High hydrostatic pressure treated tumour cells – Cell death pathways and immunogenicity

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Summary:
We investigated the response of mammalian cells to high hydrostatic pressure (HHP). The characterization of the cell death pathway induced by a HHP treatment is a prerequisite for its clinical use. We observed that cells were not restricted in their viability by pressures up to 100 MPa. Mammalian cells die when treated with pressures of 200 MPa or more. But the effects of 200, 300, or 400 MPa do not follow the same pattern. At 200 MPa, cells die in an apoptotic way within a couple of hours. In contrast, the cells treated with 300 and 400 MPa die immediately following a unique necrotic pathway, since treated cells harbour high DNA degrading activities. In comparison to inactivation by other necrosis inducing treatments like heat, freeze/thaw, or chemical agents, HHP avoids generation of Maillard products, disintegration and lysis of the cells. The high viscosity of the internal matrix of a pressurised cell is reflected by the slow penetration of the low molecular compound propidium iodide and may limit the bleeding of antigen before uptake by antigen presenting cells. Taken together, HHP is an alternative method for the inactivation of mammalian cells in clinical settings.

Introduction:
Today cancer causes the death of every forth human world-wide. The classical therapies for solid cancer have limitations in prolonging the live span of the patients. Therefore, new therapeutic approaches are urgently needed and vaccination against cancer with autologous tumour vaccines has shown promising results in animal experiments (Ward et al., 2002, Hanna et al., 2001). Basically, inactivated autologous tumour cells are injected into the patient. This strategy depends on an efficient inactivation of the cells, which is usually accomplished by ionizing irradiation or cytostatic drugs. These methods have been shown to induce apoptosis in mammalian cells. Apoptosis is a process of programmed cell death characterised by specific morphological changes including loss of membrane asymmetry, nuclear condensation and DNA fragmentation (Wyllie et al., 1980, Wyllie, 1987, Hengartner, 2000). In contrast to necrotic cells, apoptotic cells maintain the integrity of their plasma membranes. However, a substantial feature of apoptotic cells is their low immunogenicity (Stach et al., 2000). Further threads for this therapy are the cost intense irradiation procedures and the fact that cytostatic drugs can hardly be removed completely after induction of cell death. Alternative inactivating treatments like heat often result in an unwanted complete disintegration of the cells. Here we present an alternative method for the inactivation of human tumour cell lines using high hydrostatic pressure (HHP) treatment.

Under HHP treatment the cells undergo various changes. It has been demonstrated that HHP can cause the denaturation of certain proteins (Suzuki and Suzuki, 1962). In more recent reports pressure-temperature diagrams of HHP treated proteins were presented that characterized the protein denaturation in more detail (Panick et al., 1999, Smeller, 2002, Silva et al., 2001, Boonyaratanakornkit et al., 2002). In addition, HHP treatment has been shown to disturb the inter- and intramolecular interactions of proteins leading to altered tertiary and quaternary structure (Smeller, 2002, Boonyaratanakornkit et al., 2002). For instance, enzymes subjected to HHP showed a dose dependent loss of their enzymatic activity (Boonyaratanakornkit et al., 2002, Lynch and Sligar, 2002). Furthermore, the tertiary structure of DNA has been reported to obey a pressure – temperature phase diagram (Bartlett et al., 1995, Macgregor, 1998, Macgregor, 2002). Finally, the phospholipid bilayers of the eukaryotic plasma membrane display alterations from a liquid crystal to a gel like phase, when subjected to HHP (Winter, 2002, Winter and Dzwolak, 2004). In the 50th some experiments showed the vaccinating effect of HHP inactivated bacteria (Vignais et al., 1952b, Vignais et al., 1952a). Unfortunately, the immunogenic effect was only described scarcely. Here we characterize the different cell death pathway dose dependently induced by HHP treatment. Furthermore, we demonstrate that HHP treated cells retain their immunogenic capabilities.

Material and Methods:

Cell lines and culture conditions

For the analysis of the influence of HHP on mammalian cells, we employed the non-adherent human histiocytic lymphoma cell line U-937 (ATCC CRL-1593.2) and the human Burkitt's lymphoma-derived lymphoblastoid cell line Raji (ATCC CCL-86). Cell culture was performed at 37 °C and 5.5 % CO_2 in R10 (RPMI 1640 medium (Gibco Invitrogen, Karlsruhe, Germany) supplemented with 10 % FCS (Gibco Invitrogen, Karlsruhe, Germany), 1 % glutamine (Gibco Invitrogen, Karlsruhe, Germany), 1 % penicillin-streptomycin (Gibco Invitrogen, Karlsruhe, Germany), and 1 % HEPES (10 mM, pH 7.2) (Merck KgaA, Darmstadt, Germany)).

