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STIFTUNG PROFESSOR DR. MAX CLOËTTA

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Prof. Dr. Adrian Merlo
«Pas de mythe de Sisyphe: Glioma research on the move»

Prof. Dr. Michael O. Hengartner
«Roads to ruin: Apoptotic pathways
in the nematode Caenorhabditis elegans»

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Stiftung Professor Dr. Max Cloëtta
Schaffhauserstrasse 43, Postfach, 8042 Zürich
Telefon 044 350 44 35
Telefax 044 350 44 32
E-Mail cloetta@stiftung.ch
www.cloetta-stiftung.ch

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VORWORT

Prof. Max M. Burger
Präsident des Stiftungsrates

33 Jahre, ein Drittel Jahrhundert, ist auch für eine Stiftung wie die unsere kein kurzer Weg; allerdings ist er auch noch nicht sehr lange. Wenn der Weg allein schon selber das Ziel ist, müssen unterwegs auch Stundenhalte eingeschaltet werden. Stundenhalte nicht zum Ausruhen, sondern welche, die zur Überprüfung der Ziele der Stiftung und damit wieder dem weiteren Weg dienen.

1973 gründete Dr. Antoine Cloëtta unsere Stiftung zu Ehren seines Vaters, Prof. Dr. Max Cloëtta. Glücklicherweise fasste er den Zweck der Stiftung sehr weit. Er hatte schon damals im Auge, Forschungsprojekte in Zusammenarbeit zwischen universitären Instituten und pharmazeutischen Unternehmen zu finanzieren, so Dr. Alfred Hartmann, der langjährige Stiftungsratspräsident. Der Stifter wollte dies, weil sein Vater als Pharmakologe auf diesem Wege bahnbrechende Heilmittel entwickelt hatte. Die Translation, die Übertragung des sich stetig erneuernden Wissens aus der biomedizinischen Grundlagenforschung auf den Patienten, schwebte also schon unseren Gründervätern vor.

Die durch die zweite Ölkrise ausgelöste Rezession führte zu Sparmassnahmen bei Bund und Kantonen und vor allem zur zögerlichen Besetzung von akademischen Mittelbaustellen. Mit ihren relativ beschränkten Mitteln beschloss die Stiftung Prof. Dr. Max Cloëtta deshalb, als erste in der Schweiz ein spezifisches Nachwuchsförderungsprogramm in der Biomedizin aufzubauen, um vielversprechenden jungen Forschern die Zeit bis zur Berufung auf eine Professur zu überbrücken. Der Schweizerische Nationalfonds folgte bald darauf mit seinem START-Programm (Swiss Talents in Academic Research and Teaching) und etwas später mit SCORE (Swiss Clinicians opting for Research). Aus den letzten beiden Programmen entstanden 2000 die vom SNF verge-

benen SNF-Förderprofessuren, die nicht nur die Stelle selbst abdecken, sondern nun auch die Forschungs- resp. Projektkosten mitgarantieren.

Bei jeder Stiftung ist es Aufgabe des Stiftungsrates, die Nützlichkeit ihrer Arbeit kontinuierlich zu hinterfragen/überprüfen. Die Stiftung Prof. Dr. Max Cloëtta hat in der Förderung der besten biomedizinischen und klinischen Mittelbauforschern der Schweiz auf ihrem Weg zur Professur eine Pionierrolle eingenommen. Bei der Erfüllung dieser Aufgabe darf man von ihr genauso innovatives Denken erwarten, wie sie es von ihren Gesuchstellern für ein Forschungsstipendium oder den Kandidatinnen und Kandidaten für den Cloëtta-Preis verlangt. Für das kommende Jahr sieht unsere Stiftung deshalb eine Zukunftsanalyse vor, die Klarheit darüber bringen soll, ob das Forschungsstellen-Programm neu gestaltet oder ein grundsätzlich neues Programm aufgebaut werden soll.

Wenn eine Stiftung auf ein mehrjähriges Wirken zurückblicken kann, heisst dies leider auch, irgendwann Abschied nehmen zu müssen von Menschen, die sie auf ihrem Werdegang begleitet haben. Am 31. Mai dieses Jahres verstarb nach schwerer Krankheit Professor Alex Muller im 85. Altersjahr. Professor Muller, der sich neben seiner eigenen erfolgreichen Forschungstätigkeit zeit seines Lebens sehr aktiv für die Förderung des akademischen Nachwuchses eingesetzt hat, präsidierte die Stiftung Prof. Dr. Max Cloëtta von 1996 bis 2001, nachdem er schon seit 1993 Mitglied des Stiftungsrates gewesen war. Wir haben mit ihm nicht nur einen engagierten, umsichtigen Kollegen verloren, sondern auch einen grossherzigen, charmanten Menschen, den wir in wärmster Erinnerung behalten.

Nun zu unseren beiden diesjährigen Preisträgern.
Für einen operativ tätigen Kliniker ist es nicht leicht, kompetitiv molekularbiologische Forschung zu betreiben. Wer im Operationssaal steht, kann realistischerweise kaum mit derselben Produktivität wissenschaftlich tätig sein wie zum Beispiel ein Grundlagen-Molekulargenetiker. Als leitender Arzt der Neurochirurgischen Universitätsklinik Basel dürfte Prof. Adrian Merlo nicht nur eine Ausnahme unter Schweizer Chirurgen sein, sondern zu den wenigen in Europa gehören, denen

dieser Balanceakt über Jahre hinweg erfolgreich gelang. Wenn er Gliom-Tumor-Suppressoren-Gene klont oder PTEN Mutationen sequenziert, dann weil er davon ausgeht, dass eine rationale Therapie schlussendlich nur dann entworfen und angegangen werden kann, wenn die grundlegende Molekularbiologie der Entstehung von Hirntumoren geklärt wird. Dessen ungeachtet möchte er heute schon translationelle Fortschritte in der Therapie sehen und etablierte zusammen mit Radio-chemikern und Nuklearmedizinern ein Projekt, das der Radioimmuno-Radiopeptid Brachytherapie gewidmet ist.

Die Laudatio für die Leistungen von Prof. Merlo wird Prof. Nicolas de Tribolet, Chefarzt der Klinik für Neurochirurgie des Universitätsspitals Genf, in verdankenswerter Weise halten.

Prof. Michael Hengartner wurde im zarten Alter von 35 Jahren Inhaber des neu errichteten Ernst Hadorn-Lehrstuhles am Institut für Molekularbiologie der Universität Zürich. Er gehört zu den wenigen Schweizern, die sowohl Diplom als auch Promotion an bekannten Institutionen im Ausland abgeschlossen haben; letztere am renommierten Massachusetts Institute of Technology (MIT). Seine bahnbrechenden Beiträge gelten seither in erster Linie den Mechanismen der Apoptose in Morphogenese und Entwicklungsbiologie. Einer der für die Auswahl der Preisträger beigezogenen Experten kam zum Schluss, dass Hengartners Publikationen mit seinem Doktorvater Prof. Bob Horvitz über das CED-9 Gen einer der Gründe dafür gewesen sei, dass diesem 2002 der Nobelpreis verliehen wurde.

