The impact of hypoxia on cell death pathways

Colin R. Lenihan and Cormac T. Taylor
School of Medicine and Medical Science, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland

Abstract
Hypoxia is a frequently encountered feature of the cellular microenvironment in a number of pathophysiological processes in which programmed cell death (apoptosis) affects disease progression including, but not limited to, cancer, chronic inflammation, myocardial infarction, stroke and ischaemic acute kidney injury. In these diseases, the presence of hypoxia can significantly affect the rate of cell death and thus may make a significant contribution to disease progression. In the present review, we discuss the complex relationship that exists between the presence of hypoxia and the regulation of cell death pathways.

Introduction
Apoptosis or programmed cell death is an active process that occurs in response to either internal cell stresses such as DNA damage or external ‘death signals’ generated by cytokines or immune cells. Necrosis is the other major cell death mechanism and is a passive process. Apoptosis is distinguished morphologically from necrosis by the presence of condensed fragmented nuclei, cell shrinkage and apoptotic body formation, changes that all occur prior to the loss of plasma membrane integrity [1] (Figure 1).

Pathways to apoptosis
Apoptosis is a highly regulated process that serves an important physiological function in the removal of redundant, infected, injured and transformed cells and is vital for normal development and homeostasis [2]. Cellular apoptosis can be triggered by two distinct mechanisms termed the extrinsic and intrinsic pathways respectively (Figure 2). The extrinsic pathway is activated through cell-surface death receptors such as the Fas (CD95) and TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) receptors. Activation of the extrinsic pathway is an important end point of immune surveillance, for example, cytotoxic T-cells and natural killer cells possess cell-surface death receptor ligands that allow them to cull transformed and virus-infected cells [3].

In contrast, the intrinsic pathway is primarily activated in response to cell stress or injury such as growth factor deprivation, DNA damage or toxin exposure [4]. Activation of the intrinsic apoptotic pathway depends on the cellular balance of pro- and anti-apoptotic members of the Bcl (B-cell lymphoma) protein family [4]. Anti-apoptotic Bcl proteins include Bcl-1, Bcl-2, Bcl-xL and Bcl-B and pro-apoptotic members include Bak (Bcl-2 homologous antagonist/killer), Bax (Bcl-2-associated X protein), Bad (Bcl-2-associated death promoter) and Bid (BH3-interacting domain death agonist). Bcl proteins contain functionally important α-helical segments called BH domains. All family members have a BH3 domain, whereas other multidomain members also contain BH1, BH2 or BH4 domains. The BH-domains of anti-apoptotic Bcl proteins form a hydrophobic pocket that binds the BH3 domain of their pro-apoptotic counterparts [5]. This interaction effectively sequesters the pro-apoptotic proteins and renders them inactive. The initiation of apoptosis depends on the ratio of pro-apoptotic to anti-apoptotic Bcl members [6].

Bax and Bak are both multidomain pro-apoptotic proteins and are responsible for mitochondrial permeabilization, which is the defining step in intrinsic pathway mediated apoptosis [7]. Bax/Bak-deficient cells do not have a functional...
Apoptosis is triggered via either external cell-surface stimuli such as TNF (extrinsic pathway) or internal stimuli such as DNA damage (intrinsic pathway). Both pathways end in activation of the caspase family of proteolytic enzymes and an organized disassembly of the cell. Apoptosis is characterized by nuclear and cell shrinkage, the break-off of plasma-membrane-lined cell fragments called apoptotic bodies and the preservation of membrane integrity. Apoptotic cells are ultimately removed by neighbouring cells and phagocytes. The maintenance of plasma membrane prevents local leakage of potentially injurious cellular materials. In contrast, necrosis is a passive process that can occur in response to multiple cellular insults including ischaemia and toxin exposure. Necrosis is a chaotic process that often results in leakage of cellular contents (including lysosomal enzymes) into the surrounding area. Necrosis is characterized by cellular and mitochondrial swelling, calcium influx, activation of calcium-dependent proteases and early loss of plasma membrane integrity.

Once activated, cellular apoptosis is executed by a family of cysteine proteases called the caspases. The caspases are activated as a cascade that begins with the cleavage of ‘activator caspases’, which cleave downstream ‘effector caspases’. Effector caspases target and cleave numerous cellular proteins and give rise to the characteristic pattern of apoptotic cell death [11].