High hydrostatic pressure (HHP) treatment of mammalian cells

For HHP treatment, the suspended cells were filled to cryogenic vials with a volume of 1.8 ml (Greiner bio-one GmbH, Frickenhausen, Germany) and then closed tightly. The vials were filled without any headspace to avoid bursting. Additionally, the filled vials were sealed tightly with PARAFILM™ (American National Can, Chicago, USA) before being placed in the HHP-autoclave of the high-pressure apparatus (Record Maschinenbau GmbH, Königsee, Germany). The pressure treatment was performed with an increment of 10 MPa/s, followed by a pressure holding phase of 300 s and a decrease of 10 MPa/s.

Induction of cell death

For control purposes, cell death was induced by alternative stimuli. So apoptosis and necrosis were induced by irradiation with UV-B (240 mJ/cm²) and incubation for 30 min at 56°C, respectively.

Measurement of cellular morphology

Cells undergoing distinct cell death pathways have been described to change their morphology, which can be detected by scatter alterations in flow cytometry (Elstein and Zucker, 1994). An indication for apoptosis is a loss of the cellular volume and an increased cytoplasmic granularity and vacuolization. Consecutively, apoptotic cells are detected as a population with decreased FSc and increased SSc (Hagenhofer et al., 1998).

Detection of the cellular exposure of phosphatidylserine (PS)
From the early stages of apoptotic death cells expose PS on their cytoplasmic membranes (van Engeland et al., 1997). We used FITC-labelled recombinant chicken Annexin V (AxV; responsif GmbH, Erlangen, Germany) to detect the exposure of PS. Hundred µl cell suspension with 10^5 cells/ml were incubated with 400 µl freshly prepared AxV – propidium iodide (PI) staining solution (2 µg/ml AxV FITC and 2 µg/ml PI in Ringer's solution) at 4 °C for 30 min. The cells were subsequently analyzed by cytofluorometry. PI was added to differentiate between apoptotic (AxV positive, PI negative), necrotic (AxV positive, PI positive) and viable (AxV negative, PI negative) cells (Vermes et al., 1995).

Detection of cells with sub-G1 DNA content
During apoptosis an enzymatic system is cleaving and degrading the nuclear DNA. We measured the nuclear DNA content by propidium iodide (PI; Sigma, Munich, Germany) staining in the presence of the Triton-X100 (Nicoletti et al., 1991). Hundred µl of cell suspension were treated with 300 µl PI-Triton staining solution (0.1 % sodium citrate, 0.1 % Triton-X100, and 1 mg/ml PI) and incubated at 4 °C in the dark for at least 24 h. Finally, the samples were measured by flow cytometry and the PI-fluorescence (FL4) of the nuclei was recorded.

Detection of permeability of the cytoplasm membrane
Propidium iodide (PI) does not cross the plasma membranes of viable cells. However, PI penetrates the holey membrane of necrotic cells and intercalates into double stranded nucleic acids, which causes a 50 fold increase in PI fluorescence (Waring, 1965, Arndt-Jovin and Jovin, 1989). For detection of ruptured membranes the cell suspension was incubated with 1 µg/ml PI in Ringer's solution in the dark at room temperature. To block Ca^{++} dependent enzymes during the staining 5 mM EDTA was added to the staining solution. The samples are measured by flow cytometry at various time points.

Immunisation of mice
Control Raji cells or HHP-treated Raji cells were used for immunisation of female C57B/6N mice (Charles River Wiga, Sulzfeld, Germany). Ten to the six cells were resuspended in 500 µl and injected intraperitoneally (i.p.). At day 25 the animals were subjected to a booster immunisation. Sixty-three days after the first immunisation blood was drawn, and the serum was stored at –20 °C. Preimmune sera and sera from mice injected with Ringer's solution served as negative controls. Raji cells ($1.2x10^5$) were incubated with 5 µl murine serum (diluted 1:10 with PBS containing 1 % BSA and 0.1 % NaN3) for 1 h at 4 °C. After washing the cells twice with FACS-PBS 5 µl FITC labelled anti-mouse-IgG serum was added for 30 minutes at 4 °C. The bound fluorescence was measured using a cytofluorometer.

Results:

Morphology and viability of cells after HHP treatment

Figure 1 demonstrates the direct effects on cells of HHP treatment and those only occuring during a subsequent culture period. Viable cells (population 1) display a high forward scatter (FSc) and a low side scatter (SSc). Upon induction of cell death the FSc decreases and the SSc increases (population 2). Mock treated cells and cells treated with a pressure of 100 MPa do barely change either parameter during the observation period of 48 h.

