

APOPTOSIS: MR CONSEQUENCES

Risto. A. Kauppinen
School of Sport and Exercise Sciences
University of Birmingham, Birmingham, UK
R.A.Kauppinen@bham.ac.uk

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 ¹. 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* ². The effort gains great clinical motivations from the fact that a number of anti-cancer drugs as well as radiation therapy cause apoptosis ³ and an early tumour response to these therapies predicts long-term treatment outcome ⁴. 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 ⁵. Similarly, unnecessary and sometimes laborious chemotherapy sessions could be avoided ⁶. Furthermore, scrutinised information of molecular events of PCD may offer guidelines for anticancer drug development ⁷. 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 ⁸, optical ⁹ MR-visible tags ¹⁰, 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 (Table 1).

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 ^{18}F -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, fructose-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.

^{13}C 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 pO_2 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. ^{31}P 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 ^{31}P 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 correlated²². A recent study on breast tumour cases, using *in vivo* spectroscopy at 4 T, observed that ¹H NMR –detected CCMs decrease 24 hours after chemotherapy in responding tumours, but not in non-responders²³. This is a very intriguing observation suggesting that CCM may be a sensitive ¹H NMR indicator for very early events in tumour eradication in mammary tumours, possibly through apoptosis.

¹H chemical shift dispersion of individual choline-subspecies is so small that they can not be separated *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 ¹H NMR spectrum acquired with short TE²⁴. In gene therapy - induced apoptosis of rodent glioma, the ¹H NMR peak at 3.2 ppm²⁵ appears to behave very differently to that seen in chemotherapy-treated breast cancer²³. The CCM peak, as detected at 4.7 T *in vivo*, decreases only in advanced cell kill when tumour cell density has decreased by ~70%²⁵. Recently, using high-resolution magic angle spinning ¹H NMR of glioma *ex vivo*, Valonen and co-workers reported that PC and GPC concentrations increase in the early phase of apoptosis when tumours show DNA breakdown products²⁶. In this tumour Cho+PC+GPC stays unchanged despite >70% decrease in cell density due to PCD, but Tau decreases by ~50%. These data indicate that the ¹H NMR peak at 3.2 ppm *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^{18,19}, in fact, it has been shown that intracellular acidosis can result in apoptosis in HL-60 cells²⁷. Tumours have close to neutral or alkaline intracellular pH, yet the extracellular space appears to have lower pH by up to 0.5 pH units²⁸. NMR –based methods have been introduced to determine extracellular pH in tumours^{29,30} and it would be tempting to combine these with either ³¹P NMR or recently described MRI techniques to image intracellular pH³¹ in apoptotic tumours as well.

¹H NMR detectable lipids

Cell studies have shown that induction of apoptosis by pharmacological or other means results in subtle increase in ¹H NMR detectable lipid signals³²⁻³⁴. ¹H 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 lipids³⁴. Because NMR detects lipids only in specific cellular compartments, i.e. lipid vesicles^{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 ¹H NMR lipids, most notable polyunsaturated fatty acids (PUFA) concomitantly with generation of intracellular lipid bodies^{37,38}. Recently, ¹H NMR lipids have been shown to increase in experimental lymphomas³⁹ and neuroblastomas during drug-induced apoptosis⁴⁰. It appears that increase in ¹H NMR detected PUFAs is an early sign of apoptotic process preceding tumour growth arrest³⁸. 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 ^1H NMR spectroscopy before expression of cell death. Accumulating PUFAs have chemical structure found in lipids present in mitochondrial membranes ³⁸ suggesting that ^1H NMR detected lipids originate from membrane breakdown products.

Other metabolites in apoptosis

It is not unexpected that many other ^1H NMR detectable metabolite changes occur in apoptotic cells ²⁵. 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 ².

Diffusion MRI

A remarkable observation that diffusion-weighted MR signal increases in the early phase of anti-cancer drug treatment ⁴¹ facilitated use of DWI for monitoring of treatment in experimental tumour models through apoptosis ⁴². DWI has recently been successfully used in assessment of brain tumour treatment in humans ⁴³.