The extrinsic and intrinsic signalling pathways utilize a number of distinct caspase family members. Extrinsic signalling culminates in the formation of the death-inducing signalling complex and the activation of caspase 8 with consequent activation of downstream effector caspases [12]. In intrinsic apoptosis, the externalization of mitochondrial cytochrome c allows it to interact with APAF-1 (apoptotic protease-activating factor-1) and caspase 9 to form the ‘apoptosome’, the resulting cleavage of caspase 9 leads to downstream activation of effector caspases [11].

Although the extrinsic and intrinsic pathways respond to different triggers and utilize distinct caspases, there is significant cross-talk between the pathways. For example, the extrinsic pathway is amplified by caspase-8-mediated cleavage and activation of Bid, which results in downstream mitochondrial permeabilization [13].

Hypoxia
Hypoxia arises when oxygen supply fails to meet physiological requirements. In normal healthy humans, there is broad intra-tissue variability in oxygen supply and demand. Different tissues have different hypoxic thresholds; for instance, a partial pressure of oxygen that is normal for the renal medulla (∼10 mmHg) would be pathologically low in the myocardium [14,15]. Hypoxia limits the ability of aerobic cells to generate ATP through oxidative phosphorylation and complicates many common disease processes. Hypoxia is a key pathophysiological feature of
numerous diseases, including ischaemia/reperfusion injury, cancer and inflammatory bowel disease.

Transcriptional response to hypoxia

**HIF (hypoxia-inducible factor)**

Hypoxia leads to changes in transcription that help a cell to adapt to low oxygen concentrations. Acute hypoxic adaptation is largely mediated through the transcription factor HIF-1α. HIF-1α mRNA and protein are produced continuously and at a relatively high level in all cells; however, in the presence of available oxygen, it is instantaneously hydroxylated by the PHD (prolyl hydroxylase) enzymes and rapidly degraded via the ubiquitin–proteasome pathway [16]. When oxygen concentrations decrease, the oxygen-dependent PHD s are inactivated and HIF-1α protein accumulates and translocates to the nucleus, where it interacts with HIF-1β/ARNT (aryl hydrocarbon receptor nuclear translocator) and p300 and binds HREs (hypoxia-response elements) in the promoter regions of target genes [17,18]. HIF-1 target genes improve cellular oxygenation by improving oxygen delivery through enhanced angiogenesis (vascular endothelial growth factor), haematopoiesis (erythropoietin) and local vasodilatation (iNOS; inducible nitric oxide synthase) and bolster anaerobic ATP production through enhanced expression of genes that promote glycolysis (e.g. glucose transporter 1 and pyruvate dehydrogenase kinase) [16]. HIF-1 also reduces cellular workload by inducing cell-cycle arrest through displacement of the oncoprotein c-Myc from its promoter on the p21 gene [19].

The HIF-2α transcription factor shares significant sequence homology with HIF-1α, but differs in a number of important respects. In contrast with HIF-1, HIF-2 is not ubiquitously expressed; it interacts with distinct cofactors and transactivates a different pattern of genes [20]. For instance, HIF-2 does not regulate genes involved in glycolysis, but is the main regulator of erythropoietin in the adult kidney and, in direct contrast with HIF-1, increases cell proliferation through c-Myc [21,22].

**NF-κB (nuclear factor κB)**

NF-κB is central to the function of the human immune system and regulates the expression of cytokines, growth factors, immune-effector enzymes and anti-apoptotic factors in response to cellular signals [23]. Under unstimulated
In unstimulated cells, NF-κB is sequestered in the cytoplasm by IκB. Inflammatory stimuli such as TNFα, IL-1 and LPS (lipopolysaccharide) result in the phosphorylation and activation of the enzyme IKKβ. Activated IKKβ results in the phosphorylation and degradation of IκB. Liberated NF-κB subunits are then free to translocate to the nucleus. Under normoxic conditions, IKKβ phosphorylation is inhibited in a PHD-dependent manner. Hypoxia releases IKKβ from this inhibition resulting in enhanced NF-κB.