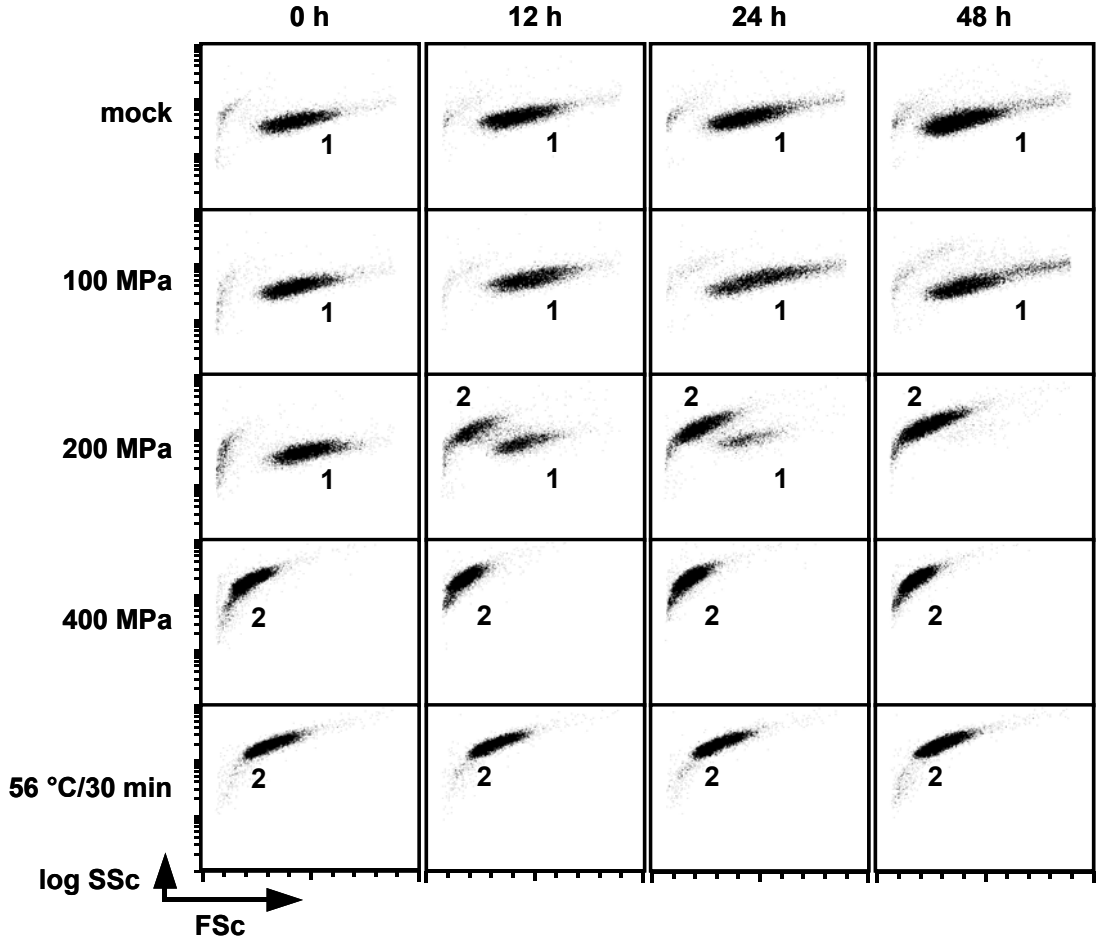

Figure 1: High hydrostatic pressure (HHP) changes the morphology of U-937 cells. The dot blots show the viability according to FSc / SSc properties of cells after the treatments and during culture time as indicated in the figure. The different populations can be distinguished: (1) viable cells and (2) dead cells. The experiment was performed in triplicates.

Directly after the treatment with 200 MPa, the cells looked pretty viable. However, the cells underwent progressive morphological changes during the consecutive culture period. 48 h after HHP all cells displayed a morphology typical for dead cells. The course of the morphological changes is similar to that experienced by cells treated with UV-B radiation. These cells display a constantly decreasing FSc due to the reduction of their volume caused by the release of vesicles (blebs) from their surfaces. Concomitantly they show an increase of their SSc, which is mainly due to chromatin clumping, nuclear fragmentation, and vacuolisation (not shown). HHP treatment with more than 300 MPa, exemplified by the 400 MPa data, induced an immediate death of almost all cells. The FSc values strongly decreased, whereas the SSc values increased. These cells were even smaller then the heat-induced necrotic cells generated by a 30 min standard treatment with 56 °C.

