Death by Dozens of Cuts

Protein-cutting enzymes called caspases are at the core of the cell's suicide machinery. Researchers are beginning to understand how they work and how they may be harnessed for treating disease

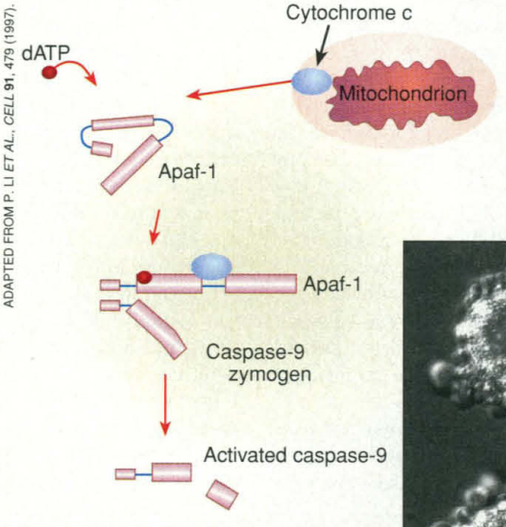
Every cell contains the makings of its own demise: a set of proteins that can kill it from within. Once unleashed, these proteins direct other molecules to shatter the cell's nucleus and chop up its chromosomes, digest the internal skeleton that gives the cell its shape, and break up the cell itself into small fragments, ready for disposal.

This self-immolation, called programmed cell death or apoptosis, is essential for the good of the organism. Cells must die, for instance, as tissues are sculpted during embryonic development or when they have been infected with harmful viruses. But either too much or not enough apoptosis can be catastrophic. Many cancers, for instance, are hard to kill because they fail to respond to apoptosis signals, while in neurodegenerative conditions such as Parkinson's disease, or following the oxygen starvation caused by stroke, excess apoptosis may kill off brain neurons. Now researchers are learning about the inner core of the death machinery—a family of protein-cleaving enzymes known as caspases—and are getting hints about how science might someday use this knowledge to intervene when apoptosis goes wrong (see sidebar).

They have found, for example, that caspases act at two levels to mete out death. Initiator caspases pronounce the death sentence. They are activated in response to signals indicating that the cell has been stressed or damaged or has received an order to die. They clip and activate another family of caspases, the executioners, which go on to make selected cuts in key proteins that then dismantle the cell. Researchers have also identified proteins that keep the caspases in check except when they are needed—a necessity for enzymes that pack the destructive wallop that these do. "There has been exponential progress," says apoptosis researcher John Reed, of the Burnham Institute in La Jolla, California. "The picture has begun to fill in."

The first hint that proteases, as protein-cleaving enzymes are called, play a central role in apoptosis came about 6 years ago from Robert Horvitz's lab at the Massachusetts Institute of Technology (MIT). Horvitz's team had found a mutation in the roundworm *Caenorhabditis elegans* that prevents normal cell death from occurring during embryonic development. The mutant gene, *ced-3*, turned out to encode a protease that is closely related to a mammalian enzyme called ICE, which until then was known only for its role in promoting inflammation.

Soon after, in 1994, Junying Yuan of Harvard showed that artificially activated ICE causes cell death in cultured mammalian cells. ICE itself is apparently not normally involved in apoptosis because it does not respond to natural cell-death signals, but Horvitz's and Yuan's findings touched off a search that turned up a whole family of ICE-related proteases, at least seven of which normally participate in apoptosis.



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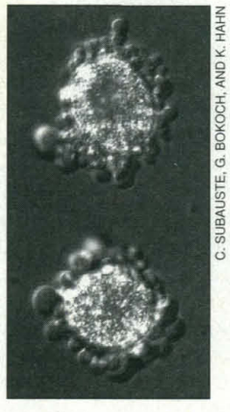


C. SUBAUSTE, G. BOKOCH, AND K. HAHN

Causal chain. Cytochrome c released from mitochondria binds to Apaf-1, allowing it to bind and activate caspase-9. This triggers apoptosis, marked by stereotyped features such as the membrane blisters on these cells *(inset)*.

These enzymes were named caspases (for cysteine-containing aspartate-specific proteases) because they all contain the amino acid cysteine in their active sites and clip their protein targets next to the amino acid aspartate. Among those targets are the caspases themselves, which start out as inactive proteins called zymogens that must be clipped to become fully active.

Recognition that the zymogens contain the exact same sequence of amino acids that the caspases are designed to snip suggested, says Reed, that "if you could get a caspase activated somehow, a cascade of proteolysis would ensue," with active caspases cutting and activating more caspases. Investigators have accumulated evidence showing that the enzymes are indeed turned on in this way, in a sequence of cutting that begins with initiator caspases and ends with proteins that kill the cell.