Two pathways, termed canonical and non-canonical, result in NF-κB nuclear accumulation and gene transcription; however, only the canonical pathway is activated by hypoxia [24]. The key step in activation of the canonical NF-κB activation pathway is the phosphorylation of IκB by the IKK (IκB kinase) enzyme complex. IKKβ is a member of a complex of three IKK enzymes (IKKα, IKKβ and IKKγ), which are all involved in NF-κB regulation. IKKβ is activated by the NIK (NF-κB-inducible kinase) phosphorylation of its Ser177 and Ser181 residues. NIK activation occurs as a downstream response to a number of inflammatory stimuli such as TNFα (tumour necrosis factor α) and IL-1 (interleukin-1) [23] (Figure 3).

Mechanisms linking hypoxia to NF-κB-dependent inflammatory processes have until recently remained poorly understood [25]. However, IKKβ is both phosphorylated and induced at low oxygen tensions. Cellular hypoxia leads to the activation of IKKβ and subsequent phosphorylation and degradation of IκB. The resultant increase in NRE (NF-κB/Rel enhancer) binding is hydroxylase-dependent. The relative contributions of the three PHD enzymes to NF-κB activation were assessed using isoform-specific knockdowns and revealed PHD1 knockdown to be the most potent stimulus. Whether IKKβ is a direct target of the PHDs remains to be determined; however, sequence analysis of IKKβ reveals a consensus motif resembling the hydroxylation site on HIF-1α (LXXLAP) and mutation of this site results in loss of IKKβ induction in hypoxia [25].

**Hypoxia and cell death**

A key question with important implications is: does hypoxia in solution affect cell death and, if so, through what mechanism? Cell viability experiments using different cell types and oxygen concentrations have unsurprisingly yielded various results. Anoxia or near anoxia (O2 <0.1%) induces both necrosis and apoptosis in cultured cells [26,27]. Anoxia-induced apoptosis is associated with increased mitochondrial permeability and leakage of cytochrome c into the cytoplasm and is dependent on the presence of caspase 9, but independent of caspase 8, suggesting intrinsic apoptotic pathway activation [28].

However, it is remarkable how well many cell types tolerate prolonged periods of low oxygen tension (1% O2), often requiring the presence of additional stressors such as serum deprivation or acidosis before cell death occurs [29–31]. This capacity for cells to resist cell death in the setting of low oxygen tensions is the result of a well-developed...
cellular adaptive response, a phenomenon that is important in many disease processes. This adaptive response is largely mediated by HIF-1. Hypoxia also influences a number of other transcription factors that are important in the cell death decision-making process such as the HIF-2, NF-κB and p53 transcription factors. In many complex disease processes, it is the development of this hypoxic phenotype rather than the direct lethality of hypoxia itself that has the most effect on cell survival and disease natural history.

**HIF and apoptosis**

Although studies display some heterogeneity, probably as a result of different cell types and oxygen tensions studied, the balance of evidence supports the inhibitory role of HIF in the regulation of hypoxic cell death. Importantly, in cell culture, the survival benefit attributable to HIF-1 is lost under anoxic or near anoxic conditions (<0.01%), since the pro-survival effect of HIF is presumably contingent on there being some environmental oxygen to conserve [27,32]. However, in moderate hypoxia (O₂ = 2%), wild-type cells sustain higher intracellular oxygen concentrations than their HIF-1-deficient counterparts and this oxygen conservation translates into increased cell survival when viability is compared in a culture system with finite oxygen availability [33]. Similarly, cells transfected with short hairpin RNA targeting HIF-1 have significantly reduced cell survival under hypoxic conditions compared with sham-transfected controls [34]. However, HIF-1 activation also reduces cell death in response to many other known inducers of apoptosis including chemotherapeutic agents, an effect that promotes resistance to chemotherapy-induced apoptosis [45]. The anti-apoptotic Bcl family member Bcl-xL is up-regulated in constitutive HIF-1-expressing prostate cancer cells. Bcl-xL up-regulation is reversed with HIF-1 knockdown and correlates with enhanced UV radiation-induced apoptosis [46].

