with 0.5 ml penicillin/streptomycin, 0.48 ml l-glutamine, 2.4 ml horse serum, 8 ml fetal calf serum and 0.24 ml ITS supplement (SIGMA, Taufkirchen, Germany)], (ii) supplemented William's E medium without serum, and (iii) supplemented Dulbecco's modified Eagle's medium (DMEM) without serum [50 ml of DMEM or William's E medium with 0.6 ml l-glutamine, 0.5 ml penicillin/streptomycin and 0.34 ml ITS supplement; media all from Biochrom, Berlin, Germany]. Three different volumes of the three media (0.5 ml, 1 ml, 1.5 ml) were tested. The medium was changed after 30 min and again after 24 h. After every hour, tissue slices were removed and fixed in 10% neutral buffered formalin and also in 2.5% glutaraldehyde for histological and ultrastructural investigations. Aliquots of the culture medium were assayed for lactate dehydrogenase (LDH) and alanine aminotransferase (ALAT) by standard procedures. All experiments were done in duplicate.

Morphological assessment. The formalin-fixed PCTS were embedded in paraffin and histological slides were produced according to standard procedures. For morphological examination, one slide of each PCTS was stained with H&E.

For detection of apoptosis, one slide of each PCTS was stained immunohistologically with an antibody for cytokeratin 8 cleavage products (M30Cytodeath, Boehringer/Ingelheim, Ingelheim, Germany). Antigen retrieval was achieved by microwave treatment with 650 W in 0.01 M citrate buffer, pH 6.0 three times for 5 min. After blocking of the endogenous peroxidase (30% H_2O_2; 30 min at room temperature [RT]) and blocking of endogenous biotin (Avidin/Biotin blocking kit; Vector, Burlingame, CA, USA), the slides were incubated with the primary antibody at a dilution of 1:50 overnight. A rabbit anti-mouse antibody (DAKO, Glostrup, Denmark) was used as secondary antibody (30 min, RT). Visualization was performed with the avidin/biotin complex with horseradish peroxidase (Vector) using DAB (Vector) as substrate.

For assessment of proliferation, the detection of Ki-67 antigen was used (MIB-5, DAKO). Antigen retrieval was achieved by microwave treatment two times for 7 min with 650 W in 0.01 M citrate buffer, pH 6.0. After blocking of endogenous peroxidase and endogenous biotin, the slides were incubated with the primary antibody at a dilution of 1:50 overnight. A goat anti-rabbit antibody (DAKO) was used as secondary antibody (30 min, RT). Visualization was done as described above.

Ultrastructural analysis was performed by transmission electron microscopy (CEM 902 Zeiss, Oberkochern, Germany) after fixation of small particles of the PCTS in 2.5% glutaraldehyde at 4˚C and embedding in araldite.

For evaluation of the tissue perfusion tissue stain 10 μl/ml medium (WAK Chemie, Steinfurt, Germany) was added. After every 10 min, slices were removed and snap-frozen. Frozen sections were made and stained for H&E.

Cell viability assay. At specified time points, 3-(4,5-dimethyldiazol-2-yl)-2,5-dipenyltetrazolium bromide (MTT; SIGMA) was added to the incubation media and incubated for 1 h, as described by Mossman *et al.* (23). Acid-isopropanol was added to the slices to dissolve the reduced MTT. Absorbence was determined using a U200 spectrophotometer (Hitachi, Tokyo, Japan) at 570 nm.

Stimulation of apoptosis. Apoptosis was induced using recombinant human tumor necrosis factor (TNF) alpha (TEBU, Offenbach, Germany) and actinomycin D (Lyovac; Merck, Harleem, The Netherlands). The following concentrations were added to the slices in 1 ml of DMEM medium: (i) TNF alpha 10 ng/ml, (ii) TNF alpha 1 ng/ml + 1 μg/ml actinomycin D, (iii) TNF alpha 10 ng/ml + 1 μg/ml actinomycin D, (iv) TNF alpha 10 ng/ml + 10 μg/ml actinomycin D, and (v) actinomycin D 10 μg/ml.

The slices were cultured up to 24 h. At specified time points the slices were removed and fixed in formalin. These experiments were performed in triplicate.

