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Caspase-8 as a Regulator of Tumor Cell Motility

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Abstract

The caspases are a family of ubiquitously expressed cysteine proteases best known for their roles in programmed cell death. However, caspases play a number of other roles in vertebrates. In the case of caspase-8, loss of expression is an embryonic lethal phenotype, and caspase-8 plays roles in suppressing cellular necrosis, promoting differentiation and immune signaling, regulating autophagy, and promoting cellular migration. Apoptosis and migration require localization of caspase-8 in the periphery of the cells, where caspase-8 acts as part of distinct biosensory complexes that either promote migration in appropriate cellular microenvironments, or cell death in inappropriate settings. In the cellular periphery, caspase-8 interacts with components of the focal adhesion complex in a tyrosine-kinase dependent manner, promoting both cell migration *in vitro* and metastasis *in vivo*. Mechanistically, caspase-8 interacts with components of both focal adhesions and early endosomes, enhancing focal adhesion turnover and promoting rapid integrin recycling to the cell surface. Clinically, this suggests that the expression of caspase-8 may not always be a positive prognostic sign, and that the role of caspase-8 in cancer progression is likely context-dependent.

Keywords

Cell Migration; Phosphorylation; Calpain; SH2 domain; Nonapoptotic; Caspase 8

Caspase-8 and the Cellular Microenvironment

Tissues may be defined by their unique “zip codes,” or specific combinations of extracellular matrix (ECM) proteins, cell adhesion molecules, and matricellular proteins [1]. The ECM proteins may be bound by cell surface receptors that include integrins, immunoglobulin superfamily members, and CD44, as well as other, often tissue specific receptors. Cellular behavior is influenced by the receptors present on a cell, in concert with the ECM components present in a tissue. When cells become detached from their ECM they undergo anoikis [2]. This cell death serves as an important mechanism to promote tissue homeostasis by keeping cells types confined to their correct micro-environmental niches. However, cancer cells often evade anoikis, allowing them to metastasize to distant organs. The exact mechanism underlying this process is not well understood.

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Anoikis is a special form of apoptosis, a programmed cell death that has been studied extensively [2, 3]. Apoptotic cell death is characterized by the activation of a signaling cascade of cysteine proteases known as caspases [4]. The activation of an upstream initiator caspase leads to the activation of downstream target executioner caspases, which then perform specific cleavage events that disable some cellular signaling systems, enable others, and accelerate the cell toward death characterized by cellular blebbing, chromatin condensation, nuclear fragmentation, and lack of a strong immune response [5]. Another special form of apoptosis is induced by unligated or antagonized integrins and is termed: integrin-mediated death (IMD). In contrast to anoikis, cells remain attached to the ECM while initiating cell death in microenvironments lacking the necessary ligands for their integrins. For example, IMD inhibits neuroblastoma cell metastasis *in vivo* [6, 7].

A key mediator for apoptosis and IMD is caspase-8, a protein originally identified as the most upstream enzymatic component of the extrinsic apoptotic pathway. Under normal conditions, caspase-8 is present as a monomeric procaspase zymogen; it is composed of a catalytic domain and two death effector domains comprising the prodomain. The prodomain is important for recruitment to membrane-associated death receptor signaling complexes, of which the death inducing signaling complex (DISC) is the best studied [8, 9]. Binding of caspase-8 to this complex facilitates maturation of the zymogen by proximity induced dimerization and proteolytic cleavage [10, 11]. In the last decade, it has become evident that the zymogen can also be activated by other signals, including those from toll-like receptors [12], antigen receptors [13], microtubule scaffolding of death domains [14], and as mentioned above, unligated integrins.