That left the question of how the first caspase gets turned on. It now appears that some initiator caspase zymogens can cut and activate each other, even though they have at most only one-fiftieth of the protein-splitting ability of the active enzymes. Reports within the past several months from Vishva Dixit's team at Genentech Inc. in South San Francisco, working with Guy Salvesen at the Burnham Institute, David Baltimore's group at MIT, and Donald Nicholson's at Merck Frosst Canada Inc. in Pointe Claire–Dorval, Quebec, have all shown that molecules of some caspase zymogens brought into close contact in the test tube become activated.

The finding could explain how Fas, a cell surface receptor that causes cells to commit suicide when it receives the right signals from the immune system, does its work. Three years ago, Dixit's team, then at the University of Michigan Medical School in Ann Arbor, and David Wallach's team at the Weizmann Institute for Science in Rehovot, Israel, both found that Fas can draw caspase-8 zymogen molecules together. It now seems that this proximity allows the caspase precursors to activate one another.

But apoptosis isn't always triggered by receptors receiving specific signals from outside the cell. During development, for example, cells commit suicide if they are deprived of growth factors. Cells mortally damaged by radiation, oxidizing agents, or other harmful chemicals often undergo apoptosis rather than dying a less controlled death that is potentially more damaging to the organism. And some cells kill themselves when they just sense that things aren't going right, such as when control of the cell-division cycle has gone haywire. Such a diversity of death triggers means drawing caspase zymogens to a receptor can't be the only way to activate them. Some additional ways are now coming to light.

The mitochondrial connection

In 1996, Xiaodong Wang and his colleagues at the University of Texas Southwestern Medical Center at Dallas made a surprising

Caspase Work Points to Possible New Therapies

Death is a part of life, and in the life of any healthy organism, some cells are marked to die by programmed cell death, known as apoptosis. But sometimes cells refuse the order to die, resulting in cancer. Other cells die when they shouldn't, causing neurodegenerative problems such as Parkinson's disease or contributing to the brain damage of stroke. Now, researchers hope that what they are learning about inhibitors of the protein-splitting enzymes called caspases, at the core of the cell's death machinery, will lead to better therapies for some of these conditions.

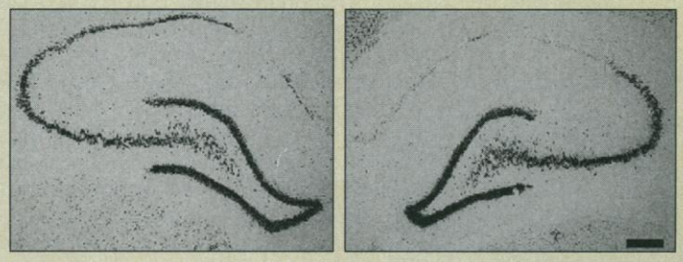
In the case of cancer, their goal is reactivating the responses to stress and other signals that normally trigger cell death. Last year, cancer biologist Dario Altieri and his colleagues at Yale Medical School discovered one reason why cancer cells are deaf to these signals. They found that tumor cells, but not normal adult cells, contain high levels of a protein they call survivin, which blocks apoptosis. "We haven't found one tumor that is negative for survivin," says Altieri.

The researchers do not yet know how survivin works, but it may inhibit the caspases, as its structure reveals that it is related to the IAPs, a group of proteins known to block caspase action. But whatever survivin does, Altieri says, "the implications are enormous in terms of susceptibility to therapy." Radiation and chemotherapy are both designed to stress the cell in ways that would normally induce apoptosis. If researchers can find a way to inactivate survivin, cancer cells might be made more susceptible to such treatments.

Saving cells from apoptosis, in contrast, could limit the damage in neurodegenerative diseases and stroke. Until recently, some researchers worried that blocking apoptosis could make matters worse if the injury that triggered the apoptosis in the first place—for example, the lack of oxygen caused by a stroke—had irreversibly damaged the cell. Cells undergoing apoptosis don't just fall apart but succumb in a neat and tidy way, breaking apart into self-contained packages to be scarfed up by patrolling scavenger cells. If apoptosis were blocked, damaged cells might be left with no alternative but to die in a way that spills the cell's contents, triggering harmful inflammation and spreading the damage even further.

"That is something the field has been wondering about over the past year or so," says Donald Nicholson, of Merck Frosst Canada Inc. in Pointe Claire–Dorval, Quebec. But some recent experiments, he says, provide hope that when apoptosis is blocked, cells can sometimes recover to lead healthy lives.

A report last September by George Robertson at the University of Ottawa and his colleagues supported this idea by showing that mice whose brains were engineered to make extra IAP proteins lost fewer neurons when they had strokes. This suggests that the IAPs protected some neurons from apoptosis, and that those neurons were able to overcome the shock of the stroke. Robertson says his group is now testing the surviving neurons to make sure they can still function normally.