Prof. Walter Schaffner, Direktor des Institutes für Molekularbiologie der Universität Zürich, wird Prof. Hengartners Beiträge würdigen. Wir sind ihm dankbar dafür.

Last but not least möchte ich meinen Kollegen im medizinischen Ausschuss der Stiftung, Prof. Marco Baggiolini, Prof. Bernard Rossier, Prof. Michel Vallotton und Prof. Jürgen Zapf, für die fachlich interessante und menschlich sehr angenehme Zusammenarbeit danken. Ebenso den übrigen Mitgliedern des Stiftungsrates, den Herren Dr. Hans Bollmann, Antoine Cramer und Dr. Hans-Ulrich Doerig. Wir alle

wirken im Milizsystem und sind deshalb auf eine effiziente und zuverlässige Geschäftsstelle angewiesen. Frau Brigitt Küttel, der Geschäftsführerin, und Frau Irène Thomann sowie ihrem Team sei deshalb für ihre unentbehrliche Hilfe im Namen aller Kollegen herzlich gedankt.

DER CLOËTTA-PREIS 2006
WIRD VERLIEHEN

HERRN PROFESSOR DOKTOR

ADRIAN ULRICO MERLO

GEBOREN 1957, VON BRUSIO (GR)
PROFESSOR FÜR NEUROCHIRURGIE
AN DER UNIVERSITÄT BASEL UND LEITENDER ARZT DER
NEUROCHIRURGISCHEN KLINIK DES
BASLER UNIVERSITÄTSSPITALS
FÜR SEINE INTERNATIONAL ERFOLGREICHEN
WISSENSCHAFTLICHEN ARBEITEN
SOWOHL ALS KLINIKER ALS AUCH ALS MOLEKULARBIOLOGE
AUF DEM GEBIET DER
ONKOLOGISCHEN NEUROCHIRURGIE.

ZÜRICH, 24. NOVEMBER 2006

FÜR DEN STIFTUNGSRAT:

DER PRÄSIDENT

DER VIZEPRÄSIDENT

EIN MITGLIED

Prof. Dr. Adrian Merlo

CURRICULUM VITAE

Name:	Adrian Ulrico Merlo
Place and date of birth	Flawil/SG, Switzerland; 26 April 1957
Citizenship	Brusio/GR, Switzerland
Marital status	Married, 3 children

Education and Training

1977	Matura, Type A, Kantonsschule Baden/AG, Switzerland
1983	Final Federal Examination, University of Zurich, Switzerland
1984–1989	Clinical Training in General Surgery, Internal Medicine, Neurology and Neurosurgery
1988	Doctoral Thesis, University of Zurich, Switzerland
1990–1992	Research Fellow in CNS Tumor Immunology, Laboratory of Surgical Research, University Hospital Basel
1993	Board-certified Neurosurgeon FMH (Swiss Medical Association)
1993–1995	Post-doctoral Fellowship in Cancer Genetics with David Sidransky, Johns Hopkins Hospital, Baltimore, MD, USA
1998	Habilitation/venia docendi, University of Basel, in the field of Neurosurgery

1999	Training Course National Cardiovascular Center, Neurovascular Surgery Branch, Osaka, Japan
2001	Visiting Professor, Neurosurgical Clinic, Emory University, Atlanta, USA

Appointments

1995	Staff position in Neurosurgery at the University Hospital, Basel, Switzerland, heading clinical and preclinical brain tumor research projects
1998	Permanent staff position in Neurosurgery at the University Hospital, Basel, Switzerland
2001	Full professorship (personal extraordinariate)

Awards and Prizes

1993	European Assoc. of Neurol. Surgeons-Research Prize (3rd)
1994	SIAK/Essex Prize for Applied Cancer Research
1994	Amerbach Prize University of Basel
2002	Robert Bing Prize, Swiss Academy of Medical Sciences
2003	Springer Prize for the best clinical paper of the year 2002 in the Eur J Nucl Med (29, 486–493) awarded by the European Association of Nuclear Medicine
2006	Prize Prof. Dr. Max Cloëtta

Memberships and Committees

1983	*foederatio medicorum Helvetiae (FMH,* Swiss Medical Association)
1992	Swiss Society of Neurosurgeons
1992	Swiss Society of Neurology
1994	American Association for the Advancement of Science
1994	American Association for Cancer Research
1998	Swiss Society for Neuroscience
2003	European Association of Neurosurgical Societies
2003	Swiss Archives for Neurology and Psychiatry
2002–2005	Curriculum Commission, Medical Faculty, University of Basel
2003	Delegate of the Medical Faculty, University of Basel, to the Swiss Academy of Medical Sciences

External reviewer for:
Clinical Cancer Research, Cancer Research, Oncogene, International Journal of Cancer, Swiss National Science Foundation, Swiss Cancer League/Oncosuisse, Alberta Cancer Foundation (Canada)

KEY PUBLICATIONS

1. Stefan Kneifel, Dominik Cordier, Stephan Good, Mihai C. S. Ionescu, Anthony Ghaffari, Silvia Hofer, Martin Kretzschmar, Markus Tolnay, Christos Apostolidis, Beatrice Waser, Marlene Arnold, Jan Mueller-Brand, Helmut R. Maecke, Jean Claude Reubi, and Adrian Merlo. Local Targeting of Malignant Gliomas by the Diffusible Peptidic Vector 1,4,7,10-Tetraazacyclododecane-1-Glutaric Acid-4,7,10-Triacetic Acid-Substance P. Clin. Cancer Res 12: 3843–3850, 2006.

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PAS DE MYTHE DE SISYPHE: GLIOMA RESEARCH ON THE MOVE

Adrian Merlo

A myth of futility

In Greek mythology, Sisyphos, king of Corinth, was punished in Hades for his trickery against Thanatos, the God of death. When his time had come and Thanatos came to fetch him, Sisyphos had him chained up so that no one on earth died until Ares, the God of War, came to free Thanatos. Before Sisyphos was taken to the underworld with the help of Ares, he asked his wife to leave his body unburied. When he reached Hades he was permitted to go back to earth to punish his wife, and he lived to a ripe old age before dying a second time. His trickery resulted in his eternal punishment in Hades. The cunning Sisyphos had to roll a huge stone up a mountain over and over again (Britannica Concise Encyclopedia 2006).