Exposure of Phosphatidylserine (PS) and membrane permeability

The cell count and the binding of FITC labelled AxV and PI permeable cells were measured by flow cytometry. In figure 2 the different fractions of viable, apoptotic, necrotic and degraded cells summarized to 100 % are given by the bar charts. A pressure treatment of 100 MPa did not affect the cells at all (fig. 2).

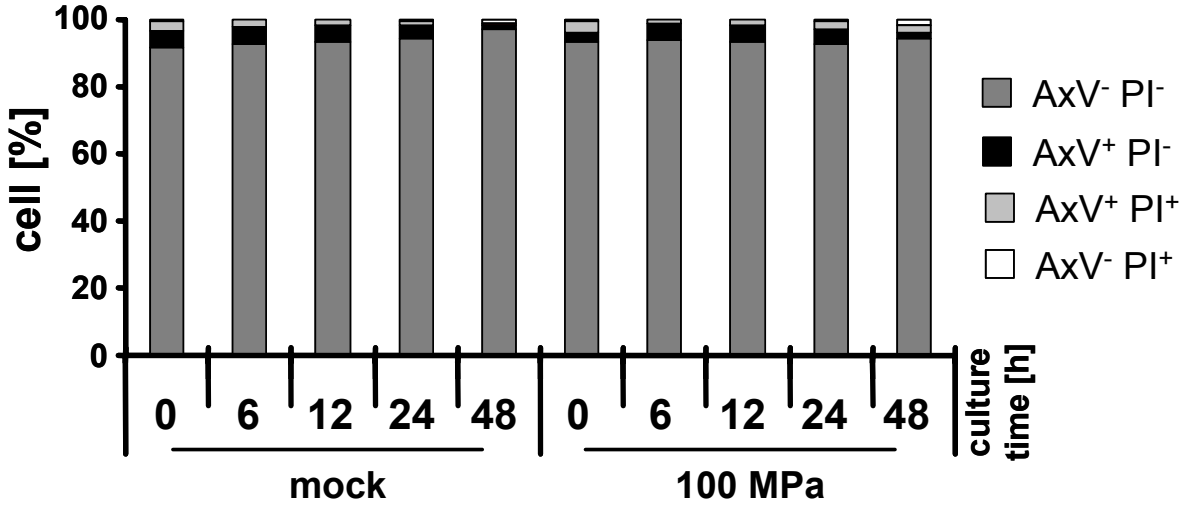

Figure 2: Annexin V (AxV) / PI staining after high hydrostatic pressure (HHP) treatment. AxV binds to apoptotic and necrotic cells while PI specifically stains necrotic cells. The bar charts represent the percents of the cell death pathways after the treatments indicated in the figure: mock treated (w/o) and 100 MPa pressure treatment. The experiment was performed in pentaplicates.

Only a small fraction of apoptotic and necrotic cells was to be detected. Cells treated with UV-B radiation or with 200 MPa showed a time dependent increase of apoptotic and necrotic cells (fig. 3). However, the time flow was faster in the cells that had been treated with 200 MPa.

