Quantification and comparison of time-dependent changes in mobile lipid resonances with cellular processes.

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Introduction: Mobile lipid resonances (MLRs) are observed in the ^{1}H magnetic resonance (^{1}H MRS) spectra of many cancers *in vivo* and *in vitro*. Increase in the MLR signals has been correlated with suppressed proliferation[1], ongoing apoptosis[2,3] or growth arrest[4] while necrosis observed *in vitro* does not show accumulation of lipids but a reduction in most intracellular metabolites including MLRs[5,3]. However, despite increasing evidence that MLRs are involved in tumour cell physiology, growth and invasiveness it is unclear whether their nature and degree of saturation change in relation to phase of the cell cycle and to cellular processes. The aim of this study, therefore, was to document changes in nature and saturation of MLRs associated with proliferation, apoptosis, growth arrest and necrosis in Hela cells.

Methods: HeLa cells were grown as adherent cells at 37°C in an incubator with 5% CO_2 in DMEM medium (Gibco), supplemented with 10% fetal calf serum (Gibco), 1% sodium pyruvate (Gibco), and 1 % penicillin-streptamycin solution (Gibco). In all experiments attention was given to ensure that cells were not deprived of nutrients at any stage of growth.

Apoptosis was induced by exposing the cells to etoposide or paclitaxel. Experiments with etoposide (Sigma) were performed by exposing cells 24 hours after seeding to 10µM etoposide in 0.5% DMSO for 12 and 24 hours. Experiments with paclitaxel (Sigma) were conducted with 1µM paclitaxel in 99% ethanol for 7, 12 and 24 hours. Growth arrest was induced at 12 and 24 h with 1µM apicidin (Sigma) in 0.5% DMSO. Necrosis was induced at 24h with 10µM cytochalasin B in 99% ethanol. The occurrence of induced processes was validated using flow cytometry (using DAPI staining), fluorescence microscopy (with Hoechst 33258 staining) and trypan blue exclusion test. All lipid spectra were collected on a 500 MHz Bruker Avance spectrometer (Bruker Biospin, Germany), using a HR-MAS probe spun at 3000 Hz and 4°C. A stimulated-echo pulse sequence with bipolar gradient pulses was applied[6]. This eliminates signals from low molecular weight metabolites, which have high diffusion coefficients. Application of the sequence was validated using a monounsaturated lipid – oleic acid (18:1) as a model to ensure that diffusion-weighted sequence did not distort the relative intensities of the different lipid resonances. Data for lipid resonances at 0.9 ppm ($C\underline{H}_3$-), 1.3 ppm (-$C\underline{H}_2$-), 2.0 ppm (allyl methylene; =CH-$C\underline{H}_2$-), 3.0 ppm (diallyl methylene; =CH-$C\underline{H}_2$-CH=), and 5.3 ppm (vinyl; =CH) were measured. A ratio of 1.3/0.9 ppm and 2.0/3.0 were used as they reflect CH_2/CH_3 and contains information about the degree of polyunsaturation respectively.

Results: *Proliferation.* Hela cells in the exponential phase of growth were characterised by relatively low levels of mobile lipids. CH_2/CH_3 ratio was constant (1.47) but increased at confluence to 2.55. Also the ratio of 2.0/3.0 ppm lipids increased when passing from a proliferative to a confluent state (from 3.42 to 4.27). An increase of vinyl signal at 5.3 ppm was also recorded when cells progressed to confluence. A gradual accumulation of cells in G1 phase of the cell cycle, from 49.6% to 65.2%, was observed as cells approached confluence.

Apoptosis: A general increase in MLRs was only observed 24 hours after exposure. At that timepoint the increase in the CH_2/CH_3 ratio (from 1.36 to 1.79) was significant ($p<0.01$). There was increased unsaturation of lipids (2.0 and 5.3 ppm peaks), predominantly due to significant increase in mono-unsaturated components (2.0 ppm; $p<0.02$), while the polyunsaturated component (3.0 ppm) remained constant. The majority of these cells were still viable (83% ± 5%; mean ± range). Cells exposed to paclitaxel gradually accumulated in G2/M phase of the cell cycle. Cells exposed to etoposide could not progress beyond the S phase and consequently at 24h a sub-G1 peak was observed. Characteristic apoptotic nuclear morphology such as chromatin condensation and clumping was seen using Hoechst DNA staining.