However, activation of caspase-8 by large macromolecular complexes does not always lead to cell death. Recruitment of caspase-8 to the ripoptosome, a large multi-protein complex, prevents necrotic cell death [15]. Eponymous RIP1 and RIP3 kinases promote cleavage of a variety of substrates to induce necrosis in a caspase-independent manner. Caspase-8 recruitment to the ripoptosome results in proteolytic inactivation of these kinases [15]. Caspase-8 cleavage of RIP1 and RIP3 can be inhibited by specific small molecules or by heterodimerization of caspase-8 with its close but inactive homologue FLIP [16, 17]. The inhibition of necrosis by caspase-8 appears critical during murine development, as deletion of *Ripk3* rescues embryonic lethality in *Casp8* deficient mice [17, 18]. *Casp8* knockout mice die by day 13.5 due to neural and vascular defects. Lethality is attributed to congested accumulation of erythrocytes in various tissues likely due to impaired cardiac muscle development or placental abnormalities [19]. Tissue specific knockouts in the vascular compartment duplicate these findings, suggesting a critical role for caspase-8 during embryonic vascular development [20]. This may result from placental abnormality, since caspase-8 is reported to be required for syncytial fusion [21]. Although *Casp8* $-/-$, *Ripk3* $-/-$ mice are viable, they exhibit enlarged spleens, progressive lymphoproliferative disease, and some failure to thrive. Therefore, although caspase-8 mediated apoptosis is dispensable for mouse development, (reviewed in [22]) the inhibition of necrosis at some key point(s) in murine development appears critical.

Independent of RIP3, RIP1 plays a key role in signaling downstream to the NF- κ B pathway [23], and caspase-8 is known to play a key role in NF- κ B signaling downstream of antigen

receptors [24]. As might be expected from this observation, mice deficient in caspase-8 show an early life lymphodeficient phenotype that transitions with age, yielding lymphoproliferative disease late in life [25]. The latter status is attributed to the lack of proapoptotic activity of the caspase. Together, these findings indicate that caspase-8 is coupled to a variety of signaling cascades enabling cells to react to diverse local microenvironments in a context-dependent manner.

Caspase-8 in Cancer

Prior to the elucidation of the nonapoptotic roles for caspase-8, it was generally considered that caspase-8, as an initiator of cell death, would be deleterious to tumors. This notion was bolstered by early studies in neuroblastomas and medulloblastomas showing frequent silencing or deletion of caspase-8 [26–28]. We mechanistically evaluated the role of caspase-8 regulating tumor progression using the developing chick as a surrogate model for pediatric/pre-natal growth of neuroblastoma. Interestingly, the presence of caspase-8 did not affect initial tumor growth in this model [7]. Moreover, caspase-8 reconstitution of neuroblastoma cells bearing a double deletion of the *CASP8* gene had no impact on primary tumor development. However, the expression of caspase-8 decreased the incidence of viable cells invading local tissues, and dramatically lowered the incidence of distant metastases detected [7]. Similar results were observed in Severe Combined Immunodeficiency (SCID) mouse models using human neuroblastoma cell lines. Interestingly, a decrease in metastatic incidence but increase in metastasis size was observed [29]. When these metastatic cells were cultured *in vitro*, the principle outgrowth had silenced the caspase-8 transgene by methylation, suggesting that either the presence of caspase-8 was a negative selection for metastasis, or that outgrowth *in vitro* selects for caspase-8 deficient neuroblastoma [29].

Among the neuroblastoma cells, the expression of caspase-8 was not a predictor of susceptibility to death-inducing ligands such as TNF α (Tumor Necrosis Factor), TRAIL (TNF Related Apoptosis-Inducing Ligand) or Fas *in vitro*. Rather, this activity of caspase-8 depended upon the expression of integrins, suggesting an IMD-type of mechanism [7]. In contrast to these results, the notion that caspase-8 ‘upregulation’ might benefit cancer patients was gaining traction. Enhanced apoptosis would be of benefit to a number of therapies, and in particular TRAIL-mediated killing of cancer cells [30].

Evaluating the expression of caspase-8 in other tumors, however, one does not find the profound down-regulation as seen in malignant neuroblastoma. In particular, epithelial cancers tend to maintain the expression of caspase-8, and some exhibit elevated caspase-8 expression relative to normal tissues [31]. The observation that caspase-8 is nonetheless more frequently down-regulated in pediatric cancers provides a compelling rationale to explore the possibility that some of its functions may be positively selected during the progression of epithelial tumors in adults.