Statistics. Statistical analysis was performed with SPSS (SPSS Software, Chicago, IL, USA). Comparisons were made by the Chi-square test and Student's *t*-test. A p-value <0.05 was considered statistically significant.

Results

Evaluation of PCTS-performance

Histology: Light microscopy revealed preserved histology of the liver slices for more than 24 h. The hepatocytes and the biliary epithelial cells appeared normal. Kupffer cells seemed slightly swollen. Slices cultured for 48 h showed deterioration with

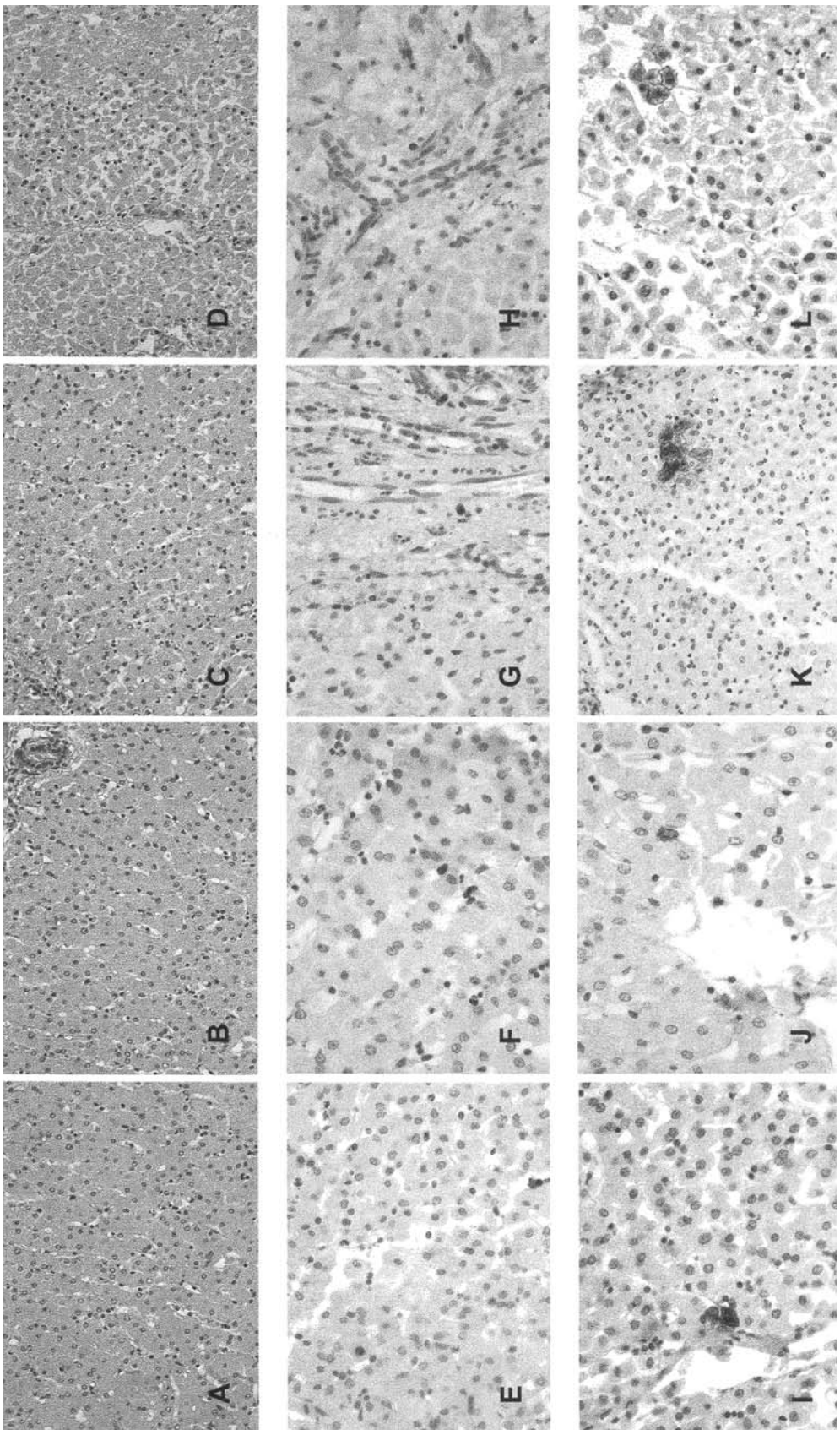

Figure 1. *Time course of morphological changes in PCTS. A-D: Histology of PCTS. A: 6 h, B: 12 h, C: 24 h, D: 48 h (200 x, H&E staining). Preserved architecture and normal cytological features could be seen in PCTS cultured up to 24 h. After 48 