APOPTOSIS: MR CONSEQUENCES

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Cancer cells have a remarkable ability to escape programmed cell death (PCD). This characteristic is often associated with aggressive growth pattern and resistance to traditional anticancer drugs. PCD, better known as apoptosis, is an active ATP-requiring cell death process involving sophisticated cellular machinery to commit suicide and to avoid recruitment of detrimental inflammatory processes, leading to clearance of dying cells by phagocytosing macrophages. PCD is triggered through two distinct pathways. Firstly, by the receptor-dependent, or extrinsic, pathway involving transmembrane signalling and recruitment of adaptor proteins and secondly, by mitochondrial-dependent, or intrinsic, apoptosis pathway involving translocation of BCL2 family members to mitochondrion and release of cytochrome c to the cytoplasm. The common domain of both pathways share is activation of intracellular caspases triggering the overall apoptosis process.

There is considerable interest to assign endogenous ‘biomarkers’ or ‘surrogate markers’ for imaging of apoptosis in cancer in vivo. The effort gains great clinical motivations from the fact that a number of anti-cancer drugs as well as radiation therapy cause apoptosis and an early tumour response to these therapies predicts long-term treatment outcome. Imaging biomarkers for apoptosis would allow to identify the patients unresponsive to a given drug soon after introduction of the therapy, because they may benefit with the use of alternative chemotherapy protocols, for instance in breast cancer. Similarly, unnecessary and sometimes laborious chemotherapy sessions could be avoided. Furthermore, scrutinised information of molecular events of PCD may offer guidelines for anticancer drug development. In this account potentials of NMR techniques to detect endogenous biomarkers or surrogate endpoint markers of apoptosis in cancer cells and tumours before tumour eradication, will be discussed. Targeted probes for early apoptotic events, such as those to externalised phosphatidylserine for revelation with radioactive, optical, MR-visible tags, are under active development, however, these methods will not be covered here.

NMR spectroscopy of apoptotic cancer cells and tumours

Due to the fact that several biochemical pathways are either directly or indirectly affected by early steps of apoptotic process, levels of several biochemical compounds change and thus they could be potential biomarkers for detection by NMR. Recent studies show that there are a number of biochemical and biophysical changes associated with early apoptotic processes that are detectable by NMR techniques serving as potential indices for PCD.
Glucose metabolism

Malignant tumours are commonly highly glycolytic and elevated lactate concentration in tumours in vivo, as detected by 1H NMR spectroscopy, has been linked to poor prognosis 11. Positron emission tomography (PET) studies observing 18F-deoxy-D-glucose (FDG) metabolism in cancer tissue in vivo have shown that both FDG uptake and phosphorylation rates decline in breast tumours responding to chemotherapy much earlier than tumour volume shrinkage 12. Smith and co-workers observed that dose-to-uptake-ratio of FDG decreased much more in the breast tumours after a single pulse of chemotherapy developing partial or complete response than in non-responders 13. Large body of chemotherapy –induced cell death is apoptotic, therefore these data indicate that glucose metabolism decreases in response to early apoptotic events well before imminent cell death.

Cell studies have revealed that apoptosis is associated with accumulation of a glycolytic intermediate, fluctose-1,6-bisphosphate (FBP) 14, 15, consistent with inhibition of glycolysis. FBP accumulation is likely to be due to inhibition of glyceraldehyde-3-phosphate dehydrogenase because of depletion of NAD.

13C NMR spectroscopy has been used in preclinical settings to study RIF-1 tumour glucose metabolism during cyclophosphamide (CP) treatment 16. It was reported that the glycolytic rate decreases by 24 hours after a single dose of CP before decline in tumour growth rate is evident. CP response was associated with increased oxidation of glucose and decreased tissue pO2 tension reflecting activation of mitochondrial oxidative metabolism in treated tumours. These data are very interesting in regard to biochemical mechanism underlying the FDG PET observations above, indicating that a shift towards oxidative metabolism could be an index of positive drug response and thus, apoptosis.

Choline –containing compounds

Choline –group containing metabolites are of special significance for the in vivo NMR spectroscopy of cancer 17. 31P NMR spectroscopy allows for separation of several choline subspecies in vivo. Cell studies have shown accumulation of CPD-choline to apoptotic cells 14, 18. The accumulation has been attributed to inhibition of phosphatidylcholine biosynthesis resulting from inhibition of cholinephosphotransferase 18. This enzyme has an alkaline pH optimum and because apoptosis is associated with cell acidification 19, it becomes inhibited in apoptotic cells. Using proton-decoupled 31P NMR spectroscopy, it has been reported that the pretherapy sums of phosphoethanolamine (PE) and phosphocholine (PC) in non-Hodgkin lymphomas 6 and head and neck tumours 20 were lower in the tumours responding to chemotherapy and radiation, respectively, than in non-responders. It has been speculated that the predictive potential of PE+PC may be related to their involvement in apoptotic signalling 6.