Overwhelming dynamics …

Not unlike Sisyphos repeatedly rolling his rock up the mountain, contemporary disciples of Askeplios engage into a fight against malignant brain tumors that repeatedly recur following surgical removal. Tumor cells rapidly proliferate and insidiously infiltrate the human brain, imposing anarchic rule onto one of the most complex and vulnerable organs. Among the various types of brain-intrinsic tumors, glioblastoma is the most malignant and prevalent form that occurs at an annual frequency of some 300 new cases in Switzerland. Mean patient survival is less than 1 year. Neurosurgeons can control the rapidly proliferating tumor mass by repetitive resections, reducing increased intracranial pressure and thereby delaying imminent death. Despite usage of most modern tools of the operating theatre, such as online magnetic resonance imaging or computer-assisted navigation, the infiltrative nature of

the disease eventually puts an end to these efforts. The problem of glioblastoma can be reduced to the fact that there is no specific treatment for tumor infiltrating cells that migrate throughout wide areas of the brain into regions where the blood barrier is still intact. Moreover, it is the blood brain barrier that represents a major obstacle for most cancer drugs to reach their target following systemic application.

Attacking the problem

From a scientific point of view, I will subdivide the problem "glioblastoma" to 4 major subtopics: *i)* the molecular genetics and biology of the tumor cell, *ii)* the complex interactions between the tumor cell and the host organ and its immune cells, neurons and astrocytes, *iii)* the specific nature of the blood brain barrier which imposes unique requirements on the biodistrution of candidate drugs, and *iv)* resistance of GBM cells to cancer drugs. In recent years, the development of lipophilic drugs that penetrate the blood brain barrier to a certain degree has been complemented by local neurosurgical strategies to directly deliver drugs into the tumor mass and adjacent areas of tumor cell infiltration. The four topics cover important aspects of the new field of preclinical and clinical neuro-oncology, a new discipline that is interconnected with fields such as neuro-surgery & neurology, radio-oncology, oncology, basic and applied cancer research, developmental biology and especially neuroscience.

The malignant cell

Human malignant gliomas arise from neural progenitor cells or dedifferentiated astrocytes [1]. Among the countless mutations in the genome of tumor cells, a few key genes and associated pathways have been well characterized. This allowed to create several distinct murine glioma models that faithfully reiterate the histological hallmarks of the disease. This similarity with the human disease phenotype does not prove that these models also exhaustively mimick the altered genotype of the human disease. That is the reason why animal models for cancer are limited to test therapeutic concepts for clinical use. Nonetheless,

these models can be utilized to test a biological hypothesis and help to define a hierarchy of genetic alterations involved in tumorigenesis. For instance, activated PKB/Akt and Ras alleles alone could not induce glioblastomas, but the combined activation did [2]. Likewise, oncogenic EGFR did only induce glioblastomas in a background genotype characterized by tumorigenic deletions of the *Ink4a/arf* locus [3]. Interestingly, astrocytes harboring homozygous deletions at the *Ink4a/arf* locus displayed accelerated growth curves similar to neural stem cells.

Cancer is a stochastic disease in which mutation patterns vary between different tumors. The relatively monotonous histological phenotype is caused by a great variety of ever new combinations of genetic alterations. Mutations that convey a growth advantage to a cell are selected for, leading to overgrowth of precursor cell populations with increasingly malignant tumor cell clones [4]. We now understand that a limited number of mutations are sufficient to give rise to brain tumors. Growth control pathways (EGFR, PDGFR, c-Met and others) activating mitogen activated kinases are deregulated [1]. The activity of a central signaling protein, the protein kinase B (PKB/Akt), is frequently out of balance, either due to mutations of an upstream regulatory protein, e.g. phosphatidylinositol-3 kinase (PI3KCA) [5], the tumor suppressor PTEN [6,7,] or the regulatory factor CTMP [8,9]. A mutation of the PTEN gene in GBM cells is shown that leads to activation of the protein kinase B (PKB) (Figure 1). PKB is a central signaling molecule that is interconnected to key cellular functions like survival, migration or energy metabolism.

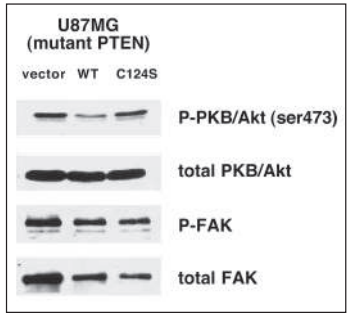

Figure 1
PTEN reduces PKB/Akt phosphorylation (Ser-473) in U87MG cells and does not modulate FAK phosphorylation in cell adhesion assays. Cellular lysates from U87MG cells transfected with empty vector (Vector), vector containing wild-type PTEN (WT), or mutant PTEN (C124S) were immunoblotted with anti-phospho PKB/Akt (Ser473, top panel) and reprobed with anti-PKB/Akt (second panel, top). [CANCER RESEARCH 59, 5479–5482, 1999]

As protection against physical and chemical stress factors, Nature bestowed a safeguard mechanism to every cell for the sake of the entire organism. Cancer cells are characterized by resistance to pro-apoptotic stimuli. If a cell accumulates genetic damage which is beyond its repair capacity, a highly controlled program of self-destruction (also called cellular suicide or apoptosis) is elicited. Tumor cells carry mutations in the regulatory network of apoptosis. For example, many cancer cells harbor mutations in the p53 gene or in functionally related genes like $p14^{ARF}$ or HDM2 [1,12]. The structural organization of the $p14^{ARF}/p16^{INK4a}$ locus on chromosome 9p21 is curious because the same nucleotide sequence gives rise to 2 distinct protein products by using a different promoter associated with a single nucleotide frame shift [10]. The multi-facetted p53 protein can be viewed as a cellular "apopostat" that sets the threshold for the induction of the cell-intrinsic death program [11]. Pro-apoptotic stimuli (physical energy, radiation, cytotoxic drugs, targeted interventions) can still induce cell death, however, higher amounts of energy or drug doses have to be used to destroy these cells in comparison to tumor cells that are wild-type for the p53 pathway. Mutations of the p53 gene have not found to be frequent in GBM, however, $p14^{INK4a}$ – an upstream regulatory protein of p53 – is often inactivated [12].

In cancer cells, stop signals controlling cell proliferation are inactivated. For example, the onset of the S-phase of the cell cycle to synthesize new DNA is hold in check by a multi-protein complex consisting of $p16^{INK4a}$, cyclin D, the cyclin-dependent kinase 4 and the retinoblastoma protein Rb. In GBM, genetic alterations can be found in at least one of these four cell cycle regulatory factors. The tumor suppressor $p16^{INK4a}$ is frequently inactivated by homozygous deletions in many cancers [12-14]. $p16^{INK4a}$ can also be inactivated by epigenetic alterations. In this case, the gene promoter which lies within a dense CpG island, is hypermethylated resulting in gene silencing [15,16].

