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Visions & Reflections (Minireview)

'Heated' Debates in Apoptosis

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Abstract. Hippocrates' assertion that 'what the lance does not heal, fire will' underscores the fact that for thousands of years heat has been used to treat a variety of diseases, including cancer. Indeed, spontaneous tumor remission has been observed in patients following feverish infection [1], and expression of activated oncogenes, such as Ras, can render tumor cells sensitive to heat compared with normal cells [2, 3]. In the past, a primary drawback to the use of heat as a clinical therapy was the inability to selectively focus heat to tumors *in situ*. Of late, however, several approaches have been devised to deliver heat more precisely, including the use of heated nanoparticles, making hyperthermia a more clinically tractable treatment option [4, 5]. Despite these practical advances, the mechanisms responsible for heat shock-induced cell death remain controversial and ill-defined. In this *Visions and Reflections* we discuss recent findings surrounding the initiation of heat shock-induced apoptosis, and propose future areas of research.

Keywords. Hyperthermia, apoptosis, caspases, Hsp70, c-Jun N-terminal kinases.

Heat shock induces of variety of stress responses in mammalian cells, depending upon the temperature and length of exposure. For example, mild heat shock (38 – 42 °C) induces the expression of heat shock proteins (hsps), such as Hsp27, Hsp70, and Hsp90, and these protein chaperones often times render cells thermotolerant and resistant to subsequent stressful stimuli, including chemotherapeutic agents [6]. However, these compensatory mechanisms fail to prevent cell death following exposure to more intense or prolonged heat shock. Therefore, heat shock has been used effectively in combination with chemo- and radiotherapy for the treatment of various cancers [7, 8]. In general, cancer cells exposed to temperatures >42 °C will undergo cell death, but as the temperature rises, the percentage of cells undergoing apoptosis decreases with a concomitant increase in necrosis [9]. Slight variations in this response can occur, depending upon other factors, such as prior exposure to heat, type of tumor, cell line, and/or stage of the cell cycle [9 – 11]. Apoptotic pathways are usually triggered when a stimulus activates an apical or 'initiator' cysteinyl aspartate-specific protease (caspase), and the extrinsic or intrinsic pathway unfolds. In the extrinsic pathway, death receptors located in the plasma membrane, such as Fas, death receptors-4 or -5 (DR4/5), and tumor necrosis factor (TNF) receptor 1 (TNFR1) are activated by their cognate ligands: Fas ligand (FasL), TNF-related apoptosis-inducing ligand

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(TRAIL), and TNF, respectively. Upon activation, the adapter protein Fas-associated death domain (FADD) is directly recruited to most death receptors to form complexes referred to as the 'death-inducing signaling complexes' (DISCs), which in turn recruit and activate the initiator caspases-8 and -10 [12, 13]. The intrinsic pathway is triggered by stress-inducing stimuli which activate proapoptotic Bcl-2 family members, such as Bax and Bak, and in turn stimulate mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c [14]. Once in the cytoplasm, cytochrome c binds to apoptotic protease-activating factor-1 (Apaf-1), along with dATP or ATP, and induces oligomerization of Apaf-1 into a large heptameric complex (referred to as the 'apoptosome'), which subsequently recruits and activates the initiator caspase-9 [15]. Following their activation, caspases-8 and -9 then process and activate the downstream 'effector' caspases-3, -6, and -7, which are responsible for proteolytically dismantling the cell.

Previous studies have implicated both the extrinsic and intrinsic pathways in heat shock-induced apoptosis, and many have focused in particular on the synergistic 'cross-talk' that exists between death receptors, chemotherapeutics, and heat shock responses. For example, downregulation of heat shock factor-1 (HSF-1) by TNF or RNA interference sensitizes some cancer cell lines to treatment with heat shock and/or cisplatin [16, 17], whereas heat shock sensitizes cells to FasL by downregulating FLIP, a dominant negative inhibitor of caspase-8 activation within the DISC [18]. Although these reports are clearly relevant to combination therapy, more recent work now suggests that heat alone may induce apoptosis through more novel pathways. Using a biotinylated analog of the polycaspase inhibitor VAD-fmk, Tu and colleagues isolated caspase-2 as the apical protease following heat shock and found that it was activated upstream of mitochondria, since both Bcl-2 and Bcl-x_L (antiapoptotic Bcl-2 family members) failed to prevent the activation of caspase-2 [19]. Moreover, they implicated both caspase-2 and its adapter protein RIP-associated ICH-1/CED-3-homologous protein with a death domain (RAIDD) in heat shock-induced apoptosis, since both activated splenocytes and mouse embryonic fibroblasts (MEFs) from *caspase-2*$^{-/-}$ and *raidd*$^{-/-}$ mice were largely resistant to heat shock [19]. As a result, the authors proposed a model wherein the upstream activation of caspase-2 resulted in MOMP and initiation of the classical intrinsic pathway (Fig. 1). To account for the residual cell death, they (and others) proposed that caspase-8 was also activated through a paracrine feedback loop involving Fas-FasL [19, 20] and that it could partially compensate for a loss in caspase-2 activity [19]. Indeed, addition of soluble Fas-Fc to the culture medium of heat-shocked cells inhibited caspase-8 activation in *caspase-2*$^{-/-}$ splenocytes (but surprisingly not in *raidd*$^{-/-}$ splenocytes) and further suppressed cell death in the *caspase-2*$^{-/-}$ MEFs [19].

