Re-examining the role of cytochrome *c* in cell death

Eric Solary, Fabrizio Giordanetto & Guido Kroemer

When cytochrome *c* is released from mitochondria, it interacts with Apaf-1 to activate death-promoting caspases. Now, a gain-of-function mutation affecting cytochrome *c* with enhanced caspase-stimulatory activity is shown to have no other consequences for human health than a subclinical thrombocytopenia, showing that, in most settings, enhanced cytochrome *c* activity *per se* is not sufficient to disturb normal tissue homeostasis.

Most, if not all, proteins involved in cell death control have a vital function unrelated to cell death[1,2]. Cytochrome *c* is not an exception to this rule. In healthy cells, cytochrome *c* is a heme protein that is exclusively present in the mitochondrial intermembrane space, where it is loosely associated with the inner membrane of the mitochondrion and shuttles electrons between the respiratory complexes III and IV. However, when cells undergo apoptosis, the subcellular localization and function of cytochrome *c* change radically[3]. Specifically, apoptotic signaling triggers mitochondrial outer membrane permeabilization (MOMP), releasing cytochrome *c* from mitochondria[4]. Once liberated, cytochrome *c* interacts with a constitutively cytosolic protein, Apaf-1, to create a wheel-shaped heptameric complex, the apoptosome, which recruits and allosterically activates caspase-9 in its center, thus triggering the caspase activation cascade[5,6]. Effector caspases such as caspase-3 then participate in the apoptotic dismantling of cellular structures.

Morison *et al.*[7], reporting on page 387 of this issue, studied a family with an autosomal dominant form of thrombocytopenia, and discovered a mutation affecting cytochrome *c* (G41S) that is neutral with regard to its bioenergetic function, yet mediates a gain-of-function phenotype affecting its caspase-stimulatory function. Specifically, recombinant G41S cytochrome *c* has a normal redox potential, and cells expressing the mutant protein respire normally. However, when recombinant G41S cytochrome *c* is added to cytosolic extracts containing Apaf-1 and inactive caspases, it is more efficient than the wild-type protein in triggering caspase activation[7]. G41S and wild-type cytochrome *c* have a similar fold, as determined by nuclear magnetic resonance[7]. Although the side chain introduced by the amino acid substitution does not contribute to the cytochrome *c*–Apaf-1 binding interface[8], the G41S substitution enhances the stability of the cytochrome *c*–Apaf-1 interaction, as determined by molecular modeling (**Fig. 1**). Thus, a change in the affinity of cytochrome *c* for Apaf-1 may explain the increased caspase-stimulatory function of the G41S mutant. In addition, or alternatively, it is possible that the entire hepta-heteromeric G41S cytochrome *c*–Apaf-1 complex undergoes a subtle conformational change that improves its action as an allosteric activator of caspase-9, a question that will need to be resolved by future studies.

Walking the line

There is no doubt that mitochondria and their products have a key role in delimiting the frontier between death and life, both in physiological and pathological scenarios of cellular demise[4]. Nonetheless, there are two distinct schools of thought on the details of the 'point of no return'—the molecular event that marks the decisive step beyond which cells cannot recover and hence are doomed to die. According to one school of thought, cell death is determined by the release of cytochrome *c* because it is coupled to caspase

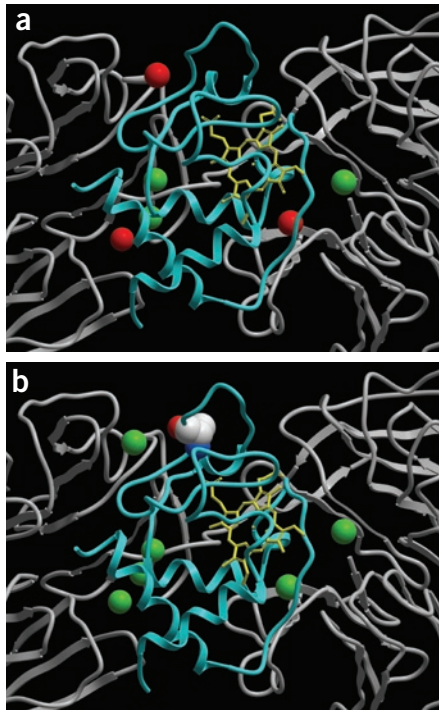

Figure 1 Modeling interactions between cytochrome *c* and Apaf-1. (**a**,**b**) Models of the interaction of Apaf-1 (gray) with wild type (**a**) and G41S (**b**) cytochrome *c* (protein in cyan; heme in yellow). Selected electrostatic interactions between cytochrome *c* and Apaf-1 with less than 70% occupancy are shown as red spheres (K39-E1045, E61-N1219/N1237, N70-S745). The S41G variant, shown as the space-filling model in **b**, stabilizes the cytochrome *c*–Apaf-1 interaction, on the basis of reduced structural fluctuations (root-mean-square deviations (RMQS) of 1.72 Å and 0.92 Å for wild-type cytochrome *c*–Apaf-1 and G41S cytochrome *c*–Apaf-1 interactions, respectively) and increased occupancy for electrostatic interactions between the two proteins (51% and 79% for wild-type cytochrome *c*–Apaf-1 and G41S cytochrome *c*–Apaf-1 interactions, respectively).