Invasiveness of tumor cells is regulated by a number of factors, including EGFR and focal adhesion kinase (FAK) [17] and the tumor suppressor PTEN [6,7,14]. A curiosity of cancer is that malignant cells carry a severe mutator phenotype [18]. Genes that control the genomic integrity

of a cell can also be mutated, leading to the accumulation of countless epigenetic and genetic alterations. A rare example is the mutation of mismatch repair genes MSH2 or MLH1 in Turcot's syndrome, which is defined by the co-manifestation of a GBM and a colorectal cancer [19]. Genes that control genomic integrity in ordinary GBM are still to be discovered.

The altered microenvironment in the brain

The vision to use a patient's own immune defense system to fight cancer cells is also being tested in malignant brain tumors. In an early study, we analyzed the T cell receptor profile in tumor-derived lymphocytes [20] and the gene expression profile of critical cytokines [21]. Interestingly, transcription of genes encoding for inhibitory factors (IL-10, TGF-b2) appears to prevail in primary brain neoplasm. Comparing the patterns of subpopulations of T-cells by characterizing their molecular composition disclosed distinct enrichment of T-cell within the tumor as compared to the distribution pattern in peripheral blood lymphocytes, indicating a selection of some T-cell families possibly due to recognition of tumor antigens. *Ex vivo* expanded tumor infiltrating lymphocytes that were harvested following stimulation with interleukin-2 and antibodies against CD3 showed nearly complete loss of their cytotoxic potential, likely to be a consequence of the expression of immunosuppressive factor by the tumor cells. It became clear that cellular adoptive immune therapy has to overcome functional inactivation of these cytotoxic T cells in order to become a useful anti-tumor tool.

The invasion of healthy brain tissue by tumor cells proceeds along the extracellular matrix of white matter tracts and the perivascular space, which contain ligands for the integrin family of transmembrane receptors. Focal adhesion kinase (FAK) is required for integrin-dependent signaling and modulates cellular adhesion, migration, and survival. By blocking FAK activity, levels of activated caspase-3, a cell death effector, were found to be increased which sensitized tumor cells to apoptotic stimuli and reduced their invasiveness [17]. Loss of substrate

attachment in normal cells leads to the induction of apoptosis, or anoikis (greek: homeless). Tumor cells are generally resistant to anoikis, reflecting independence from integrin signaling. We found that introducing functional PTEN into glioma cells was sufficient to restore the anoikis reaction by reverting activating phosphorylation of PKB/Akt on Ser-473 [6].

Tenascin-C, an extracellular matrix glycoprotein, is expressed in the stroma of most solid tumors, including GBM [22]. Acting like an oncogene, tenascin-C stimulates tumor growth by various mechanisms including promotion of proliferation, escaping immuno-surveillance and positively influencing angiogenesis. Clinically, radiolabelled monoclonal antibodies against tenascin-C are being evaluated as new therapeutic options for GBM patients [23,24]. In a large genetic study comparing deletion pattern on chromosome 1p between glioblastoma and oligodendroglioma (a much less malignant brain-intrinsic tumor with a mean survival of some 10 years), we detected a unique and highly consistent recombination site on 1p11 that involved the developmental gene Notch2. By studying the role of Notch2 in brain tumorigenesis, we found that gene expression of tenascin-C is regulated by Notch2. Notch2 mediates this effect via the canonical transcription factor CSL. We have detected a mutation in an malignant oligodendroglioma cell line that inactivates the binding domain between Notch2 and CSL (so called ram23 domain). This mutation leads to a significant reduction of tenascin-C expression. Inactivation of Notch2 appears to be linked to a glioma subtype (oligodendroglioma) that has a much better prognosis than GBM and does not express tenascin-C. We found in a large epidemiological study that the prognosis of brain tumor patients is linked to expression of Notch2 and Tenascin-C (Figure 2). In GBM, both proteins are abundantly expressed while they are absent in the more benign oligodendroglioma that usually have lost chromosomal arms 1p and 19q (manuscript in review).

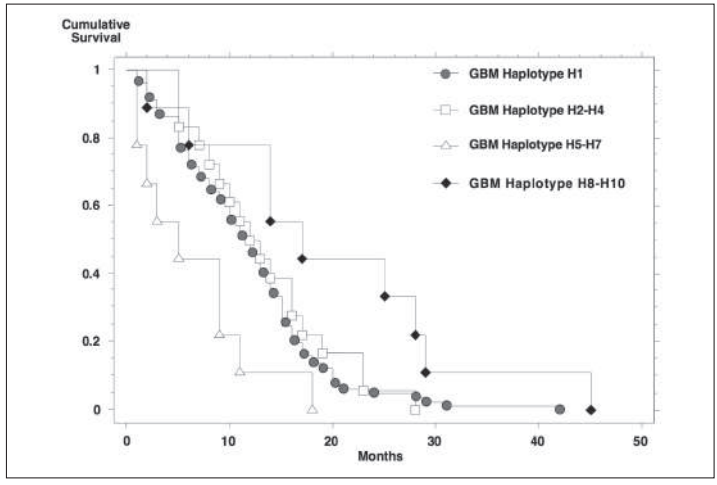

Figure 2
Glioma patients with centromeric 1p allelic loss show better survival. Deletion patterns on chromosome 1p in GBM. Somatic deletion mapping in 118 GBM was performed using 43 microsatellite markers. Four markers at deletion hotspots (D1S2845 at 1p36.3, D1S507 at 1p36.1, D1S216 at 1p22 and D1S2696 at 1p11) were selected to define chromosome 1p haplotypes. In GBM, 10 haplotypes were grouped into tumors with centromeric (H8-H10), interstitial (H5-H7) and telomeric (H2-H4) deletion patterns. Kaplan-Meier cumulative survival curve of the haplotype groups shows significantly better survival for tumors with centromeric deletions. [Manuscript in review]

Beta- and alphaKnife: targeted radiopeptides for malignant gliomas

There is no satisfactory surgical therapy for malignant gliomas because of their infiltrative nature and exponential growth rate that annihilates the best surgical result. Furthermore, malignant brain tumors are often critically located adjacent to or within functionally important areas that lead to severe neurological morbidity when aggressively treated by debulking surgery. The general principle of tumor surgery, which demands resection around the tumor mass respecting a safety margin of 1–2 cm, cannot be respected in neurosurgery for obvious reasons. In a longstanding collaboration with the Clinic and Institute of Nuclear