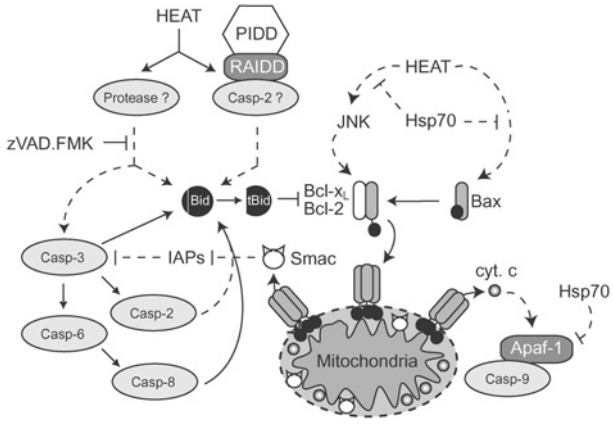

Figure 1. Model of heat shock-induced apoptosis. Heat shock activates an apical protease, positioned above mitochondria, which in turn mediates MOMP in part through the cleavage and activation of Bid. The identity of this apical protease remains unclear, but may be procaspase-2. Heat may also directly activate Bax or Bak or may do so indirectly through the activation of JNKs. How JNKs are activated in response to heat, how they activate Bax, and how Hsp70 inhibits this process remains unclear. Both Bcl-2 and Bcl-x_L inhibit heat shock-induced MOMP and cell death by antagonizing Bax or Bak. Interestingly, however, neither caspase-9 nor its adapter protein Apaf-1 are required for cell death, suggesting that release of IAP antagonists, such as Smac, may be important for promoting caspase activity and cell death through inhibition of IAPs.

In concurrent studies, however, our group found no evidence for increased resistance of *caspase-2*$^{-/-}$ or *raidd*$^{-/-}$ MEFs to heat shock-induced apoptosis [21] [S. Mahajan and S. B. Bratton, unpublished observations]. Heat shock also failed to induce formation of a stable RAIDD●caspase-2 complex in human Jurkat T cells, and neither depletion of caspase-2 by RNA interference (RNAi) nor pharmacological inhibition of caspase-2 with Z-VDVAD-fmk prevented MOMP or cell death. Furthermore, FADD and caspase-8-deficient Jurkat T cells remained sensitive to heat shock, and knock-down of caspase-2 expression in the caspase-8-deficient cells failed to inhibit cell death, indicating that at least in these cells, caspase-8 was not required for cell death and did not compensate for caspase-2 [21]. However, Z-VAD-fmk did attenuate heat shock-induced MOMP and completely inhibited cell death. Thus, there was general agreement that heat shock activated a Z-VAD-fmk-inhibitable apical protease, upstream of mitochondria, but disagree-

ment over the requirement of caspases-2 and -8 for MOMP and cell death (Fig. 1).