Survival booster. Five days after a stroke, brains from rats making high levels of the apoptosis inhibitor NAIP had more surviving neurons (*left*) than brains from controls (*right*).

D. G. XU *ET AL., NATURE MEDICINE* **3**, 997 (1997).

Hermann Steller's team at the Massachusetts Institute of Technology has shown something similar: They started with fruit flies that had retinitis pigmentosa (RP), a leading cause of blindness in humans. In humans and flies, RP is caused by a mutation in the light-sensitive protein rhodopsin. Over time, the alteration stresses the retina's photoreceptor cells, triggering apoptosis. But when Steller's team engineered some of these flies to make a potent caspase inhibitor called p35, the retinal neurons survived and continued to function normally.

Steller says his and Robertson's experiments show that "in some degenerative conditions, the cell is a little too wimpy, a little too sensitive. It perceives a problem and dies too readily." In those cases, says Steller, "if you block apoptosis, you get a permanent, long-term rescue." **–M.B.**

discovery about the identity of one caspase activator. Working with a "cell-free" apoptosis system—an extract prepared from a cultured human cell line—they found that a critical protein component of mitochondria, the organelles that produce most of the cell's energy, can activate one of the executioner caspases, caspase-3. The idea that this mitochondrial protein, cytochrome c, would moonlight as an apoptosis trigger was "totally unexpected," says Wang, and too bizarre to be believable at first.

But by late last year, Wang's team had a fix on just how cytochrome c triggers cell death. It binds to a protein they discovered called apoptotic protease activating factor–1 (Apaf-1). That binding allows Apaf-1 to link up with and somehow activate caspase-9, an initiator caspase which then activates caspase-3. Wang says he doesn't yet know how Apaf-1 turns on caspase-9, but his "favorite hypothesis" is that it acts as Fas appears to: by bringing together multiple caspase zymogens, which then activate each other.

Cytochrome c makes sense as an apoptosis trigger, says cell death researcher Hermann Steller of MIT, given that various forms of cell damage can harm mitochondria, even causing their outer membranes to rupture. The release of cytochrome c under such conditions could serve as "a stress sensor," says Steller, telling the cell it has received a fatal insult, and so "it is time to die."

Other death triggers may also discharge cytochrome c. Cell biologist Donald Newmeyer of the La Jolla Institute for Allergy and Immunology, Sally Kornbluth of Duke University Medical Center in Durham, North Carolina, and their colleagues have found that after caspase-8 is activated by the death receptor, Fas, it causes mitochondria to dump their cytochrome c. "Even though caspase-8 can activate other caspases directly," says Newmeyer, "this cytochrome-c pathway is more efficient. It works at much lower levels of caspase-8." Consequently, it can amplify a death signal that on its own is too weak to cause death.

The executioners' targets

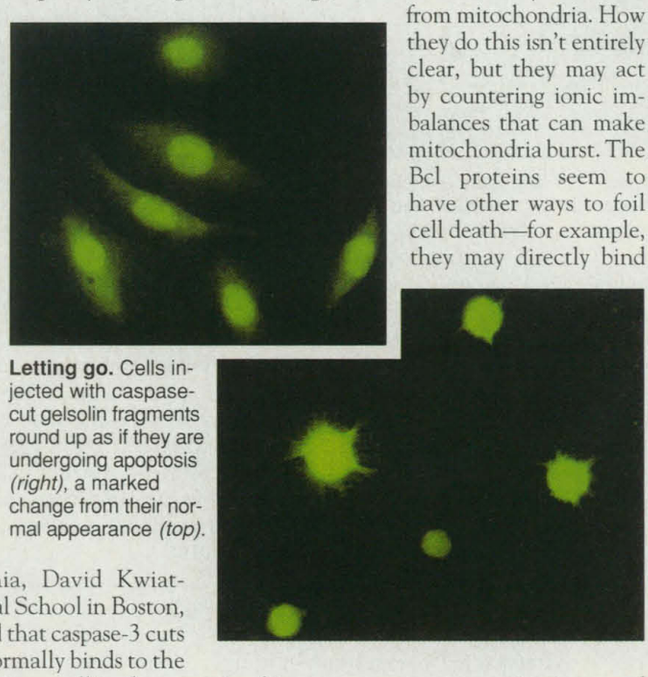
Once the executioner caspases are activated, they start cutting other proteins. So far, researchers have identified more than two dozen of these caspase targets, but until recently their link to cell death in most cases was unclear or at best indirect. But recently researchers have found caspase targets whose cleavage clearly triggers specific well-known steps in apoptosis.