Medicine and the Radiochemistry Unit, we have advanced technology that allows neoadjuvant tumor treatment prior to resection. Initially, the octreotide analog DOTATOC was used which was not found to be suitable for the treatment of prevalent GBM because of inconsistent expression of somatostatin receptors type 2 [25,26]. We therefore developed a modified version of the 11-mer peptide substance P that is conjugated to DOTAGA, a chelator that entraps the radionuclides Yttrium-90 (pure beta-emitter, mean tissue range 5 mm) or Bismuth-213 (alpha-emitter, mean range 84 micrometers). Such an approach could have several advantages over conventional debulking strategies [27]. First of all, the tumor mass is saturated with the therapeutic agent by local injection prior to resection which induces widespread tumor necrosis and tumor shrinkage (Figure 3). Secondly, spreading of malignant and invasive tumor cells by the surgical procedure (violating the principle of circumferential tumor surgery) can be significantly reduced. Furthermore, tumors at critical location can be treated with ultra low range-alpha emitters sparing adjacent neuronal tissue. Preliminary experience using peptidic vectors labeled with alpha- and beta-emitting metallic radionuclides has shown that many of these goals

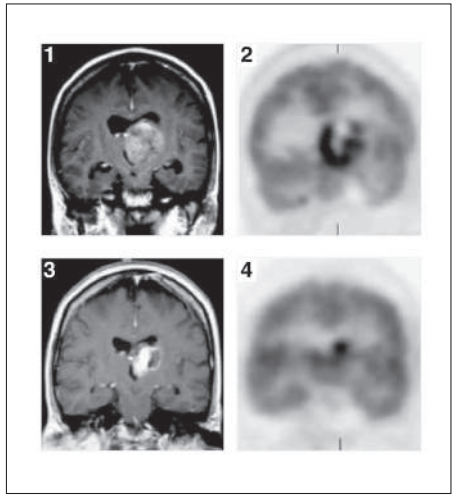

Figure 3
Radiological response in deep-seated GBM. Coronal planes of T1-weighted MR-images with the contrast agent gadolinium-DOTA (1, 3) and of [18F]-deoxy-glucose-PET images (2, 4) show an inoperable large GBM in the left thalamus (case 12, Table 1). A partial response (3, 4) was observed 3 months after targeted intratumoral β-radiotherapy using [90Y]-DOTAGA-SP, leading to clinical improvement (15 to 65 points in Barthel-Index). [Clin Cancer Res 2006;12, 2006]

can be achieved [28]. Using diffusible, small drug like vectors, development of a new dosimetry protocol allows to perform precise dosimetry on a Gray/voxel basis, representing the scientific basis for future calculations of dose-response curves (Kneifel et al, submitted). Glioblastomas are highly vascularized lesions that tend to bleed during resection, rendering3D-orientation difficult for the surgeon. Neoadjuvant targeting of NK-1 receptor positive tumor cells that also grow around the neo-vasculature induces an additional anti-angiogenic effect that transforms the highly vascularized tumor into a non-hemorrhagic radionecrotic mass that forms a pseudocapsule. Neoadjuvant treatment ("betaKnife") thus improves respectability of glioblastoma. For critically located tumors, alpha protocols are being established ("alphaKnife").

New systemic approaches as combinatorial strategy

In collaboration with Novartis Oncology, we have tested a number of new cancer drugs as single agents and specifically in drug combinations, looking for additive or synergistic effects. Many regulatory genes are epigenetically silenced by methylation and acetylation. Cardinal feature of malignant gliomas, as well as many other cancers, is a high rate of ATP consumption via upregulation of glycolysis. These metabolic as well as the classical cancer pathways represent potential targets for interference with specific drugs. Recently, small lipo-soluble molecules were designed as a new class of drugs more specifically targeting these cancer pathways. These drugs were applied to human GBM cells for induction of apoptosis as a readout of drug efficacy. Single applications of the protein kinase inhibitors against EGFR, PDGFR and mTOR were tested as single drugs and in various combinations. Even though these new drugs showed cytostatic effects to variable degrees, apoptosis of tumor cells could only be induced if an additional cytotoxic compound was added. This observation supports the argument that blocking signalling of well established cancer pathways may not be sufficient to achieve a lasting effect. Induction of cell death may be a prerequisite to obtain a profound effect.

In addition, the glycolytic inhibitor 2-deoxyglucose (2-DG) was used to exert a metabolic stress onto the cancer cells, reducing the availability of ATP. 2-DG alone was not able to induce cell death and had only a cytostatic effect at dose ranges from 1–25 mM. In contrast, cytotoxic drugs like the microtubule inhibitor EPO906 or histone deacetylase inhibitors like trichostatin A, sodium butyrate and LAQ824 were able to efficiently trigger apoptosis, but are known to lead to toxicity with increased dose ranges in mice and humans. We therefore tested combinations of low doses of cytotoxic drugs with cytostatic drugs, 2-DG and indomethacin. As p21 was shown to be re-expressed upon exposure to histone deacetylase inhibitors and to protect cancer cells against apoptotic stimuli, 2-DG was used as a sensitizer for apoptosis following treatment with histone deacetylase inhibitors, since 2-DG strongly inhibits protein expression including p21. Synergistic induction of apoptosis was observed in glioma and other cancer cell lines upon this combined treatment (Figure 4). Thus, drug combinations that inflict a

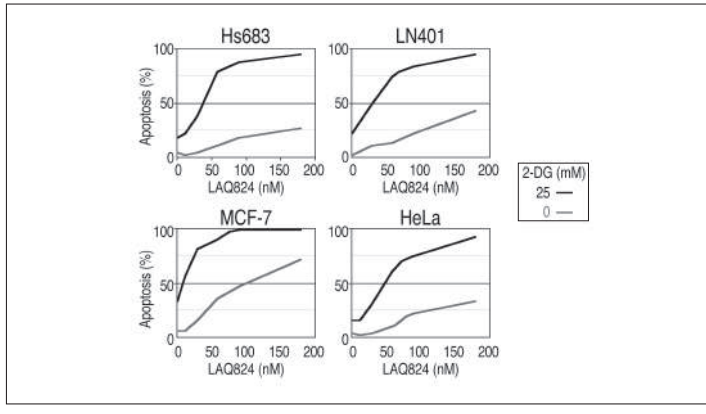

Figure 4
2-DG and HDAC inhibitor LAQ824 synergize to induce apoptosis in cancer cell lines. Hs683 and LN401 glioma and MCF-7 and HeLa cancer cell lines were cultured in triplicates in standard medium during 48h. Cells got fresh medium before to be treated with increasing doses of LAQ824 (10, 30, 60, 90, 180nM), and/or 25mM 2-DG during 72h after which time floating and viable cells were pooled and cell death was measured using FACS analysis (pro-G1 phase). HeLa cell line was exposed to 25mM 2-DG and 60nM for 72h.

cytotoxic stress to cancer cells by inducing widespread re-expression of silenced genes (which leads to expression of p21 of the stressed cell to halt the cell cycle machinery) and simultaneously reduce the availability of ATP (thwarting the p21 response) may trigger a profound pro-apoptotic effect selectively to cancer cells. The combination of an anti-metabolic and cytotoxic stress may represent a promising new way to treat certain cancers in the future including glioblastoma.