In rat tumour models, correlating histological changes of apoptosis and cell death with MRI ⁴⁴⁻⁴⁶, 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 ⁴⁶. 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 T_2 , as quantified with Hahn single echo MRI, increase in concert ^{45, 46}. 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 T_2 MRI with comparable sensitivity. In some other tumours, where absolute DWI, but only T_2 -weighted MRI has been exploited, diffusion has shown greater sensitivity to cell death than conventional T_2 MRI ⁴⁷. 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_{1ρ} MRI used for imaging of apoptosis in a glioma model ^{48,49}. T_{1ρ} 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_{1ρ} to (chemical) exchange processes ^{50,51}.

Several studies using Hahn single echo T₂ 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 T₂ signal offering improved sensitivity for detection of PCD ⁴⁹. The study by Gröhn *et al* demonstrates that T₂ 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 T₂ and diffusion MRI, bear great clinical potentials. In future molecular imaging, exploiting either ‘smart contrast agents’ (8-10) or hyperpolarised ¹³C 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

| Substance/Variable | Biomarker | Surrogate marker | Reference |
|--|---------------|------------------|-----------|
| Fructose-1,6-bisphosphate | Increase | | 14 |
| CDP-choline | Increase | | 18 |
| Intracellular pH | acidification | | 18,19 |
| ¹ H detected lipids | Increase | | 32,34,38 |
| PUFA | Increase | | 37,38 |
| Glucose uptake/glycolysis | Decrease | | 12,13,16 |
| T _{1ρ} MRI contrast | Increase (?) | Increase | 48, 49 |
| Creatine/taurine/glycine | | Decrease | 25,26 |
| Cholines | | Decrease | 23 |
| ADC | | Increase | 41,43,44 |
| T ₂ or CP-T ₂ MRI contrast | | Increase | 45,49 |

References

1. Schmitt CA. *Nat Rev Cancer*. 2003; **3**: 286-295.
2. Hakumaki JM, Brindle KM. *Trends Pharmacol Sci*. 2003; **24**: 146-149.
3. Thompson CB. *Science*. 1995; **267**: 1456-1462.
4. Sapunar F, Smith IE. *Ann Med*. 2000; **32**: 43-50.

5. Heys SD, Eremin JM, Sarkar TK, Hutcheon AW, Ah-See A, Eremin O. *J Am Coll Surg*. 1994; **179**: 493-504.
6. Arias-Mendoza F, Smith MR, Brown TR. *Acad Radiol*. 2004; **11**: 368-376.
7. Denicourt C, Dowdy SF. *Science*. 2004; **305**: 1411-1413.
8. Blankenberg FG, Katsikis PD, Tait JF *et al*. *Proc Natl Acad Sci USA*. 1998; **95**: 6349-6354.
9. Schellenberger EA, Sosnovik D, Weissleder R, Josephson L. *Bioconjug Chem*. 2004; **15**: 1062-1067.
10. Zhao M, Bearegard DA, Loizou L, Davletov B, Brindle KM. *Nat Med*. 2001; **7**: 1241-1244.
11. Tedeschi G, Lundbom N, Raman R *et al*. *J Neurosurg*. 1997; **87**: 516-524.
12. Wahl RL, Zasadny K, Helvie M, Hutchins GD, Weber B, Cody R. *J Clin Oncol*. 1993; **11**: 2101-2111.
13. Smith IC, Welch AE, Hutcheon AW *et al*. *J Clin Oncol*. 2000; **18**: 1676-1688.
14. Williams SN, Anthony ML, Brindle KM. *Magn Reson Med*. 1998; **40**: 411-420.
15. Ronen SM, DiStefano F, McCoy CL *et al*. *Br J Cancer*. 1999; **80**: 1035-1041.
16. Poptani H, Bansal N, Jenkins WT *et al*. *Cancer Res*. 2003; **63**: 8813-8820.
17. Podo F. *NMR Biomed*. 1999; **12**: 413-439.
18. Anthony ML, Zhao M, Brindle KM. *J Biol Chem*. 1999; **274**: 19686-19692.
19. Barry MA, Eastman A. *Biochem Biophys Res Commun*. 1992; **186**: 782-789.
20. Shukla-Dave A, Poptani H, Loevner LA *et al*. *Acad Radiol*. 2002; **9**: 688-694.
21. Bolan PJ, Meisamy S, Baker EH *et al*. *Magn Reson Med*. 2003; **50**: 1134-1143.
22. Gupta RK, Sinha U, Cloughesy TF, Alger JR. *Magn Reson Med*. 1999; **41**: 2-7.
23. Meisamy S, Bolan PJ, Baker EH *et al*. *Radiology*. 2004; **233**: 424-431.
24. Millis K, Weybright P, Campbell N *et al*. *Magn Reson Med*. 1999; **41**: 257-267.
25. Lehtimäki KK, Valonen PK, Griffin JL *et al*. *J Biol Chem*. 2003; **278**: 45915-45923.
26. Valonen PK, Griffin JL, Lehtimäki KK *et al*. *NMR Biomed*. 2005; **18**: 252-259.
27. Park HJ, Makepeace CM, Lyons, J.C., Song CW. *Eur J Cancer*. 1996; **32A**: 540-546.
28. Gillies RJ, Bhujwala ZM, Evelhoch J *et al*. *Neoplasia*. 2000; **2**: 139-51.
29. van Sluis R, Bhujwala ZM, Raghunand N *et al*. *Magn Reson Med*. 1999; **41**: 743-750.
30. Garcia-Martin ML, Herigault G, Remy C *et al*. *Cancer Res*. 2001; **61**: 6524-6531.
31. Zhou J, Payen JF, Wilson DA, Traystman RJ, Van Zijl PC. *Nat Med*. 2003; **9**: 1085-1090.
32. Blankenberg FG, Storrs RW, Naumovski L, Goralski T, Spielman D. *Blood*. 1996; **87**: 1951-1956.
33. Al-Saffar MMS, Titley JC, Robertson D *et al*. *Br J Cancer*. 2001; **86**: 963-970.
34. Delikatny EJ, Cooper WA, Brammah S, Sathasivam N, Rideout DC. *Cancer Res*. 2002; **62**: 1394-1400.
35. Barba I, Cabanas ME, Arus C. *Cancer Res*. 1999; **59**: 1861-1868.
36. Hakumäki JM, Kauppinen RA. *Trends Biochem Sci*. 2000; **25**: 357-362.
37. Hakumäki JM, Poptani H, Sandmair A-M, Ylä-Herttuala S, Kauppinen RA. *Nature Med*. 1999; **5**: 1323-1327.
38. Griffin JL, Lehtimäki KK, Valonen PK *et al*. *Cancer Res*. 2003; **63**: 3195-3201.
39. Schmitz JE, Kettunen MI, Hu DE, Brindle KM. *Magn Reson Med*. 2005; **54**: 43-50.
40. Lindskog M, Spenger C, Jarvet J, Graslund A, Kogner P. *J Natl Cancer Inst*. 2004; **96**: 1457-1466.