In addition, p53, a tumour suppressor central to the regulation of the cell cycle, DNA repair and apoptosis is phosphorylated and stabilized in response to a number of cell stressors including hypoxia [47]. Hypoxic p53 stabilization is a HIF-1-dependent process that results in apoptosis through the repression of as yet unidentified pro-survival factors [48,49].

Data on HIF-2 are more limited in regard to apoptosis. HIF-2 stabilization has been shown to inhibit apoptosis through a variety of mechanisms that include the up-regulation of the anti-apoptotic Bcl-xL and the enhanced degradation of p53 [50,51]. However, in HIF-2-expressing tumours, the anti-apoptotic effect is not prominent and tends to be overshadowed by its dramatic effect on cell proliferation [52].

However, the pro-apoptotic effects of BNIP3 and BNIP3L have been called into question with experimental overexpression of BNIP3, BNIP3L or indeed HIF-1 failing to induce apoptosis in many cell lines [27,43]. Such experimental inconsistency and the somewhat counterintuitive notion that the pro-survival transcription factor HIF-1 would induce pro-apoptotic gene products has led to a reappraisal of the function of BNIP and BNIP3L and an interest in their role in hypoxic autophagy [43]. Autophagy is a process where cellular organelles are degraded by the cell’s own lysosomal machinery in an organized manner; the protein beclin is the key initiator of autophagy and is usually sequestered inactive in the cytoplasm by Bcl-2 protein [44]. In hypoxia, BNIP3 and BNIP3L are induced and compete with the Bcl-2–beclin complex to release free beclin and initiate autophagy. Preventing autophagy by silencing beclin actually increases cell death in hypoxia [43]. Therefore cellular autophagy seems to be acting to streamline cellular organelles, reduce metabolic demand and improve hypoxic tolerance and is probably a further example of a HIF-1-dependent pro-survival effect.

HIF-1 has also been reported to regulate some other Bcl family members. In hypoxic colon cancer cell lines, the expression of the pro-apoptotic Bcl family member Bid is repressed in a HIF-1-dependent manner, an effect that promotes resistance to chemotherapy-induced apoptosis [45]. The anti-apoptotic Bcl family member Bcl-xL is up-regulated in constitutive HIF-1-expressing prostate cancer cells. Bcl-xL up-regulation is reversed with HIF-1 knockdown and correlates with enhanced UV radiation-induced apoptosis [46].

NF-κB and apoptosis

NF-κB is a key regulator of inflammation and cell death decision-making and its regulation by PHD1 represents an important link between hypoxia and cell death regulation [23,25]. NF-κB activation, with certain exceptions, exerts a potent anti-apoptotic effect.

In cells stimulated by TNFα, TNFR1-binding activates the extrinsic apoptotic pathway. However, cellular TNFα stimulation rarely results in apoptosis because concurrent activation of NF-κB and expression of its gene product c-FLIP
(cellular FLICE [FADD (Fas-associated death domain)-like IL-1β-converting enzyme]-inhibitory protein), a potent inhibitor of caspase 8, effectively blocks the extrinsic pathway apoptosis. Notably, TNFα does become a potent inducer of apoptosis in cells either lacking c-FLIP or a functional canonical NF-κB pathway, further supporting the importance of cross-talk between the NF-κB and extrinsic apoptotic signalling pathways [53].

NF-κB also inhibits caspase-independent cell death signalling via its cross-talk with the JNK (c-Jun-N-terminal kinase)/MAPK (mitogen-activated protein kinase) pathway. The JNK/MAPK cascade is activated by TNFα and a range of other toxic cell stimuli and has divergent biological roles in cell development and growth; however, its most prominent function is the induction of cell death either through apoptosis or necrosis [54]. However, mirroring its inhibitory effect on TNFα-induced extrinsic apoptosis, activation of NF-κB results in inhibition of JNK signalling and blocks the downstream cell-death stimulus [55]. In the absence of a functional NF-κB pathway TNFα results in unopposed JNK activation and cell death [56].

NF-κB also results in the expression of a number of anti-apoptotic Bcl proteins including Bcl-2, Bcl-xL and NR13, which bind and sequester pro-apoptotic Bcl family members and increase the cell’s apoptotic threshold [57]. In addition, NF-κB results in the expression of a number of members of the IAP (inhibitor of apoptosis protein) family including c-IAP (cellular IAP) 1, c-IAP2 and x-IAP (X-linked IAP) [58]. IAPs directly block the cleavage (and activation) of the caspases, thus inhibiting apoptosis [59]. Notably, c-IAP2 is up-regulated in hypoxia in an NF-κB-dependent manner [60].