For example, one hallmark of apoptosis is

the disintegration of the cell's nucleus: First the chromosomes are chopped up, and then the nucleus itself breaks into small pieces. Last April, Wang and his co-workers reported that they had found a protein from cultured human cells that once cut by caspase-3 triggers chromosome breakup like that seen in apoptosis. The researchers also found that the uncut protein is ordinarily paired with another protein. But they didn't know the role of the other protein, or how the caspase action leads to the breakup of the DNA.

Then, early this year, Shigekazu Nagata and his colleagues at the Osaka University Medical School in Japan provided an answer. They found the same protein pair in mouse cells and showed that the caspase target's partner is a DNA-cleaving enzyme known as an endonuclease. When the caspase target is clipped, it frees the endonuclease to enter the nucleus and start chopping DNA. "That is a pretty amazing story," says the Burnham Institute's Reed. "This is the first time you can link an endonuclease directly to the caspases."

Another key element in apoptosis—changes in the cell's outer membrane—has been linked recently to two caspase targets. Cells dying by apoptosis go through predictable membrane changes: They lose their normal shape, becoming more rounded and forming blisterlike bumps on their surfaces. Last year, Lewis Williams of Chiron Corp. in Emeryville, California, David Kwiatkowski of Harvard Medical School in Boston, and their colleagues found that caspase-3 cuts gelsolin, a protein that normally binds to the actin filaments that help give a cell its shape. One of the gelsolin pieces then severs actin filaments, says Williams, an effect the team confirmed by injecting caspase-cut gelsolin fragments into cells. Cells treated this way round up and "look like they are undergoing apoptosis," Williams says.



S. KOTHAKOTA *ET AL.*, *SCIENCE* **278**, 294 (1997).

Letting go. Cells injected with caspase-cut gelsolin fragments round up as if they are undergoing apoptosis *(right)*, a marked change from their normal appearance *(top)*.

After losing their shape, cells dying by apoptosis break into membrane-encased "apoptotic bodies" that are gobbled up by roving scavenger cells. Last spring, Gary Bokoch and Thomas Rudel of The Scripps Research Institute in La Jolla identified one caspase target that is necessary for formation of the bodies: an enzyme called p21-activated kinase-2 (PAK2) that regulates the activity of other proteins by adding phosphate groups to them.

Bokoch and Rudel found that caspase-3 cuts and activates the enzyme. Then, by engineering cells to make a form of PAK2 that can't be activated, they confirmed the crucial role of the enzyme in the dismantling of the cell. "Even though [the engineered cells] still underwent DNA fragmentation" in response to appropriate apoptotic stimuli, Bokoch says, "they didn't fragment into apoptotic bodies." PAK2 helps regulate the cell's internal skeleton, but just how it causes dying cells to break up is not known.

Controlling the death machine

Given the incredible power of caspases to direct the dismantling of cells, cells need to keep the powerful enzymes under control at times when it's not appropriate to die. Two proteins that seem to aid this cause in multiple ways are Bcl-2 and Bcl-x, which, among other things, block the release of cytochrome c from mitochondria. How they do this isn't entirely clear, but they may act by countering ionic imbalances that can make mitochondria burst. The Bcl proteins seem to have other ways to foil cell death—for example, they may directly bind Apaf-1 to prevent caspase activation—and their amounts in a cell help determine how vulnerable it is to apoptosis. In fact, excess Bcl-2 can convert normal cells into cancer cells that refuse to die upon receiving death commands, and anti–Bcl-2 therapies have shown success in clinical trials for some types of cancer.

Another set of proteins called inhibitors of apoptosis (IAPs) seems to apply the brakes to apoptosis by inhibiting caspases directly. IAPs were discovered by Lois Miller's team at the University of Georgia, Athens, in 1993. The researchers found that viruses deploy these proteins to keep host cells alive while the viruses replicate and spread. In 1994, Alex MacKenzie's group at the University of Ottawa found the first cellular IAP, a protein that inhibits apoptosis in nerve cells. Since then, researchers have found five more IAPs in mammalian cells alone.

Several teams, including MacKenzie's, Reed's, and Nicholson's at Merck Frosst Canada Inc., have evidence that IAPs bind to caspases and block their activity. But this may be too simple a picture; like the Bcl proteins, IAPs may work in more than one way. Miller's team found that IAPs apparently can arrest apoptosis before the caspases are involved. "There is no point in jumping to conclusions yet," says Miller. "It is a complex story."

Indeed, the same might be said about virtually all aspects of caspase function and control. Researchers wonder, for example, what other proteins besides cytochrome c may trigger caspase activation. They puzzle over how the cell maintains its checks on the enzymes—then turns off these safeguards when it is time for the cell to die. And of course they are looking for more levers like Bcl-2 that will enable them to manipulate apoptosis for disease treatments. So although the learning curve has been steep over the past year, it looks like researchers in this field won't run out of big questions anytime soon.

–Marcia Barinaga

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