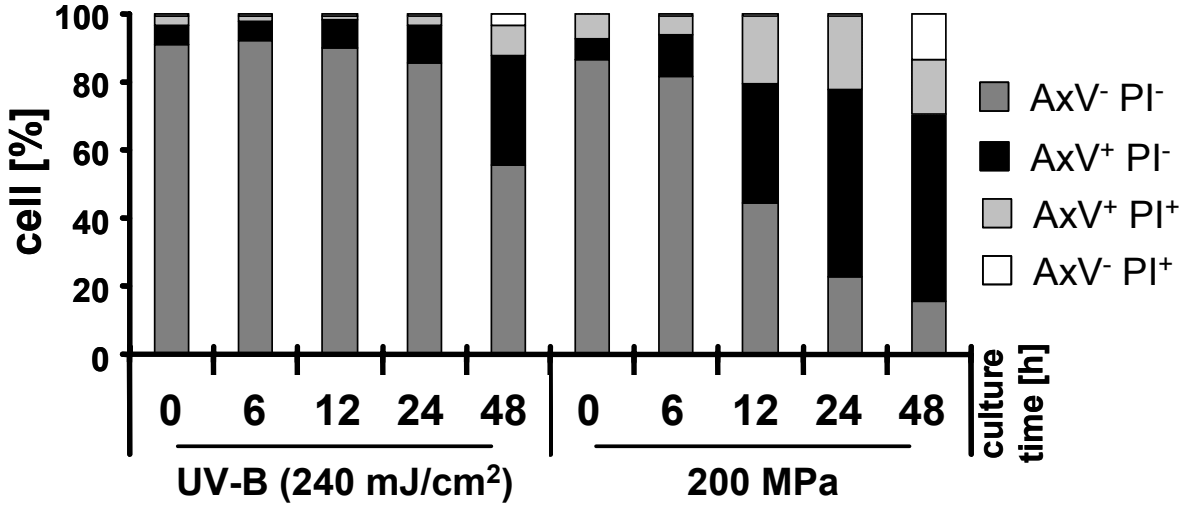

Figure 3: Annexin V (AxV) / PI staining after high hydrostatic pressure (HHP) treatment. AxV binds to apoptotic and necrotic cells while PI specifically stains necrotic cells. The bar charts represent the percents of the cell death pathways after the treatments indicated in the figure: UV-B irradiation with 240 mJ/cm^2 and the 200 MPa pressure treatment. The experiment was performed in pentaplicates.

A treatment with 56 °C for 30 min necrotizes all cells and permeabilizes the cytoplasm membrane. All cells show a high binding of AxV and a high PI signal (fig. 4). Immediately after HHP treatment with 400 MPa the cells showed a similar behaviour like cells treated with 56 °C for 30 min. In contrast to heat necrotized cells, those that had been pressurized with 400 MPa displayed a drastically reduced PI staining, while the AxV signal was virtually unchanged.

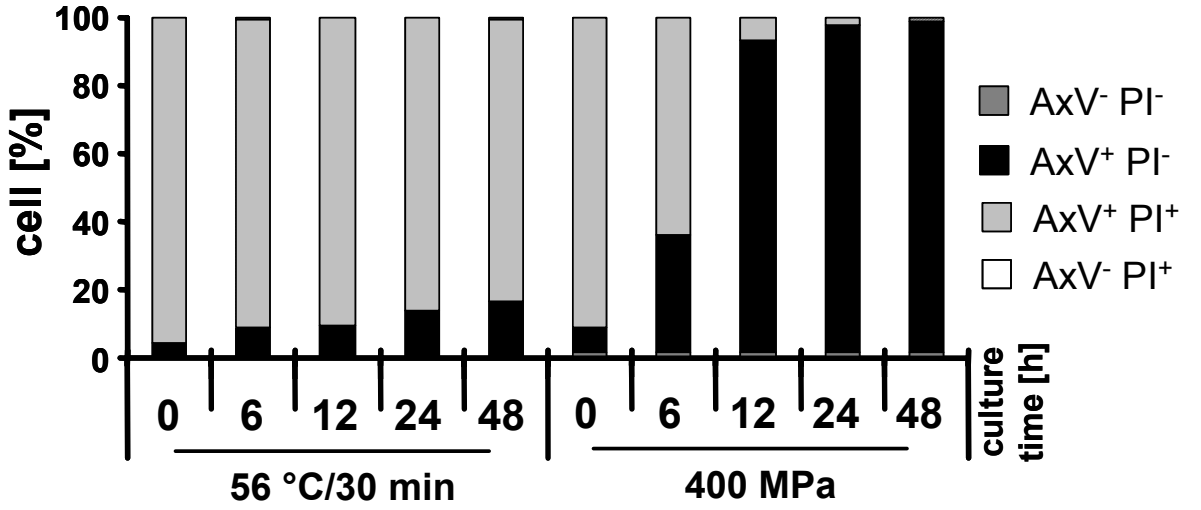

Figure 4: Annexin V (AxV) / PI staining after high hydrostatic pressure (HHP) treatment. AxV binds to apoptotic and necrotic cells while PI specifically stains necrotic cells. The bar charts represent the percents of the cell death pathways after the treatments indicated in the figure: heat necrosis (30 min at 56 °C) and the 400 MPa pressure treatment. The experiment was performed in pentaplicates.

This resembled an apoptotic phenotype, however, the cells showed a complete breakdown of their mitochondrial membrane potential (data not shown). Therefore, a repair of the permeable cytoplasm membrane is unlikely. Furthermore, we excluded a disintegration of the cells during the culture time, since the cell count did not change. We conclude that this “pseudo-apoptotic” phenotype is due to the fast degradation of the nucleic DNA in the cells treated with 400 MPa.

Detection of cells with sub-G1 DNA content

The nucleic DNA content can be measured after staining with PI in the presence of the detergent Triton-X100. The analysis of the amount of DNA defines the DNA degradation during apoptosis of individual cells. As shown in figure 5 viable (mock treated) and heat necrotized cells did not show degradation of their nuclei. In contrast, cells induced to undergo apoptosis (200 MPa or UV-B) showed a time dependent nuclear degradation. Surprisingly, the cells necrotized by 400 MPa showed a similar nuclear degradation.