Caspase-8 Expression Promotes Cell Migration

Among its nonapoptotic roles, such as promoting NF- κ B signaling and suppressing RIPK3 activity, an enhanced capacity for cell migration is observed among cells expressing

caspase-8 [6, 32–36]. Although caspase-8 is not essential for migration, it enhances a pre-existing migration response via modulators of cellular actin dynamics such as Rac [33, 35], the activation of calpain 2, a calcium-dependent protease which promotes focal adhesion turnover [37–40], and Rab-5, a small GTPase that directs early endosome trafficking [35, 36]. These events appear to be coordinately regulated and interdependent [41]. However, the molecular mechanisms by which procaspase-8 influences these events have only recently begun to be appreciated.

Caspase-8 localizes to the leading edge of cellular lamellae [32]. Although the catalytic domain is sufficient for this activity, the prodomains have been suggested to contribute as well. This has been attributed to classical interaction motifs (RXDL) associated with the prodomain-mediated recruitment to membrane complexes [42], and is supported by biochemical, but not subcellular localization studies [43]. The recruitment of the catalytic domain, on the other hand, is dependent upon tyrosine residues, and is compromised by mutations substituting phenylalanines for tyrosines, suggesting a requirement for phosphorylation [32]. Caspase-8 has 12 tyrosine residues in its catalytic domain; significantly more than the 4 found in its nearest human homolog, caspase-10 (Fig. 1). Several tyrosine residues are present in the core caspase fold of the protein, but the majority are present on loop-type structures on the periphery of the procaspase. The tyrosine residue at position 380 is phosphorylated by nonreceptor tyrosine kinases of the Src family [11, 44, 45] and this potentially alters recognition of the maturation cleavage motifs comprising aspartates 374 and 384 within the catalytic domain [11]. Additional tyrosines are located within the dimerization interface of caspase-8, and phosphorylation on these residues might influence the ability to dimerize. Therefore, tyrosine phosphorylation provides one potential mechanism of committing caspase-8 to nonapoptotic pathways, both *in vitro* [11, 44] and *in vivo* [44], possibly by compromising dimerization/maturation [11].

Aside from regulating caspase-8 maturation and homodimerization, phosphorylation on specific residues has the potential to influence caspase-8 interactions with other proteins. One example would be its close homologue c-FLIP. Although generally acknowledged as a caspase-8 ‘inhibitor’, c-Flip forms a dimer with caspase-8 that exhibits high levels of proteolytic activity [47]. Several tyrosine residues fall in the interface between these two proteins (Fig. 2), and phosphorylation at any of these sites would be predicted to prevent dimerization.

Phosphorylation of Caspase-8 Influences its Recruitment to the Cellular Periphery

Besides inhibiting protein-protein interactions, phosphorylation of caspase-8 can also provide sites for phosphotyrosine-binding protein domains such as those of the PTB and SH2 domain families. The SH2 domains in particular have been demonstrated to interact with caspase-8. A phosphorylated caspase-8 peptide encompassing Y380 was shown to bind a number of SH2 domains [34]. Binding might sequester the intersubunit linker containing the Y380 and prevent maturation of the enzyme. This creates an interesting model wherein the oncogenic activation of tyrosine kinases at the membrane could compromise the activation of the most important pool of caspase-8 in the cell, those in the periphery.

Studying caspase-8 phosphorylation by Src *in vitro*, we have now determined that caspase-8 can be phosphorylated on residues other than Y380. Initial clues came from our unpublished observations that caspase-8 phosphorylation can be observed on the mature form of the catalytic domain, in which the fragment containing Y380 is excised by cleavage of the aspartic acid residues at 374 and 384. Both the p18 and p10 subunits, showed reactivity with phosphotyrosine specific reagents. Analysis of literature databases suggests at least one other caspase-8 residue capable of being phosphorylated: Y334 [49]. Together with point mutation experiments on Y293 and Y448 [45], these data collectively suggest that caspase-8 may be phosphorylated on multiple residues. If several SH2 proteins bind to phosphocaspase-8 simultaneously it could be considered a *de facto* adaptor protein.