The value of 1H NMR peak centred at 3.2 ppm, with large contributions from choline –containing metabolites (CCM), as a ‘biomarker’ of apoptosis is under research. CCMs are high in cancerous tissue 17 and for instance, breast lesions with CCM > 4 mM almost exclusively are malignant tumours 21. Tumour cellularity and CCM concentration are...
strongly correlated\textsuperscript{22}. A recent study on breast tumour cases, using in vivo spectroscopy at 4 T, observed that $^1$H NMR–detected CCMs decrease 24 hours after chemotherapy in responding tumours, but not in non-responders\textsuperscript{23}. This is a very intriguing observation suggesting that CCM may be a sensitive $^1$H NMR indicator for very early events in tumour eradication in mammary tumours, possibly through apoptosis.

$^1$H chemical shift dispersion of individual choline-subspecies is so small that they can not be separated \textit{in vivo} within the peak centred at 3.2 ppm. In addition, several other molecules, such as taurine, myo-inositol, phosphatidylcholine and macromolecules, overlap the peak in a high field $^1$H NMR spectrum acquired with short TE\textsuperscript{24}. In gene therapy–induced apoptosis of rodent glioma, the $^1$H NMR peak at 3.2 ppm\textsuperscript{25} appears to behave very differently to that seen in chemotherapy-treated breast cancer\textsuperscript{23}. The CCM peak, as detected at 4.7 T \textit{in vivo}, decreases only in advanced cell kill when tumour cell density has decreased by $\sim$70\%\textsuperscript{25}. Recently, using high-resolution magic angle spinning $^1$H NMR of glioma \textit{ex vivo}, Valonen and co-workers reported that PC and GPC concentrations increase in the early phase of apoptosis when tumours show DNA breakdown products\textsuperscript{26}. In this tumour Cho+PC+GPC stays unchanged despite $>$70\% decrease in cell density due to PCD, but Tau decreases by $\sim$50\%. These data indicate that the $^1$H NMR peak at 3.2 ppm \textit{in vivo} may be indicative of cellular processes involved in apoptosis, however, the underlying biochemical changes may greatly vary. Thus, it appears that CCM can not be regarded as universal biomarkers of early phase of apoptosis, but rather surrogate endpoint marker of (apoptotic) cell kill in cancer.

**Intracellular pH**

It is a common observation from cell studies that apoptosis is associated with intracellular acidification\textsuperscript{18,19}, in fact, it has been shown that intracellular acidosis can result in apoptosis in HL-60 cells\textsuperscript{27}. Tumours have close to neutral or alkaline intracellular pH, yet the extracellular space appears to have lower pH by up to 0.5 pH units\textsuperscript{28}. NMR–based methods have been introduced to determine extracellular pH in tumours\textsuperscript{29,30} and it would be tempting to combine these with either $^{31}$P NMR or recently described MRI techniques to image intracellular pH\textsuperscript{31} in apoptotic tumours as well.

**$^1$H NMR detectable lipids**

Cell studies have shown that induction of apoptosis by pharmacological or other means results in subtle increase in $^1$H NMR detectable lipid signals\textsuperscript{32-34}. $^1$H NMR detectable lipids do not appear in Jurkat cells during necrotic cell death, but in human HBL-100 breast cancer cells also necrosis leads to accumulation of lipids\textsuperscript{34}. Because NMR detects lipids only in specific cellular compartments, i.e. lipid vesicles\textsuperscript{35,36}, NMR would allow to monitor lipid body formation in apoptosing tumours. In rat glioma apoptosis has been shown to be associated with accumulation of $^1$H NMR lipids, most notable polyunsaturated fatty acids (PUFA) concomitantly with generation of intracellular lipid bodies\textsuperscript{37,38}. Recently, $^1$H NMR lipids have been shown to increase in experimental lymphomas\textsuperscript{39} and neuroblastomas during drug-induced apoptosis\textsuperscript{40}. It appears that increase in $^1$H NMR detected PUFAs is an early sign of apoptotic process preceding tumour growth arrest\textsuperscript{38}. Pattern recognition
methods have shown that vinyl-, bis-allylic- and methyl-resonances provide the greatest contributions to discriminate apoptotic tumours from non-apoptotic ones by $^1$H NMR spectroscopy before expression of cell death. Accumulating PUFAs have chemical structure found in lipids present in mitochondrial membranes suggesting that $^1$H NMR detected lipids originate from membrane breakdown products.

Other metabolites in apoptosis

It is not unexpected that many other $^1$H NMR detectable metabolite changes occur in apoptotic cells. Glycine, creatine and alanine show a correlation with cell density, thus serving as potential surrogate endpoint markers for the severity of cell death.

MRI and apoptosis in tumours

Several MRI techniques may provide information useful for detection of PCD in tumours in vivo (Table 1). Much better spatial resolution by MRI over MRS makes it certainly very attractive to be exploited for imaging of consequences of apoptosis and monitoring of cancer treatment response.