Growth arrest: Exposure of Hela cells to apicidin resulted in growth arrest in G1 phase of the cell cycle (up to 75% at 24h). There was no significant change in CH_2/CH_3 peak ratios (1.24±0.05; $p>0.05$) in comparison to control cells at 12h but at 24h CH_2/CH_3 ratio increased significantly (1.76±0.1; $p<0.05$). A significant increase ($p<0.001$) in peak intensities at 2.0/3.0 ppm was also observed and an increase at 5.3 ppm indicated accumulation of unsaturated fatty acids.

Necrosis: The lipid profile of Hela cells exposed to cytochalasin B revealed a decrease in the intensities of major mobile lipid peaks at 24h. Almost no changes in the CH_2/CH_3 and 2.0/3.0 peak ratios and 5.3 ppm peak were observed. Viability of the cells exposed to cytochalasin B after 24h was 64% ± 8.4 (mean ± range). Hoechst DNA staining did not indicate apoptotic nuclear morphology in cells exposed to apicidin or cytochalasin B.

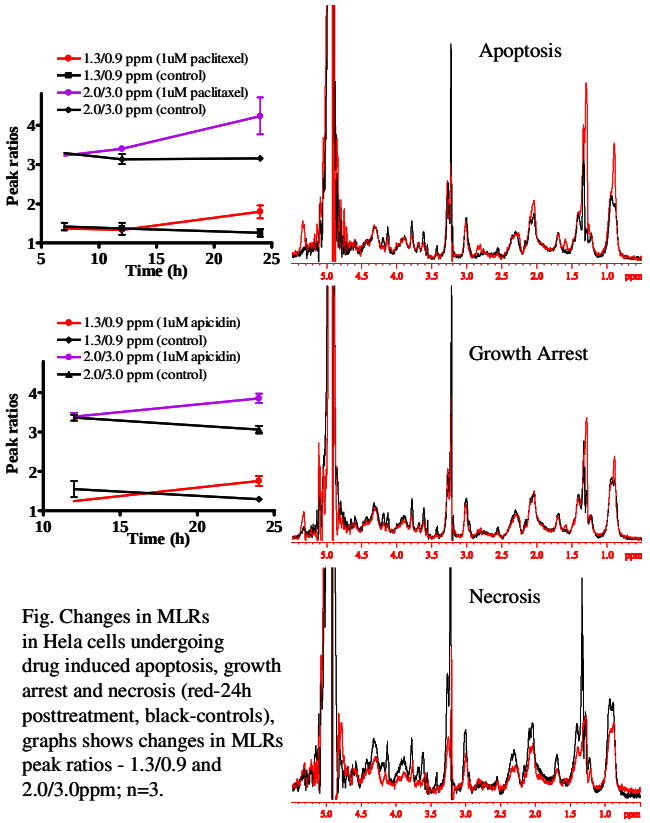

Fig. Changes in MLRs in Hela cells undergoing drug induced apoptosis, growth arrest and necrosis (red-24h posttreatment, black-controls), graphs shows changes in MLRs peak ratios - 1.3/0.9 and 2.0/3.0ppm; n=3.

Conclusion: Changes in MLRs in Hela cells exposed to drugs inducing apoptosis, growth arrest and necrosis occur at 24h and are much more rapid than changes observed when cultured cells are progressing from exponential phase to confluent and postconfluent stages (days). Ratios of 1.3/0.9 ppm and 2.0/3.0 ppm and 5.3 ppm peak change significantly in case of growth arrest and apoptosis. Lipid profiles of growth arrested and apoptotic cells are almost indistinguishable with an increase in unsaturated chains. Flow cytometry data suggests that these MLRs changes are not cell cycle dependent as cells exposed to paclitaxel (arrests cells in G2/M phase of the cell cycle) and to apicidin (arrests Hela cells in G1 phase of the cell cycle) display similar lipid profiles.

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