In fact, we found that the Src-phosphorylated catalytic domain was able to interact with a greater number of SH2 domains than the isolated phosphopeptide corresponding to the D374–D384 region of caspase-8 (Fig. 3), identifying additional SH2 domains which are present in signaling molecules and adaptor proteins. In particular, interactions were frequently seen with Src family kinases (except Lyn), though interactions with Ras-GAP, PLC γ , Shc and p85 α were also observed. The interaction with p85 α , the regulatory adaptor protein associated with p110, was previously observed for the isolated phosphopeptide [34]. This interaction is significant, since binding could stabilize active p110 [50], promoting the interactions of other domains of p85 α with more proteins.

If so, this raises the possibility that activation of tyrosine kinases may usurp the apoptotic function of caspase-8, switching it from an apoptosis-initiator to a supporting member of the tyrosine kinase or lipid kinase signaling cascades. In support of such a notion, cellular stimulation with growth factors or by attachment to extracellular matrix proteins results in transient phosphorylation of caspase-8 [32]. Since phosphorylated caspase-8 has been observed in human tumor sections [44], this alteration of function may be physiologically relevant to tumor development and progression.

Rac Activation is Rab5 and Caspase-8 Dependent

Signaling downstream of tyrosine kinases can promote tumor cell motility, and growth factors such as HGF [51], EGF [52] and PDGF [53] are motogens. Although caspase-8 expression is associated with increased accumulation of GTP-loaded Rac [33], it has not been clear precisely how this occurs. The capacity to interact with p85 α following phosphorylation may provide avenues for Rac activation through PI3K. The p85 α protein is composed of two SH2 domains as well as an SH3, iSH2 (inter-SH2) and GAP (GTPase Activating Protein) domain. While the SH2 domains bind phosphotyrosines and the SH3 bind PXXP motifs, the iSH2 region is responsible for binding with the catalytic p110 protein [54]. Consistent with pull down studies performed in neuroblastoma cell lines [32, 34], caspase-8 can form a complex that includes the p85 α subunit, and this interaction is abrogated by mutagenesis of the tyrosine 380 residue on caspase-8 to phenylalanine [36].

The GAP domain of p85 α possesses Rab-GAP activity [50], with the highest levels of activity against Rab4 and Rab5 [54]. The Rab family of GTPases control the trafficking of endosomes, and are primary mediators of compartmental targeting/specificity in eukaryotic

cells [55]. Rab5 is present on early endosomes in a GTP-loaded form, and hydrolyzes to GDP precisely during the maturation of the vesicle to 'late' endosome status, which is then hallmarked by the presence of GTP-loaded Rab7. By contrast, GTP-loaded Rab4 is a key mediator of recycling of endocytic components to the cell surface [54].

Investigating the potential effects of caspase-8 expression on Rab activity, an increased fraction of Rab5 remains GTP-loaded in caspase-8 expressing cells, and this corresponded to increased peripheral distribution of Rab5 positive endosomes [36]. Although no role for Rab4 was demonstrated, increased recycling of integrins to the surface of cells was observed in a caspase-8 dependent manner. This could be suppressed by ectopically increasing the expression of p85 α , or by suppressing caspase-8 expression. This regulation of Rab5 activity is of interest, because activation of Rab5 on early endosomes can activate Rac1 as a downstream target via the activation of TIAM-1 [56] and possibly other GTPase exchange factors. Importantly, disruption of the Rab5 activation similarly disrupted activation of Rac1 [35], supporting the concept that the activation of these two small GTPases is linked, or codependent.

These effects were only demonstrated in cells attached to an extracellular matrix via integrins, and were not seen in anchorage-free cells or in those attached to surfaces coated with anti-CD44 monoclonal antibodies [36]. The results suggest a dependence upon integrin signaling and interaction with the focal adhesion complex. The focal adhesion complex is a cellular organelle enriched in receptor tyrosine kinases, non-receptor tyrosine kinases, serine/threonine kinases, lipid kinases, other SH2-containing adaptor proteins, and many small GTPase binding proteins and effectors [41, 57–59]. It remains unclear, however, whether the effects of caspases-8 are dependent upon the nascent formation of focal adhesions via integrin recycling, turnover of these complexes via endocytosis, or both processes. The turnover of focal adhesions is key to motility in many cells, permitting mature focal adhesion components to recycle, forming new contacts and releasing old contact points [37].