Diffusion MRI

A remarkable observation that diffusion-weighted MR signal increases in the early phase of anti-cancer drug treatment facilitated use of DWI for monitoring of treatment in experimental tumour models through apoptosis. DWI has recently been successfully used in assessment of brain tumour treatment in humans.

In rat tumour models, correlating histological changes of apoptosis and cell death with MRI, have shown that apparent diffusion coefficient (ADC) increases briefly before or close to the time when tumour growth ceases. A massive increase in ADC takes place during subsequent days along the tumour eradication. Histological analyses of apoptosis markers and cell count in association with quantitative MRI have shown that the initial ADC increase coincides appearance of TUNEL positivity and decline in cell density. These events are associated with increase in spin density as a result of net water accumulation to apoptotic tumour. Interestingly, in the gliomas undergoing gene therapy-induced apoptosis, both ADC and T2, as quantified with Hahn single echo MRI, increase in concert. It appears that loss of intracellular volume, increase in extracellular volume and net water gain are the key cellular factors leading to revelation of apoptotic cell death with DW and T2 MRI with comparable sensitivity. In some other tumours, where absolute DWI, but only T2-weighted MRI has been exploited, diffusion has shown greater sensitivity to cell death than conventional T2 MRI. DWI is considered a surrogate endpoint marker for monitoring of tumour apoptosis and cell death both in experimental and clinical settings.

$T_1$, $T_1\rho$ and $T_2$ MRI

$T_1$ MRI is commonly used for ‘anatomical’ imaging of tumours. It appears that absolute $T_1$ increases in apoptosing gliomas parallel to tumour volume shrinkage, suggesting that $T_1$
has a low sensitivity to early events of tumour cell death \(^{48}\). Exciting data have been reported from \(T_{1\rho}\) MRI used for imaging of apoptosis in a glioma model \(^{48,49}\). \(T_{1\rho}\) contrast appears in parallel to DNA degradation, as quantified with TUNEL-staining, well before decline in cell density or arrest of tumour growth. The contrast in apoptotic tumours may be owing to the inherent sensitivity of \(T_{1\rho}\) to (chemical) exchange processes \(^{50,51}\).

Several studies using Hahn single echo \(T_2\) MRI have shown that apoptosis can be revealed with this contrast well before tumour volume shrinkage \(^{45,48,52}\). Carr-Purcell type of multi-echo method, incorporating adiabatic pulses \(^{53}\), has been introduced for exploitation of dynamic dephasing processes for MRI contrast. Using this method Gröhn et al. recently showed that in gene therapy –induced apoptosis of rat glioma, dynamic dephasing signal increases much before conventional \(T_2\) signal offering improved sensitivity for detection of PCD \(^{49}\). The study by Gröhn et al demonstrates that \(T_2\) contrast can be tailored for improved sensitivity to pathology for potential clinical exploitation.

**Conclusions**

Several endogenous biomarkers and surrogate markers for apoptotic process are amenable for detection by multimodal NMR methods *in vivo*. Some of these, such as absolute \(T_2\) and diffusion MRI, bear great clinical potentials. In future molecular imaging, exploiting either ‘smart contrast agents’ (8-10) or hyperpolarised \(^{13}\)C substrates \(^{54}\), is expected to become feasible for monitoring of apoptosis *in vivo*.

**Table 1.** Putative biomarkers and surrogate endpoint markers of apoptosis for detection by NMR methods

<table>
<thead>
<tr>
<th>Substance/Variable</th>
<th>Biomarker</th>
<th>Surrogate marker</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose-1,6-bisphosphate</td>
<td>Increase</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>CDP-choline</td>
<td>Increase</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Intracellular pH</td>
<td>acidification</td>
<td></td>
<td>18,19</td>
</tr>
<tr>
<td>(^1)H detected lipids</td>
<td>Increase</td>
<td></td>
<td>32,34,38</td>
</tr>
<tr>
<td>PUFA</td>
<td>Increase</td>
<td></td>
<td>37,38</td>
</tr>
<tr>
<td>Glucose uptake/glycolysis</td>
<td>Decrease</td>
<td></td>
<td>12,13,16</td>
</tr>
<tr>
<td>(T_{1\rho}) MRI contrast</td>
<td>Increase (?)</td>
<td>Increase</td>
<td>48,49</td>
</tr>
<tr>
<td>Creatine/taurine/glycine</td>
<td>Decrease</td>
<td></td>
<td>25,26</td>
</tr>
<tr>
<td>Cholines</td>
<td>Decrease</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>ADC</td>
<td>Increase</td>
<td></td>
<td>41,43,44</td>
</tr>
<tr>
<td>(T_2) or CP-(T_2) MRI contrast</td>
<td>Increase</td>
<td></td>
<td>45,49</td>
</tr>
</tbody>
</table>

**References**

34. Delikatny EJ, Cooper WA, Brammah S, Sathasivam N, Rideout DC. Cancer Res. 2002; **62**: 1394-1400.