Outlook

Instead of living the myth of Sisyphos, new building blocks are constantly integrated into the growing wall of scientific progress that will eventually lead to a new house representing the future way to attack the clinical problem of glioblastoma. Local treatment modalities will improve resectability of these tumors. The goal is to reduce the visible tumor mass to less than 1 cm^3, a value which is proven to be critical to obtain lasting clinical responses in medulloblastoma, a malignant brain tumor of children. New systemic approaches will eventually lead to a better control of the infiltrative component of the disease. Given the combinatorial "genius" of the system GBM (mutator phenotype), it is likely that singular approaches will rapidly be thwarted by emergence of resistance mechanisms. For this reason, combinatorial strategies have to be tested and set up to tackle the problem from several angles. Targeted biological interventions will sensitize tumor cells to physical and chemical stress (irradiation, cytotoxic agents), enhancing the degree of tumor cell apoptosis that will lead to lasting clinical responses.

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I wish to express my deep gratitude to the Prof. Dr. Max Cloetta Foundation for the great honour to receive this award. It distinguishes an intense and interdisciplinary research effort that could only be achieved by substantial support of highly dedicated technicians, nurses, Ph. D. students, postdoctoral fellows, fellow clinicians and researchers

from basic and clinical Sciences of several Faculties. I especially acknowledge the noble endurance of these many collaborators in difficult times when the fruits were hanging high. I am also indebted to the many patients struck by a most serious illness that honored our team with their confidence by conscientiously supporting this research.

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DER CLOËTTA-PREIS 2006
WIRD VERLIEHEN

HERRN PROFESSOR DOKTOR

MICHAEL O. HENGARTNER

GEBOREN 1966, VON MUOLEN (SG)
ORD. PROFESSOR FÜR MOLEKULARBIOLOGIE
AM INSTITUT FÜR MOLEKULARBIOLOGIE
DER UNIVERSITÄT ZÜRICH
FÜR SEINE HERAUSRAGENDEN
FORSCHUNGSARBEITEN ÜBER DIE REGULATION
DES PROGRAMMIERTEN ZELLTODES.

ZÜRICH, 24. NOVEMBER 2006

FÜR DEN STIFTUNGSRAT:

DER PRÄSIDENT

DER VIZEPRÄSIDENT

EIN MITGLIED

Prof. Dr. Michael O. Hengartner

CURRICULUM VITAE

Name:	Michael Otmar Hengartner
Date of Birth:	5 June 1966 in St. Gallen, Switzerland
Citizenship:	Muolen (SG) Switzerland, Canada
Civil Status:	Married, 2 children

Education and Training

Département de Biochimie & Microbiologie, Université Laval, Québec, Canada
1988 B.Sc. Biochemistry

Department of Biology, MIT, Cambridge, USA
1994 Ph.D. Biology
Thesis: Genetic and molecular analysis of programmed cell death in C. elegans
Advisor: H. Robert Horvitz

Employment

Hôtel-Dieu de Québec et Université Laval, Québec, Canada
1986–1988 Research Assistant (Summers only)
Supervisor: Dr. Guy Poirier

Cold Spring Harbor Laboratory, Cold Spring Harbor, USA
1994–1995 Staff Investigator
1995–1998 Assistant Investigator
1998–1999 Associate Investigator
1999–2002 Associate Professor

Institute of Molecular Biology, University of Zurich, Zurich, Switzerland

2001–present	Ernst Hadorn Endowed Chair in Molecular Biology and Professor of Molecular Biology
2006–present	Vice-dean, Faculty of Science

Academic Experience

SUNY Stony Brook, Stony Brook, USA

1994–2001	Faculty Member (Graduate program in Genetics)
1997–1999	Member, Admissions Committee (Genetics, MCB)
1997–2001	Member, Executive Committee (Genetics)
1999–2001	Faculty Member (Graduate program in Molecular and Cell Biology)

Cold Spring Harbor Laboratory, Cold Spring Harbor, USA

1995–2001	Head, CSHL Cancer Center Genomics Shared Resource
1996–2001	Director, Undergraduate Research Program
1998–2002	Faculty Member, Watson School of Biological Sciences (WSBS)
1998–2000	Member, WSBS Admissions Committee
1998–2001	Member, WSBS Executive Committee

University of Zurich, Zurich, Switzerland

2001–present	Member, Zurich Neuroscience Center
2002–present	Chair, Ph.D. Program in Molecular Life Sciences (MLS)
2002–present	Science faculty (MNF) representative, Committee on continuing education
2003–present	Member, MLS Admissions Committee
2003–present	Member, Steering committee, Cancer Network Zurich
2003–present	Member, MNF Research Committee (Chair, 2005–2007)
2005–present	Member, University of Zurich (UZH) Research Committee
2003–present	Member, MNF undergraduate curriculum development committee

2004–present	Coordinator, UNIZH M.Sc. program in molecular and cellular biology
2004–present	Member, MNF Information Sciences committee
2004–present	Member, Center for model organism proteomes, UZH and ETH
2005–present	Chair, Zurich Life Sciences Learning Center task force
2005–present	Member, Interfaculty MD/Ph.D commission
2005–present	Chair, Life Science Zurich Graduate School

Teaching Experience

1987–88	Departement de Biochimie & Microbiologie, Université Laval, Québec, Canada
1990–1991	Department of Biology, MIT, Cambridge, USA
1994–2000	Rockefeller University, New York City, USA
1994–2001	SUNY Stony Brook, Stony Brook, USA
1995–2003	Cold Spring Harbor Laboratory, USA Watson School of Biological Sciences
1997–2004	Gulbenkian Institute of Science, Lisbon, Portugal
2000	Max Planck Institute for Developmental Biology, Tuebingen, Germany
2003	University of Tampere, Finland
2001–present	University of Zurich, Zurich, Switzerland

Business Experience

Upstate Biotechnology, Lake Placid, USA

1997–2002	Consultant (Apoptosis Research)

Devgen, Gent, Belgium

1997	Co-Founder
1998–2003	Member of the Scientific Advisory Board

ForScience, New York, NY, USA
2000 Co-Founder
2000–2004 Member of the Advisory Board

UBS Warburg Equity Research – Health Care Group, New York City, USA
2001–2002 Member of the Scientific Advisory Board