h, deterioration with incipient single and group cell necrosis and apoptotic bodies was seen. E-H: Spontaneous proliferation in PCTS. E: 6 h, F: 12 h, G: 24 h, H: 48 h (Immunohistology, Ki-67, hemalaun, 200x): At an early stage, very low proliferation rate of hepatocytes and sinusoidal cells could be seen. Later, portal mesenchymal cells and biliary epithelial cells showed increased proliferation. I-L: Spontaneous apoptosis in PCTS. I: 6h, J: 12h, K: 24h, L: 48h (Immunohistology, M30Cytodeath, hemalaun, 200x): Up to 24 h, there was only a low apoptotic rate of hepatocytes in the tissue slices. The rate increased at 48 h.*

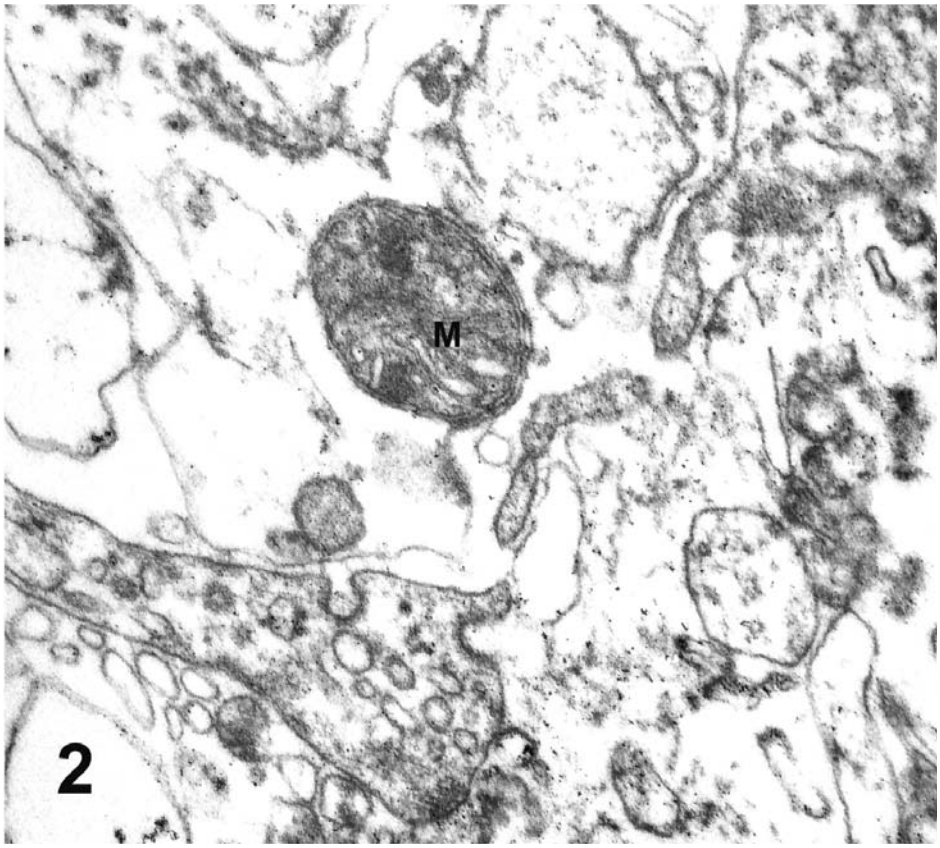

Figure 2. *Ultrastructural analysis of PCTS. Preservation of hepatocellular organelles in slices cultured for 24h (M=mitochondrion).*