41. Zhao M, Pipe JG, Bonnett J, Evelhoch JL. *Br J Cancer*. 1996; **73**: 61-64.
42. Kauppinen RA. *NMR Biomed*. 2002; **15**: 6-17.
43. Chenevert TL, Stegman LD, Taylor JM *et al*. *J Natl Cancer Inst*. 2000; **92**: 2029-2036.
44. Chenevert TL, McKeever PE, Ross BD. *Clin Cancer Res*. 1997; **3**: 1457-1466.
45. Poptani H, Puumalainen A-M, Gröhn OHJ *et al*. *Cancer Gene Ther*. 1998; **5**: 101-109.
46. Valonen PK, Lehtimäki KK, Vaisanen TH *et al*. *J Magn Reson Imag*. 2003; **19**: 389-396.
47. Ross BD, Chenevert TL, Rehemtulla A. *Eur J Cancer*. 2002; **38**: 2147-2156.
48. Hakumäki JM, Grohn OH, Tyynelä K, Valonen P, Ylä-Herttuala S, Kauppinen RA. *Cancer Gene Ther*. 2002; **9**: 338-345.
49. Grohn OHJ, Valonen PK, Lehtimäki KK *et al*. *Cancer Res*. 2003; **63**: 7571-7574.
50. Knispel RR, Thompson RT, Pintar MM. *J Magn Reson*. 1974; **14**: 44-51.
51. Mäkelä HI, Gröhn OHJ, Kettunen MI, Kauppinen RA. *Biochem Biophys Res Comm*. 2001; **289**: 813-818.
52. Poptani H, Duvvuri U, Miller GG *et al*. *Acad Radiol*. 2001; **8**: 42-47.
53. Bartha R, Michaeli S, Merkle H *et al*. *Magn Reson Med*. 2002; **47**: 742-750.
54. Ardenkjaer-Larsen JH, Fridlund B, Gram A *et al*. *Proc Natl Acad Sci U S A*. 2003; **100**: 10158-10163.