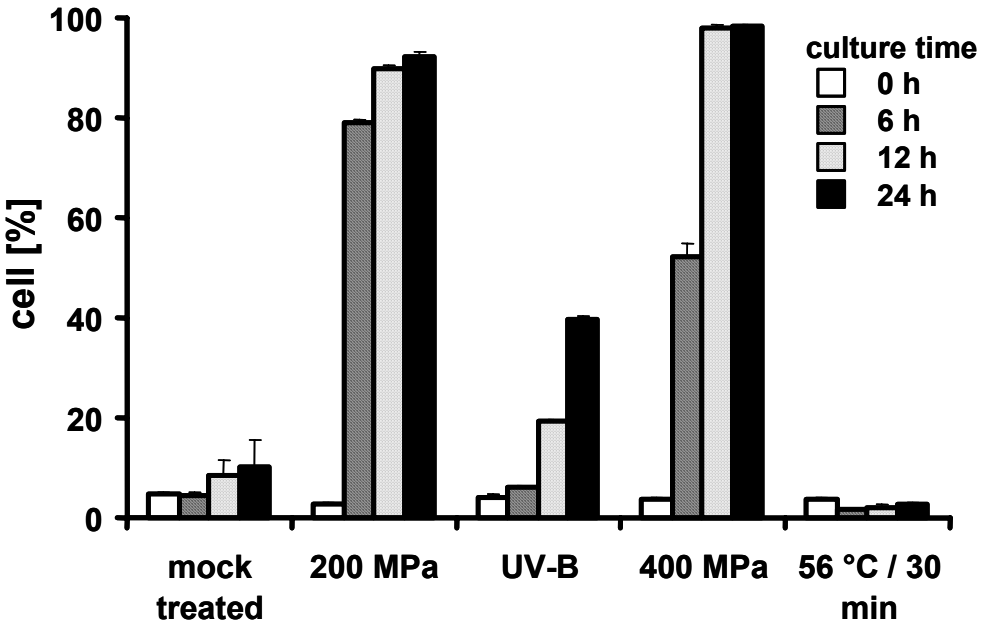

Figure 5: Cellular DNA content after high hydrostatic pressure (HHP) treatment. The PI-Triton method was used to analyse the content of cellular DNA by flow cytometry. This graph demonstrates the amount of cells with subG1 (degraded) DNA content. Treatments are indicated in the figure. The experiment was performed in pentaplicates. S.D. are indicated in the figure.

PI penetration into necrotized cells

The penetration of PI into necrotized cells (e.g. cells treated with 56 °C for 30 min) usually occurs within seconds after disruption of their cytoplasm membrane. The cells treated with a pressure of 300 MPa showed a similar property, however, the fluorescence intensity was not as high as in heat treated cells. Interestingly, necrosis induced by HHP with 400 MPa led to a decelerate penetration of PI. Within the first 400 min, the PI level never reached the PI levels of heat induced necrotic cells (fig. 6).

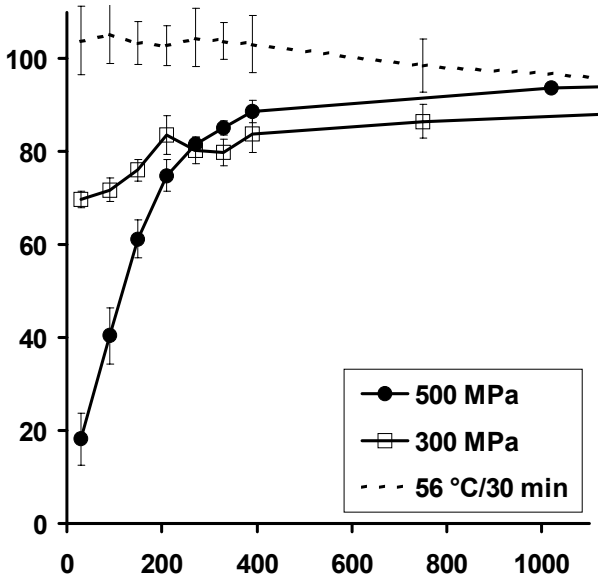

Figure 6: PI penetration after HHP treatment. The time dependence of PI fluorescence was monitored. Treatments are indicated in the figure and EDTA (5 mM) was added to the cell suspension to inactivate Ca^{++} dependent DNAses. The experiment was performed in triplicates. S.D. are indicated in the figure.

Immunogenicity of HHP treated human cells in mice

HHP treated cells were injected twice into C57/BL6 mice, to investigate whether they retain their immunogenicity. Figure 7 shows that all HHP treated cells had retained their immunogenicity. The pre-immune serum did not bind to Raji cells.