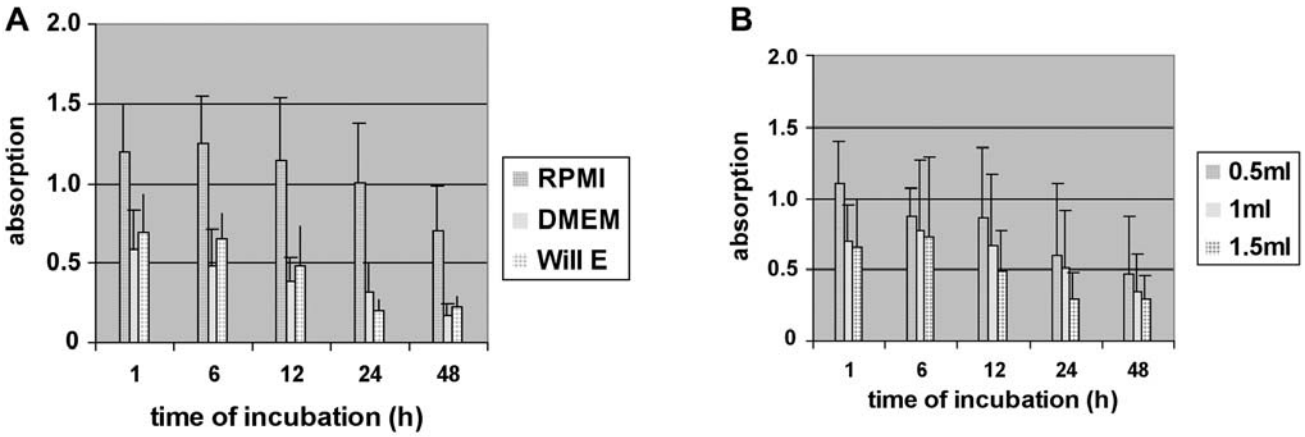

Figure 3. *Cell viability assay of PCTS (MTT-test). Preserved viability of the slices over the time of incubation is seen with a reduction after 24 h. Slices in 0.5 ml of medium and slices in RPMI with serum showed a better preservation of the reduction capability compared with the others. A: Cell viability according to the used medium. B: Cell viability according to the amount of medium.*

Table I. *Results of the viability test using different media and different amount of media.*

	1	6	12	24	48
RPMI	1.2 (±0.31)	1.25 (±0.31)	2.2 (±0.4)	0.92 (±0.37)	0.71 (±0.27)
DMEM	0.59 (±0.24)	0.48 (±0.24)	0.69 (±0.14)	0.29 (±0.18)	0.175 (±0.07)
Williams E	0.69 (±0.24)	0.445 (±0.16)	0.48 (±0.25)	0.2 (±0.07)	0.225 (±0.06)
0.5ml	0.56(±0.3)	0.435 (±0.25)	0.44 (±0.5)	0.3 (±0.5)	0.235 (±0.4)
1ml	0.7(±0.25)	1.32 (±0.49)	0.67 (±0.5)	0.48 (±0.41)	0.345 (±0.27)
1.5ml	0.9 (±0.3)	1.17 (±0.56)	0.73 (±0.49)	0.45 (±0.18)	0.443 (±0.17)