The Genetics Company, Zurich, Switzerland
2001–2003 Member of the Scientific Advisory Board

Awards and Distinctions

1988 Silver Medal of the Governor General of Canada
1988–1990 B1 Joint NSERC-FCAR Postgraduate Scholarship
1988–1992 1967 Science and Engineering Postgraduate Fellowship
1993–1994 Glaxo Academic Links Fellowship
1994–2000 Rita Allen Foundation Scholar
1995 Pharmacia & Science Prize for Young Investigators – 1995 Grand Prize
2001– present Ernst Hadorn Endowed Chair in Molecular Biology
2003 Friedrich Miescher Award
2003 Josef Steiner Cancer Research Award
2006 Swiss National Latsis Prize
2006 Prize Prof. Dr. Max Cloëtta

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ROADS TO RUIN: APOPTOTIC PATHWAYS IN THE NEMATODE CAENORHABDITIS ELEGANS

Michael O. Hengartner

Summary

Apoptosis is a cell suicide program used by animals to get rid of cells that are either superfluous or potentially dangerous. The same gene families – caspases, Apaf-1-like molecules, and Bcl-2 family members – control apoptosis in organisms as diverse as humans, *Drosophila*, and *C. elegans*, underscoring the conserved nature of the apoptotic program. There are at least three death-inducing pathways in *C. elegans*. During embryonic and larval development, cell death is specified through transcriptional regulation of the BH3 domain gene *egl-1*. Similarly, in response to DNA damage, germ cells in the adult hermaphrodite activate a p53-dependent pathway that leads to apoptosis through transcriptional upregulation of the BH3 domain proteins EGL-1 and CED-13. By contrast, death of these same oocytes during differentiation is regulated by an *egl-1*-independent pathway. Apoptotic cells are recognized and engulfed by their neighbours, in a process that requires the function of several, evolutionarily conserved engulfment proteins. These proteins act in two partially redundant signaling pathways, which coordinately regulate the activation of CED-10/Rac and the subsequent cytoskeletal rearrangements required for cell corpse engulfment. In this review, I will outline our understanding of these various apoptosis-inducing and apoptosis-induced pathways.

Introduction

Millions of our cells commit suicide every day. Why? And how? Is this process important? If yes, important for what? And what happens if it goes awry? These are the questions that drew me to study the cellular process known as programmed cell death, or apoptosis. In this review,

I wish to briefly review what we have learned about the molecular basis of apoptosis, using the simple nematode *C. elegans* as a model organism.

The process of controlled cell death was initially discovered by developmental biologists. Indeed, and rather surprisingly, development is characterized not only by rapid cell growth and cell division, but also by high rates of cell death (Glücksmann, 1951). In addition to its role in development, controlled cell death is also used extensively for homeostatic functions, or to eliminate aberrant, damaged, or harmful cells (reviewed in refs Baehrecke, 2002; Jacobson et al., 1997; Meier et al., 2000; Vaux and Korsmeyer, 1999). Although several forms of cell death have been described in multicellular organisms (see ref. Vaux and Korsmeyer, 1999), I will focus here exclusively on apoptosis. Apoptotic cells usually present characteristic morphological changes, including chromatin condensation and DNA laddering, loss of mitochondrial membrane potential and of plasma-membrane phospholipids asymmetry, and detachment from the cellular matrix (Kerr et al., 1972; Wyllie, 1980; Wyllie et al., 1980). Because dysregulation of apoptosis is associated with several human pathologies, such as cancer, autoimmune diseases and neurodegenerative disorders, a better understand-ing of apoptosis could have interesting prognostic and therapeutic implications.

Developmental cell death in C. elegans

During *C. elegans* development, exactly one hundred and thirty-one somatic cells in the hermaphrodite die as a result of apoptosis (Sulston and Horvitz, 1977; Sulston et al., 1983). Most of these deaths occur around the middle of embryogenesis, between 250 and 500 minutes after fertilization. Death is a rapid and transient process: cells that die are swiftly engulfed by their neighbors and digested. Thus, by the time that embryos hatch (~800 minutes), hardly any cell corpses can be observed any more.

Genetic studies have identified many genes that function in one of multiple steps in C. elegans apoptosis (reviewed by Kinchen and

Hengartner, 2005). Epistasis analysis of these mutations allowed Horvitz and colleagues to place the genes into a largely linear pathway by which apoptosis cell death is thought to proceed (Figure 1; Metzstein et al., 1998). This pathway can be subdivided into three components – execution of apoptosis, engulfment of the apoptotic cell corpse, and degradation of the corpse within the engulfing cell. Upstream of this common core pathway are genes that influence the decision of individual cell types whether to live or die (Kinchen and Hengartner, 2005). I refer the interested reader to a recent review that covers these genes in more details (Lettre and Hengartner, 2006).

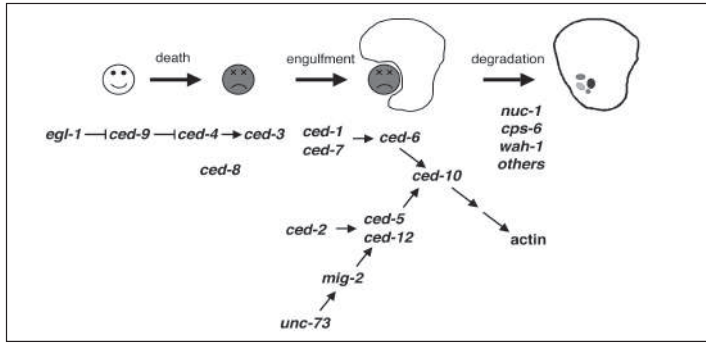

Figure 1
The genetic pathway for programmed cell death in C. elegans.
Genetic screens identified many genes that function in C. elegans apoptosis. These genes divided the apoptotic program into three distinct steps: killing of cells that need to die, recognition and phagocytic engulfment of apoptotic cells, and degradation of engulfed cells. Positive (→) and negative (⊣) genetic interactions are shown. Dashed lines represent indirect interactions. All the genes shown here act in most if not all cell deaths. Genetic screens also identified genes that control the decision of individual cells whether to live or die. These cell type-specific apoptotic regulators act upstream of the killing step (not shown).