Ironically, controversy surrounding caspase-2 and its role in apoptosis is nothing new for this enigmatic protease [22]. Numerous reports have suggested, for example, that caspase-2 is the apical protease in DNA damage-induced apoptosis [23–25] and that caspase-2 is activated within a large PIDDosome complex, composed of RAIDD and the scaffold protein, p53-induced protein with a death domain (PIDD) [24, 26]. However, an equally large number of studies have found no connection between caspase-2 and DNA damage-induced cell death [19, 27–29]. One possibility is that caspase-2 may participate in an important amplification loop in some cells. Indeed, caspase-2 is a direct substrate of caspase-3 [30], and cleavage of caspase-2 following heat shock is dependent upon caspase-3 activity [21]. Active caspase-2 does not efficiently process any caspase, other than itself, but it can weakly process and activate the proapoptotic Bcl-2 family member Bid [31, 32]. Thus, if activated by caspase-3, caspase-2 might cooperate with caspases-3 and -8 to cleave Bid and consequently induce Bax or Bak-dependent MOMP (Fig. 1). Consistent with this notion, *bid*$^{-/-}$ MEFs are resistant to cell death induced by caspase-2 overexpression and heat shock [32]. However, it has also been reported that processed but catalytically inactive caspase-2 can stimulate MOMP in isolated mitochondria [33]. Thus, the relative contribution of caspase-2 to Bid cleavage and to heat shock-induced MOMP in particular remains unclear.

Alternatively, it has been argued that procaspase-2, when bound to its adapter protein RAIDD within the PIDDosome, might exhibit catalytic activity in the absence of autoprocessing [24]. In other words, procaspase-2 might be active prior to autocatalytic cleavage or processing by caspase-3. However, since there are currently no good tools to selectively measure the catalytic activity of procaspase-2 in cells, this notion is largely based on previous studies with procaspase-9, in which non-cleavable mutants of procaspase-9 were shown to be catalytically active when bound to Apaf-1 [34, 35]. It is important to emphasize, however, that wild-type procaspase-9 is rapidly processed within the apoptosome. Thus, it appears unlikely that procaspase-2 could remain bound to RAIDD and active for long periods of time without undergoing observable autocatalytic processing. In short, though caspase-2 clearly binds to RAIDD, the role of RAIDD●caspase-2 PIDDosome complexes in promoting DNA damage or heat shock-induced apoptosis remains controversial and warrants further investigation.

With regard to heat shock-induced MOMP, Pagliari and colleagues find that heat can also directly activate recombinant Bax and Bak *in vitro* and stimulate MOMP in isolated mitochondria [36]. Moreover, they find that Bcl-x_L normally binds to and inhibits activated Bax and Bak and that truncated Bid (formed presumably *via* the action of caspase-2) is sufficient to promote MOMP by antagonizing Bcl-x_L (Fig. 1) [36]. Interestingly, Hsp70 also appears to inhibit the activation of Bax. However, Hsp70 does not directly interact with Bax either in control or heat-shocked cells, suggesting that Hsp70 indirectly suppresses Bax activation [37]. One possibility is that Hsp70 may inhibit the c-Jun N-terminal kinases (JNKs), which are known to be activated following heat shock and are proapoptotic under certain circumstances [37, 38]. Indeed, JNKs reportedly activate various 'BH3-only' proteins, including Bid, Bim, and Bmf, all of which promote the activation of Bax and induce MOMP (Fig. 1) [39–41]. Nevertheless, precisely how JNKs are activated in response to heat shock and what the specific target(s) of JNKs are in this context remains largely unknown.

It is somewhat difficult to reconcile how Hsp70 could inhibit heat shock-induced cell death by acting solely upstream of Bax, if in fact heat can directly activate Bax *in vivo*. One possibility is that Hsp70 might inhibit the activation of the Z-VAD-fmk-inhibitable apical protease (be it caspase-2 or another protease), or alternatively, Hsp70 might act downstream of MOMP. Several reports have suggested that Hsp70 can directly inhibit the formation and/or activity of the apoptosome, but these studies have recently been questioned [42–44]. Moreover, although the apoptosome may contribute to the activation of caspases, neither Apaf-1 nor caspase-9 is essential for heat shock-induced cell death, and procaspase-9 does not undergo apoptosome-dependent processing in some cells [21]. Therefore, the specific mechanisms through which Hsp70 inhibits heat shock-induced cell death are still being actively pursued. Given that MOMP is essential for heat shock-induced apoptosis, release of inhibitor of apoptosis (IAP) antagonists such as Smac/DIABLO, rather than cytochrome c, may be critical for promoting caspase activity, as has been observed during FasL-dependent signaling [21, 45].

Concluding remarks: Slight differences in temperature or duration of heat treatment can produce variations in the cell death outcome. Therefore, defining the molecular mechanisms that mediate heat shock-induced apoptosis is critical for better understanding its use as a clinical therapy, and importantly, could lead to the discovery of drugs that mimic the heat shock response and trigger a more homogeneous cell death. The recent studies described above, though at times contradictory and controversial, nevertheless serve as an important backdrop for

future studies in this intriguing and exciting area of cancer therapy.

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