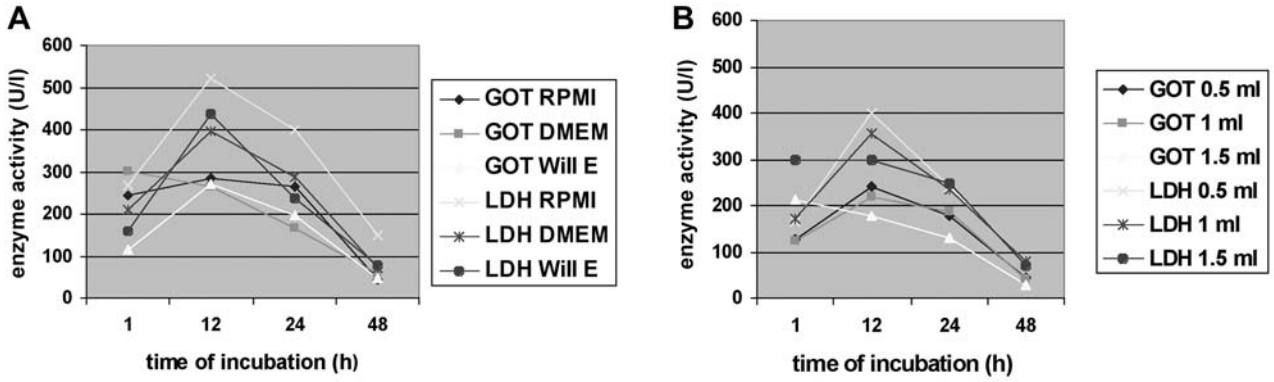

Figure 4. *Enzyme concentrations in the supernatant of the PCTS. A time-dependent leakage of LDH and ALAT in the supernatant indicating progressive cellular damage was seen during the time course of the incubation. No differences in the extent of the leakage were evident between the different types and volumes of media. A: Enzyme concentration according to the type of medium. B: Enzyme concentration according to the volume of medium.*

incipient single and group necrosis and apoptotic bodies (Figure 1). No differences were evident between the three types of media and the three volumes of media used in this study.

Ultrastructure: Using electron microscopy, preservation of cell organelles could be demonstrated in slices cultured for 24 h (Figure 2). Again, no differences were evident between the three types of media and the three volumes of media used in this study.

Perfusion: After adding the tissue stain to the medium, within 10 min the stain was seen within the portal vein branches of the slices. After 30 min, the stain was unequally distributed within the sinuses of the liver slices.

Cell viability: MTT assays showed a preserved viability of the slices over the time of incubation with a reduction up to 24 h (Figure 3). Slices in 0.5 ml of medium showed a better preservation of the reduction capability. Slices in RPMI medium with serum had a slightly higher absorbency level, thus having higher reduction capacity of MTT. This difference was not significant ($p=0.323$). The results are shown in Table I.

LDH and ALAT leakage: A time-dependent leakage of LDH and ALAT, indicating progressive cellular damage, was seen during the time course of the incubation. No differences in the extent of the leakage were evident between the three types of media and the three volumes of media ($p=0.199$) (Figure 4).

Rate of spontaneous apoptosis: Using M30 Cytodeath immunohistology, spontaneous apoptosis could be detected in the liver slices (Figure 1). The apoptotic count of hepatocytes showed a first peak at the beginning of the culture (after 1 h: 1.1±1.8 / HPF), a decrease afterwards (after 6 h: 0.71±0.8 / HPF; after 12 h: 0.71±0.8 / HPF) and an increase at the end of the culture time (24 h: 1.02±1.2 / HPF and 48 h: 1.52±2.2 / HPF). Apoptotic cells were diffusely distributed over the parenchyma (Figure 5). Biliary epithelium showed only slight spontaneous apoptosis at the end of the incubation period. The differences between the different types and volumes of medium were not significant.

Rate of spontaneous proliferation: There was only a low rate of spontaneous proliferation in the PCTS as demonstrated by Ki-67 immunohistology (Figure 1). Hepatocytes showed only at the beginning of the incubation a very low