Eric Solary is at INSERM U866, 21000 Dijon, France and Institut Gustave Roussy, 94805 Villejuif, France. Fabrizio Giordanetto is at SwedenAstraZeneca R&D Mölndal, SE-431 83 Mölndal, Sweden. Guido Kroemer is at Institut Gustave Roussy, INSERM U848 and Université Paris-Sud 11, 94805 Villejuif, France.
e-mail: kroemer@igr.fr

activation, which then seals the cell's fate[3]. Indeed, inhibition of caspases blocks most, if not all, of the phenotypic manifestations of apoptosis, such as advanced chromatin condensation, nuclear fragmentation, membrane blebbing and generation of apoptotic bodies[9]. However, caspase inhibition usually does not prevent cell death, which can manifest with a sub-apoptotic, autophagic or necrotic phenotype[10]. Therefore, another school of thought postulates that cell death is determined by MOMP, which leads to the release of cytochrome *c* but which also has other lethal consequences, including the release of caspase-independent death effectors and the cessation of the vital functions of mitochondria[11].

Although compatible with either school of thought, the paper by Morison *et al.*[7] lends support to the idea that MOMP (and not cytochrome *c* release itself, or at least not the absolute level of cytochrome *c* activity) marks the decisive step in cell death. Indeed, in a vast six-generation family, the only phenotypic manifestation of the G41S cytochrome *c* substitution is an autosomal dominant, subclinical thrombocytopenia. Thus, paradoxically, the phenotype conferred by the G41S cytochrome *c* variant involves a process in which cytochrome *c* mediates caspase activation in a nonapoptotic context. Differentiating megakaryocytes generate platelets by protruding long and thin cytoplasmic extensions called pro-platelets. Such pro-platelets are released into sinusoids, a specialized bone marrow compartment that is directly connected to the vascular system. This carefully timed process occurs without apoptosis, yet requires the localized release of cytochrome *c* from a selected subset of perinuclear mitochondria, leading to the spatially restricted proteolytic maturation of caspase-3 within cytoplasmic granules[12]. Manipulations designed to block mitochondrial permeabilization (by overexpression of Bcl-2) or inhibit caspase activation (by knockout of Apaf-1 or addition of pharmacological caspase inhibitors) reduce the formation of pro-platelets and cause thrombocytopenia[12,13]. Morison *et al.*[7] show that the G41S cytochrome *c* substitution unduly accelerates platelet production by megakaryocytes, causing the premature release of platelets into a compartment (the bone marrow space) in which they cannot reach the bloodstream. In the cell-free system, G41S-mediated differences in cytochrome *c*–driven caspase activation are only observed at low cytochrome *c* concentrations[7]. Hence, it is plausible that differentiation-associated caspase activation involves relatively low cytosolic concentrations of cytochrome *c*—concentrations at which G41S has a major impact on apoptosome assembly.

In summary, enhancing the caspase-stimulatory function of G41S cytochrome *c* does have consequences *in vivo*, in the context of thrombopoiesis, yet does not affect cell fate or differentiation in any other lineage. If cytochrome *c* itself were the rate-limiting factor of cell death processes, one might have expected that individuals expressing the G41S cytochrome *c* protein would manifest a generally increased susceptibility to cell death, with the resulting tendency to develop degenerative symptoms in susceptible tissues. In mice, cytochrome *c* has been knocked out (which obviously leads to embryonic death due to a bioenergetic crisis)[14] or altered (K72A) such that the respiratory function of cytochrome *c* is maintained, yet the molecular interaction between cytochrome *c* and Apaf-1 is abolished[15]. Mice homozygous for cytochrome *c* K72A develop hydrocephalus, pituitary gland dysmorphy and consequent cachexia with severe lymphopenia, but do not manifest any severe cell death defect. Thymocytes from cytochrome *c*–negative mice die normally in response to several MOMP inducers and can activate caspases in a cytochrome *c*–independent fashion[15]. Thus, both the gain-of-function mutation affecting human cytochrome *c* and the loss-of-function mutation affecting mouse cytochrome *c* point in the same direction and imply that the postulated essential role of cytochrome *c* in determining cell death might need to be reconsidered.

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Adding pathogens by genomic subtraction

Laura MacConaill & Matthew Meyerson

Two studies report the application of high-throughput sequencing technologies to discover infectious agents associated with diseased human tissues. These findings herald a breakthrough in the field of pathogen discovery.

Infectious pathogens are surmised to initiate many complex human diseases, but finding causative organisms has often proven difficult. Now, two independent studies report the application of high-throughput pyrosequencing to identify viruses in diseased human tissue. Feng *et al.*[1] discovered a new polyomavirus in samples of Merkel cell skin carcinoma, and Palacios *et al.*[2] identified a previously uncharacterized arenavirus associated with three transplant-related deaths. Last year, a similar approach by one of these teams established a virus as the cause of honeybee colony collapse disorder[3].

Cancers and autoimmune and inflammatory disorders, as well as acute illnesses, may arise from microbial infection. In recent years, increasingly sophisticated molecular techniques, including subtractive cloning[4], polymerase chain reaction (PCR)[5–7] and DNA microarrays[8–11], have successfully implicated previously unknown pathogens as the etiological agents of both acute and chronic diseases. Each of these methodologies has limitations, however, and most require some a priori knowledge of the pathogenic organism under investigation.

An unbiased approach, known as computational subtraction, can detect microbial

Laura MacConaill & Matthew Meyerson are in the Center for Cancer Genome Discovery and Department of Medical Oncology at the Dana-Farber Cancer Institute and the Department of Pathology at Harvard Medical School, Boston, Massachusetts 02115, USA and the Broad Institute of Harvard and the Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA.
e-mail: *matthew_meyerson@dfci.harvard.edu*