Finally, NF-κB also influences cell death decision-making through its effects on other transcription factors. NF-κB activation results in enhanced expression of Mdm2 (murine double minute 2), an E3 ubiquitin ligase and potent inhibitor of p53, causing a reduction in p53 activation [61]. In addition, NF-κB directly increases expression of HIF-1α mRNA and is necessary for physiological HIF-1α function [62].

Conclusion
Hypoxia is key in the pathophysiology of many common and important diseases where altered cell death contributes to disease progression. A better understanding of the cellular transcriptional response to hypoxia and specifically the mechanisms underlying its impact on hypoxic cell death will play a key role in developing new approaches aimed at controlling cell death for therapeutic benefit.

Funding
This work has received financial support from Science Foundation Ireland [grant number SPI/11/PI/1005 to C.T.T.].

References
2. Fuchs, Y. and Steller, H. Programmed cell death in animal development and disease. Cell 147, 742-758
12. Krammer, P.H., Arnold, R. and Lavrik, I.N. (2007) Life and death in the JNK (c-Jun-N-terminal kinase) pathway TNFα results in enhanced expression of Mdm2 (murine double minute 2), an E3 ubiquitin ligase and potent inhibitor of p53, causing a reduction in p53 activation [61]. In addition, NF-κB directly increases expression of HIF-1α mRNA and is necessary for physiological HIF-1α function [62].

Conclusion
Hypoxia is key in the pathophysiology of many common and important diseases where altered cell death contributes to disease progression. A better understanding of the cellular transcriptional response to hypoxia and specifically the mechanisms underlying its impact on hypoxic cell death will play a key role in developing new approaches aimed at controlling cell death for therapeutic benefit.

Funding
This work has received financial support from Science Foundation Ireland [grant number SPI/11/PI/1005 to C.T.T.].

References
2. Fuchs, Y. and Steller, H. Programmed cell death in animal development and disease. Cell 147, 742-758
12. Krammer, P.H., Arnold, R. and Lavrik, I.N. (2007) Life and death in the JNK (c-Jun-N-terminal kinase) pathway TNFα results in enhanced expression of Mdm2 (murine double minute 2), an E3 ubiquitin ligase and potent inhibitor of p53, causing a reduction in p53 activation [61]. In addition, NF-κB directly increases expression of HIF-1α mRNA and is necessary for physiological HIF-1α function [62].

Conclusion
Hypoxia is key in the pathophysiology of many common and important diseases where altered cell death contributes to disease progression. A better understanding of the cellular transcriptional response to hypoxia and specifically the mechanisms underlying its impact on hypoxic cell death will play a key role in developing new approaches aimed at controlling cell death for therapeutic benefit.

Funding
This work has received financial support from Science Foundation Ireland [grant number SPI/11/PI/1005 to C.T.T.].
34 Liao, D., Corle, C., Seagroves, T.N. and Johnson, R.S. (2007) Hypoxia-inducible factor-1α mediates aerobic inhibition of Beclin 1-dependent autophagy. Cell 130, 533–544
40 Mennad, H., Wernis, C., Schmid, T., Copanaki, E., Deller, T., Dehne, N. and Brune, B. Roles of hypoxia-inducible factor-1α (HIF-1α) versus HIF-2α in the survival of hepatocellular carcinoma cells. Hepatology 51, 2183–2192
46 Javelaud, D. and Besancon, F. (2001) NF-κB activation results in rapid inactivation of JNK in TNFα-treated Ewing sarcoma cells: a mechanism for the anti-apoptotic effect of NF-κB. Oncogene 20, 4365–4372
47 Kucharczak, J., Simmons, M.J., Fan, Y. and Gelin, C. (2003) To be, or not to be: NF-κB is the answer – role of Rel/NF-κB in the regulation of apoptosis. Oncogene 22, 8961–8982

Received 22nd December 2012 doi:10.1042/BST20120345