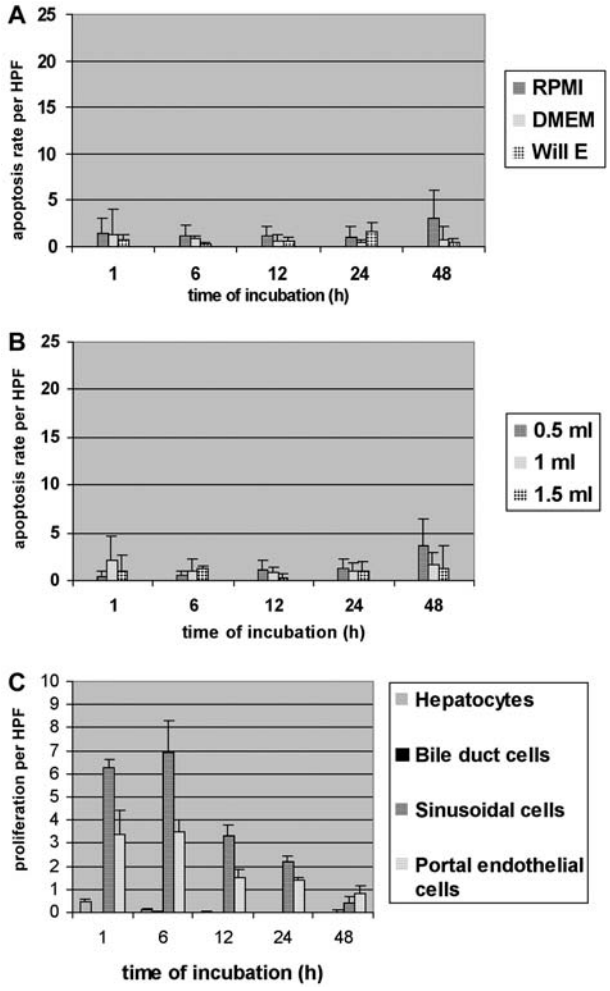

Figure 5. *Rate of spontaneous apoptosis and proliferation. A: Spontaneous apoptosis according to the type of medium. B: Spontaneous apoptosis according to the used volume of medium. C: Rate of spontaneous proliferation.*

proliferation rate and biliary epithelia after 24 h. Non-parenchymal cells, in particular the Kupffer cells, showed the highest proliferation rate (Figure 5). No differences were evident between the three types of media and the three volumes of media.

Stimulation of apoptosis. Treatment of PCTS with TNF alpha and actinomycin D led to an increase in apoptotic cells (Table II). The apoptotic cells were mainly seen in zone one of the liver lobule (Figure 6). Combination of both substances led to a higher percentage of apoptotic cells than using one drug alone. After 12 h, a significant increase in the apoptosis rate was seen using TNF alpha with actinomycin D: $p=0.08$ for TNF alpha 1 ng + actinomycin D 1 μg: $p=0.02$ for TNF alpha 10 ng and actinomycin D 10 ng, as well as using TNF alpha ($p=0.038$) or actinomycin D

Table II. *Results of stimulation of PCTS with actinomycin D (AD) and tumor necrosis factor alpha (TNF alpha) in different concentrations (Apoptosis/HPF; LK=negative control).*

	6h	12h	24h
LK	1.3	4.8	8.6
TNF alpha 1 ng + AD 1 μg/ml	2.3	18.9	13.8
TNF alpha 10 ng + AD 1 μg/ml	5.8	14.4	15.3
TNF alpha 10 ng + AD 10 μg/ml	4.8	22.8	21
TNF alpha 10 ng/ml	5.4	14.3	8.7
AD 10 μg/ml	2.6	11.6	14.3

($p=0.009$) alone. After 24 h, there was still an increase in apoptosis using TNF alpha and actinomycin D, but the differences lacked significance ($p=0.99$). The highest rate of apoptosis at all time points could be seen after incubation with 10 ng/ml TNF alpha together with 10 μg/ml actinomycin D (Figure 7).

Discussion

PCTSs overcome the limitations of other models to study apoptosis and allow the investigation of solid organ tissues with preservation of the tissue architecture and the interaction of the different cells and their matrix. Until now, they have been predominately used in pharmacological and toxicological research for metabolic studies (26). PCTS analyses are not limited to the liver. Several other tissues including lung (8), prostate (27), kidney (9), heart (3) and lymphoid tissue (33) have been used to investigate, for example, kinetics of drug metabolism (36), cocaine hepatotoxicity (5) and the protective effects of garlic extract against bromobenzene toxicity (35). Most of these studies are based on biochemical or molecular biological investigations using the supernatant, or by destroying the morphology of the PCTS. So far, only a single study has used a morphological approach for the study of apoptosis in rat liver slices (4). In human tissue, such morphological investigations are lacking.

The limitations of PCTS are mainly due to their restricted preservation time. Currently, only short-term analyses are possible (25). Since apoptosis represents a rapid process, the observation period is acceptable. Furthermore, the quality of the starting material requires stringent standardization and controls.