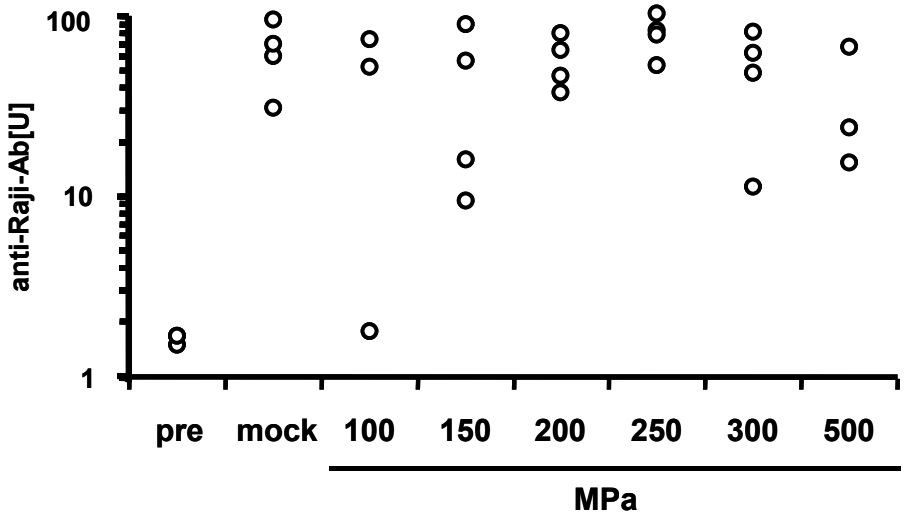

Figure 7: Immunogenicity of HHP-treated cells. Mice were immunized i.p. with differently treated Raji cells as indicated in the figure.

Discussion:

What happens to mammalian cells exposed to HHP? There are only limited published data on pressurized mammalian cells. It has been shown that mammalian cells are killed by pressures above 250 MPa. However, the pathway of cell death was not addressed (Diehl et al., 2003). Here we show that HHP induces different pathways of cell death in dependence of the applied pressure. We observed no effect on the viability of the cells for pressures up to 150 MPa. Instead, the cells underwent a transient proliferation block in the G2 phase of the cell cycle (not shown). At a pressure of 200 MPa the cells underwent a time dependent cell death. Immediately after the pressurization, the cells seemed morphologically viable and did not expose PS. However, during cell culture these cells underwent morphological and cytological changes typical for apoptosis. Pressurized cells displayed an even faster time course of the cell death program, when compared to cells irradiated with UV-B. Conversely, pressures of more than 300 MPa lead to immediate necrotic cell death (AxV and PI positive), displaying some features different from heat necrotized cells. The decrease of the PI signal observed during the culture periode of these cells is most likely due to the fact that the HHP-induced necrotic cells degrade their chromatin resulting in a “pseudo-apoptotic” phenotype (AxV positive PI negative). We confirmed the degradation of the nuclear DNA by the measurement of the nucleic DNA content of cells that had been permeabilized by detergent before staining with PI. In addition, this staining procedure confirmed the faster apoptotic time course of the cells treated with HHP (200 MPa), when compared to cells irradiated with UV-B. The kinetic of the staining of necrotized cells with PI revealed, that PI needs much more time to penetrate pressurized cells, when compared to other inducers of necrosis. This decelerated penetration of the PI is not a matter of the cytoplasm membrane, which is already ion permeable. Instead, we suppose that the diffusion or mobility of PI is reduced, if cells had been treated with HHP. This might be due to the gelification of macromolecules, a process that increases the viscosity in the cytoplasm. The gelification of proteins in solution was already described elsewhere (Totosaus et al., 2002).

Immunogenicity of inactivated tumour cells is influenced by several factors like the mechanism of killing, the levels of cell death and the local environment (Melcher et al., 1999, Sauter et al., 2000). In our experiments the HHP treated tumour cells retained their immunogenicity. The humoral immune response in a xenologous cell vaccination protocol was basically independent of the pressure and the consecutive pathway of cell death. This is in principal contrast, to the strongly reduced immunogenicity of cells rendered apoptotic by other apoptosis inducing protocols like irradiation (Voll et al., 1997, Stach et al., 2000, Bondanza et al., 2001).

Conclusion:

We conclude that this feature which separates HHP treated cells from those that had been inactivated by alternative methods may favour HHP for the inactivation of cell based vaccines. The efficient degradation of the chromatin and the gelification of the cytoplasm may further improve the safety and performance of HHP treated cells in vaccination trials.