Calpain Activation is Critical for Cell Migration

Another class of cysteine proteases that has been linked to the phenomenon of recycling focal adhesion components is the calpains. Calpains are calcium-dependent proteases [40] that play key roles in a variety of cellular processes ranging from membrane repair [60] to cell migration [61] to NF- κ B signaling [62]. The most common isoforms were originally designated μ -calpain (calpain 1) and m-calpain (calpain 2) corresponding to their requirement for ionic calcium (micromolar or millimolar, respectively). Except under pathological conditions, the concentration of calcium required for calpain activation is not present in the cell [63], permitting calpains to be suppressed by their ubiquitous endogenous inhibitor, calpastatin [39, 64].

Pharmacological and genetic inhibition of calpain 2 negatively affects migration by hindering the disassembly of focal adhesions at the rear of migrating cells, resulting in large, elongated focal adhesion complexes [37, 61, 65]. Calpain 2 is activated during the process of focal adhesion formation and remodeling downstream of ERK activation [63]. Biochemical

studies have linked calpain 2 to central components of focal adhesions, such as integrins, FAK, vinculin, paxillin and p130CAS [41]. By cleaving several substrates present in the focal adhesion, including cortactin and talin, calpain 2 plays an important role in remodeling of focal adhesion complexes [37, 65].

Calpain 2 activity has been linked to caspase-8 in some systems [6, 34]. Cells expressing caspase-8, either endogenous or ectopically, show an increase in calpain 2 cleavage, activation, and enhanced migration [6, 33]. Interestingly, this effect seems to be independent of caspase-8 catalytic activity. In contrast, attachment of cells expressing caspase-8 to fibronectin surfaces results in detectable calpastatin cleavage. This cleavage acts as an indicator of calpastatin displacement from its inhibitory site, resulting in its cleavage by its own binding partner, calpain 2. Addition of the catalytic domain of caspase-8 with the catalytic inhibiting mutation C360A to immunoprecipitates of calpain 2 resulted in a reduction of co-precipitating calpastatin [6] suggesting a physical interaction between caspase-8 and calpain 2/calpastatin that promotes calpain 2 activation. Calpastatin is composed of 4 Calp domains, which inhibit calpain dimers, as well as an L domain that associates it with L-type calcium channels [39]. Caspase-8 can be co-precipitated with calpastatin or clones of its domains, interacting most strongly with a construct containing the first Calp domain and the L domain (Fig. 4).

It is not yet clear where in the cell these specific interactions take place, but given (1) the association of caspase-8 with components of the PI3K complex [33, 35], (2) the association of calpains with phospholipids, and (3) the modification of Rab5 function, the surface of (peripheral) endosomes may certainly be involved. In some cases, colocalization of caspase-8 with calpastatin is observed at the front of migrating cells (Fig. 4). Although caspase-8 is widely touted as a cytosolic protease, we observe it to be present selectively in cellular puncta and the leading edge of migrating cells [32], suggesting that it is subject to some mechanism of spatial ordering (Fig. 5).

Outlook: Caspase-8 as an Enhancer of Cancer Therapeutics

Caspase-8 has been recognized as an effector of a variety of established and emerging therapeutics. The TRAIL pathway is of significant interest to pharma for the development of low-toxicity treatments for cancer. TRAIL signals apoptosis via caspase-8; early *in vitro* and *in vivo* studies showed TRAIL sensitivity in a wide variety of tumor cell lines, while normal cells were generally unaffected [66–69]. TRAIL itself has been of therapeutic interest for over a decade, and various monoclonal antibody agonists have also been developed to target the TRAIL pathway [70]. To date, agonists to the TRAIL pathway are generally well tolerated in the clinic. Unfortunately, resistance to this pathway has emerged as a major hurdle, and various TRAIL resistance mechanisms have been elucidated in epithelial tumors. The specific components of the TRAIL pathway consistently disabled (or not) give a good proxy for the selective advantage they confer for the tumor.