For the preparation of PCTS, two main mechanical slicing apparatuses, the Krumdieck tissue slicer and the more recent Brendel/Vitron tissue slicer, which was used in this study,

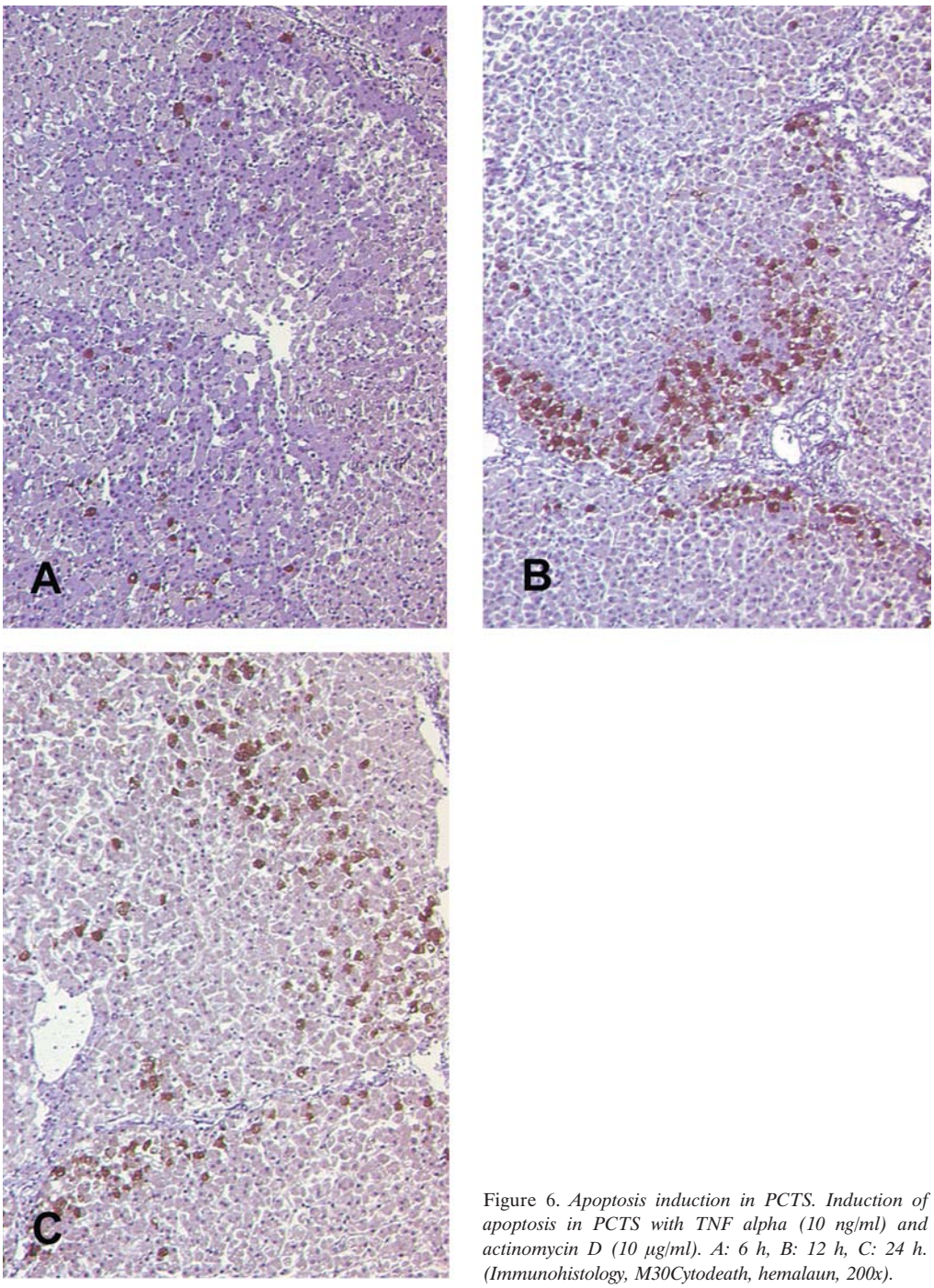

Figure 6. *Apoptosis induction in PCTS. Induction of apoptosis in PCTS with TNF alpha (10 ng/ml) and actinomycin D (10 μg/ml). A: 6 h, B: 12 h, C: 24 h. (Immunohistology, M30Cytodeath, hemalaun, 200x).*