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References:

Arndt-Jovin, D. J. and Jovin, T. M. (1989) *Methods Cell Biol,* **30,** 417-48.

Bartlett, D. H., Kato, C. and Horikoshi, K. (1995) *Res Microbiol,* **146,** 697-706.

Bondanza, A., Rovere, P., Borri, A., Caremoli, E. R., Guidetti, A., Citterio, G., Consogno, G., Zimmermann, V. S., Rugarli, C. and Manfredi, A. A. (2001) *Int J Cancer,* **91,** 713-7.

Boonyaratanakornkit, B. B., Park, C. B. and Clark, D. S. (2002) *Biochim Biophys Acta,* **1595,** 235-49.

Diehl, P., Schmitt, M., Bluemelhuber, G., Frey, B., Van Laak, S., Fischer, S., Muehlenweg, B., Meyer-Pittroff, R., Gollwitzer, H. and Mittelmeier, W. (2003) *Oncol Rep,* **10,** 1851-5.

Elstein, K. H. and Zucker, R. M. (1994) *Exp Cell Res,* **211,** 322-31.

Hagenhofer, M., Germaier, H., Hohenadl, C., Rohwer, P., Kalden, J. R. and Herrmann, M. (1998) *Apoptosis,* **3,** 123-32.

Hanna, M. G., Jr., Hoover, H. C., Jr., Vermorken, J. B., Harris, J. E. and Pinedo, H. M. (2001) *Vaccine,* **19,** 2576-82.

Hengartner, M. O. (2000) *Nature,* **407,** 770-6.

Lynch, T. W. and Sligar, S. G. (2002) *Biochim Biophys Acta,* **1595,** 277-82.

Macgregor, R. B. (2002) *Biochim Biophys Acta,* **1595,** 266-76.

Macgregor, R. B., Jr. (1998) *Biopolymers,* **48,** 253-63.

Melcher, A., Gough, M., Todryk, S. and Vile, R. (1999) *J Mol Med,* **77,** 824-33.

Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F. and Riccardi, C. (1991) *J Immunol Methods,* **139,** 271-9.

Panick, G., Vidugiris, G. J., Malessa, R., Rapp, G., Winter, R. and Royer, C. A. (1999) *Biochemistry,* **38,** 4157-64.

Sauter, B., Albert, M. L., Francisco, L., Larsson, M., Somersan, S. and Bhardwaj, N. (2000) *J Exp Med,* **191,** 423-34.
Silva, J. L., Foguel, D. and Royer, C. A. (2001) *Trends Biochem Sci,* **26,** 612-8.
Smeller, L. (2002) *Biochim Biophys Acta,* **1595,** 11-29.
Stach, C. M., Turnay, X., Voll, R. E., Kern, P. M., Kolowos, W., Beyer, T. D., Kalden, J. R. and Herrmann, M. (2000) *Cell Death Differ,* **7,** 911-5.
Suzuki, C. and Suzuki, K. (1962) *J Biochem (Tokyo),* **52,** 67-71.
Totosaus, A., Montejano, J. G., Salazar, J. A. and Guerrero, I. (2002) *Int J Food Sci Tech,* **37,** 589-601.
van Engeland, M., Kuijpers, H. J., Ramaekers, F. C., Reutelingsperger, C. P. and Schutte, B. (1997) *Exp Cell Res,* **235,** 421-30.
Vermes, I., Haanen, C., Steffens-Nakken, H. and Reutelingsperger, C. (1995) *J Immunol Methods,* **184,** 39-51.
Vignais, P., Barbu, E., Macheboeuf, M. and Basset, J. (1952a) *Bull Soc Chim Biol (Paris),* **34,** 798-805.
Vignais, P., Barbu, E., Macheboeuf, M. and Basset, M. (1952b) *Bull Soc Chim Biol (Paris),* **34,** 43-6.
Voll, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalden, J. R. and Girkontaite, I. (1997) *Nature,* **390,** 350-1.
Ward, S., Casey, D., Labarthe, M. C., Whelan, M., Dalgleish, A., Pandha, H. and Todryk, S. (2002) *Cancer Immunol Immunother,* **51,** 351-7. Epub 2002 Jun 14.
Waring, M. J. (1965) *J Mol Biol,* **13,** 269-82.
Winter, R. (2002) *Biochim Biophys Acta,* **1595,** 160-84.
Winter, R. and Dzwolak, W. (2004) *Cell Mol Biol (Noisy-le-grand),* **50,** 397-417.
Wyllie, A. H. (1987) *J Pathol,* **153,** 313-6.
Wyllie, A. H., Kerr, J. F. and Currie, A. R. (1980) *Int Rev Cytol,* **68,** 251-306.