TRAIL receptors DR4 and DR5 may be downregulated by hypermethylation [71] and accordingly, patient samples frequently show reduced DR4/DR5 protein expression in tumor tissue [72]. Upregulation of anti-apoptotic proteins BCL-2 [26, 73] Mcl-1[74] and XIAP[75,

76] have been implicated in TRAIL resistance. Rapid degradation of active caspase-3 has even been described in TRAIL resistant cell lines [77].

Surprisingly missing from these reports is the down-regulation of caspase-8, which occurs frequently in neuroblastoma progression. One explanation for this is that these cells have developed in a manner that depends upon the non-apoptotic roles of caspase-8. This has significant impacts for patient care. Since caspase-8 can have pro-tumor activity, manipulation of caspase-8 expression with global modifiers such as γ -interferon or 5-azacytidine should be carefully considered. Furthermore, if indeed secondary pathways are required to commit caspase-8 to nonapoptotic roles, then complementary inhibition of these pathways may have significant adjuvant effects for TRAIL or other therapies. Such approaches are coming closer to reality as we further understand the complexities of caspase-8 signaling.

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Figure 1.

Tyrosine residues in caspase-8 and caspase-10. The structure of the catalytic domain of procaspase-8 (pdb code: 2K7Z [46]) is shown in grey with tyrosine residues displayed in black (left). Sequence alignment of caspase-8 and caspase-10 catalytic domains with the tyrosines highlighted in black (right). The secondary structure elements as present in caspase-8 are displayed above the alignment.

Figure 2.

Tyrosine residues in the procaspase-8 | FLIP heterodimer (pdb code: 3H11 [48]) are shown. The catalytic domains of procaspase-8 and FLIP are shown in light and dark grey, respectively, with the tyrosines indicated in black. Note that several tyrosine residues fall within the interface between the two proteins. Phosphorylation at any of these could be sufficient to disrupt heterodimer formation.

Figure 3.

A representation of interacting SH2 domains and corresponding phosphorylated regions of caspase-8 using the Panomics™ SH2 screen. The size of the SH2 domain name indicates the relative strength of interaction. Non-hits are listed outside of the ovals. Shown is a compilation of data from Senft *et al.* 2007 and from our lab. Senft and colleagues probed the Panomics™ SH2 screen with a short peptide corresponding to the flexible loop linker surrounding the Y380 site on caspase-8, with a phospho-tyrosine substituted for the tyrosine on residue Y380. The data is complemented by probing the same Panomics™ SH2 screen with the whole catalytic domain of caspase-8 phosphorylated by Src kinase *in vitro* as described in [11].

Figure 4.

Interactions of caspase-8 with calpastatin. Calpastatin or constructs containing only calpastatin domains or the L domain plus a single calpastatin domain depicted in the upper panel were expressed in NB7C8 cells, immunoprecipitated with anti-Myc antibodies, and probed for the presence of coprecipitating caspase-8, as shown in the middle panel. Protein A/G beads and empty vector controls were used for specificity. Shown is an example experiment. The bottom pair of images shows confocal images of COS-7 cells spreading on fibronectin. The left panel shows staining for caspase-8, originally resolved in the 520nm channel, and the right panel shows staining for calpastatin resolved at 568nm. Arrows indicate regions of colocaliation in the cellular lamella/processes.

Figure 5.

A working model for the mechanism of caspase-8 promoted migration. Phosphorylated procaspase-8 is recruited to the leading edge of migrating cells, where it is incorporated into an insoluble complex of focal adhesion proteins. From there, procaspase-8 promotes calpain activity, and turnover of focal adhesion components. Later, procaspase-8 promotes the internalization of integrins by enhancing Rab5-GTP loading, which enhances endocytosis but prevents hand-off to later endosomal compartments. This permits integrins to recycle back to nascent focal contacts to complete the “conveyor belt” of focal adhesion turnover required for mesenchymal-type cellular migration.