were developed. Both instruments allow the production of very thin slices (approx. 200 μm) of near identical dimension without major traumatization. The Brendel/Vitron tissue slicer is easier to handle and cheaper. According to a comparative study, only minor differences exist between these two slicers (30). Dynamic and static systems have been developed (22). In the former system, the slices are floated onto roller systems under rotation in and out of the culture medium. The latter one is a submersion system, in which slices are gently agitated while submerged in wells containing the incubation medium. We have used a static multiwell system, which was found to be superior in several studies (13,

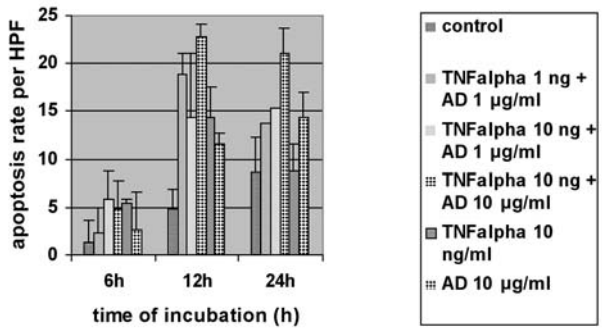

Figure 7. *Comparison of apoptosis inductor concentrations. Apoptosis was induced using TNF alpha, actinomycin D (AD) or in combination of both drugs with different concentrations for 6 h, 12 h and 24 h.*

25, 34). Different types of media are mentioned for investigations of PCTS (25, 26). We compared three media often used for maintaining cell lines of hepatic origin or primary liver cells. No significant differences were seen between these setups. Thus, conventional medium appears to be sufficient for incubation of PCTS.

The reactivity of PCTS is seen in the demonstrated spontaneous proliferation and apoptosis rate. Proliferation was mainly restricted to mesenchymal cells and was extremely rare in hepatocytes at the beginning of the culture. This is not surprising, since hepatocytes are reversible post-mitotic cells and strong stimuli are necessary to induce hepatocellular proliferation. Mesenchymal cell activation, however, is seen under different pathological conditions such as fibrosis. A low spontaneous cell death rate was also observed. These apoptotic cells were distributed throughout the parenchyma. As the apoptotic rate is higher at the beginning of the culture, it appears to be a result of cell damage during preparation of the slices. Thus, for investigations of apoptotic mechanisms, a prolonged resting phase is necessary. We recommend starting with the stimulation approx. 3 h after the first change of medium.

Fas ligand/ Fas and TNF/ TNF receptor systems play central roles in the regulation of apoptosis during inflammation and immunological processes (6, 15, 16, 29). The binding of TNF to TNF receptor 1 triggers a series of intracellular events resulting in activation of transcription factors and eventually of caspases. Hepatocytes are normally resistant to TNF toxicity, but undergo cell death from TNF in the setting of global transcriptional or translational arrest (24). We induced these conditions by adding actinomycin D. Simultaneous incubation of PCTSs with actinomycin D and TNF alpha led to substantial apoptosis of hepatocytes in a short time span (up to 12 h). Interestingly, the cell death occurred predominantly in zone 1 of the liver acinus. In most toxic liver damage, zone 3 necrosis is seen. Our investigation using PCTSs demonstrates the opposite. In viral and autoimmune hepatitis, preferentially periportal hepatocytes are known to succumb to cell death, the so-called "interphase hepatitis". These cells are in close contact to lymphocytes secreting TNF alpha. The impact of this observation with regard to the pathogenesis of viral hepatitis warrants further investigation. Deeper insight into the pathomechanism seems necessary.

From the data presented, we summarize that PCTSs are an excellent experimental tool for *in vitro* studies of liver cell apoptosis. They will allow more detailed studies without prior selection of sensitive cells. Different kinds of tissues, including human tissue, can be studied to gain new insights into the pathomechanisms of apoptosis.

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