Four genes identify a conserved genetic pathway for the induction of cell death

Genetic screens for mutants with abnormal cell death patterns identified four genes that regulate all somatic cell deaths in *C. elegans*: *ced-3*, *ced-4*, *ced-9* and *egl-1 (ced*, *ce*ll *d*eath abnormal; *egl, egg l*aying-

defective). Loss-of-function (lf) mutations in *ced-3, ced-4*, and *egl-1* result in survival of almost all (~131) doomed cells, indicating that these three genes have proapoptotic functions (Desai et al., 1988; Ellis and Horvitz, 1986). By contrast, *ced-9* has antiapoptotic activity because a *ced-9* gain-of-function (gf) mutation blocks apoptosis, whereas *ced-9*(lf) worms die during early development due to excessive cell death (Hengartner et al., 1992). Epistasis studies ordered these genes into a pathway, with *egl-1* acting as a negative regulator of *ced-9*, and *ced-9* in turn negatively regulating *ced-4* and *ced-3* (Figure 2; Metzstein et al., 1998).

The molecular identification of *ced-3*, *ced-4*, *ced-9* and *egl-1* in the

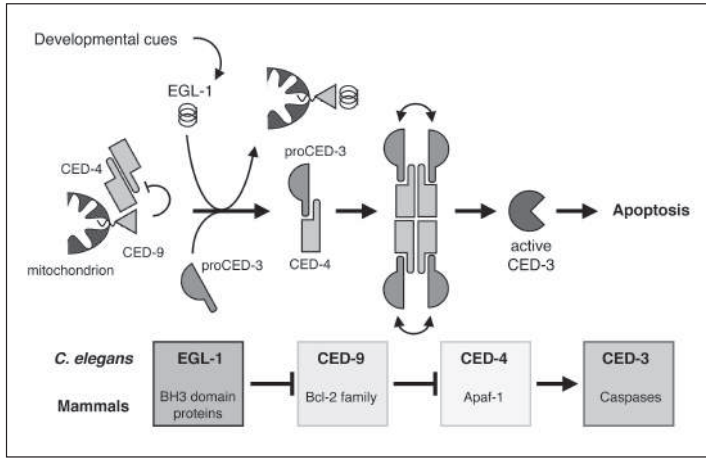

Figure 2
Activation of the C. elegans cell death machinery: a model.
Apoptosis in C. elegans is controlled by worm homologues of key regulators of apoptosis in mammals. In living cells, CED-4 dimers are sequestered by CED-9 on the outer surface of mitochondria and maintained in an inactive conformation. Cells destined to die by apoptosis express the BH3 domain protein EGL-1. Binding of proapoptotic EGL-1 to CED-9 induces a conformational change in the latter, resulting in the release of the CED-4 dimer from the CED-9/CED-4 complex. Once freed from CED-9's inhibitory interaction, two CED-4 dimers associate into a tetramer and recruit proCED-3 molecules to form the worm apoptosome. CED-3 becomes activated (through conformational change and/or proteolysis), and apoptosis is triggered.

1990s provided important clues to understand how the apoptotic machinery is engaged, not only in worms, but also in all other species. Indeed, all four genes code for conserved apoptotic regulators (Figure 2). Thus, *ced-3* encodes a protease of the caspase (**c**ysteine **asp**artyl prote**ase**) family, a group of enzymes pivotal for their role in the execution of apoptosis (Riedl and Shi, 2004; Xue et al., 1996; Yuan et al., 1993). Whereas several hundred caspase substrates have already been described in mammals, CED-3 substrates are still largely elusive (Earnshaw et al., 1999). CED-4 is an adaptor protein similar to mammalian apoptotic protease-activating factor 1 (Apaf-1; Yuan and Horvitz, 1992). Like their mammalian homologues, CED-3 and CED-4 oligomerize to form an apoptosome-like complex, and this interaction is required for CED-3 activation and apoptosis (Rodriguez and Lazebnik, 1999; Yang et al., 1998).

ced-9 and *egl-1* encode members of the Bcl-2 family: CED-9 is an anti-apoptotic protein with four Bcl-2 homology (BH) domains, whereas EGL-1 is a proapoptotic BH3-only domain protein (Conradt and Horvitz, 1998; Hengartner and Horvitz, 1994a; Hengartner and Horvitz, 1994b). In mammals, the Bcl-2 family includes pro- and antiapoptotic proteins, which all share at least one BH domain; these proteins play key roles in the control of apoptosis at the level of mitochondria (reviewed in Cory et al., 2003; Danial and Korsmeyer, 2004). The *C. elegans* genome encodes only three Bcl-2 family members: CED-9, EGL-1 and the BH3-only domain protein CED-13. In contrast to EGL-1, CED-13 does not play a role in developmental apoptosis in worms; its only known function to date is a modest role in promoting germ cell death following DNA damage (Figure 3; Schumacher et al., 2005).

Molecular model of apoptosis activation in C. elegans

The execution of apoptosis in *C. elegans* is regulated through a series of direct protein-protein interactions between the four main players described above (CED-3, CED-4, CED-9 and EGL-1; Figure 2). The immediate cause of death in C. elegans is the proteolytic cleavage of

CED-3 from its precursor form (proCED-3) into the processed, active enzyme. For this activation to occur, multiple proCED-3 molecules must associate with a CED-4 tetramer into a large oligomeric complex known as the C. elegans apoptosome. In the absence of apoptotic stimuli, however, CED-4 does not exist as a tetramer, but rather as an inactive dimer, sequestered on the outer surface of mitochondria through a direct protein-protein interaction with mitochondria-bound CED-9 (Figure 2; James et al., 1997; Spector et al., 1997; Wu et al., 1997a; Wu et al., 1997b). This sequestration (and possibly other limitations) prevents CED-4 from assembling with and activating CED-3. In the 131 cells destined to die, developmental cues lead to the expression of the proapoptotic EGL-1 protein. EGL-1 then binds via its BH3 domain to CED-9 and induces a conformational change that disrupts the CED-4/CED-9 interaction. Once released, CED-4 translocates from mitochondria to the outer surface of the nuclear membrane,

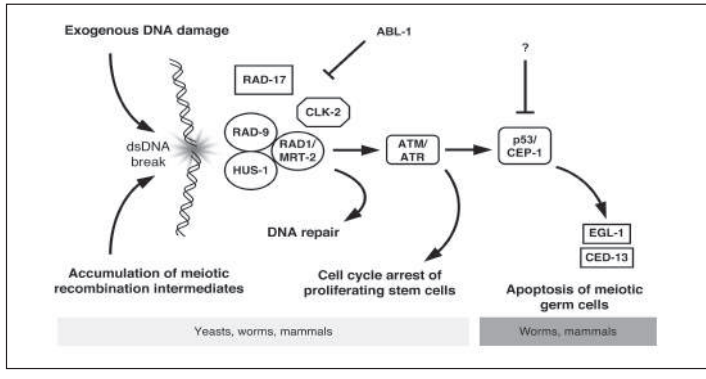

Figure 3
The pathway for dsDNA break-induced germ cell apoptosis in C. elegans.
Double-strand (ds) breaks in DNA, generated either through ionizing radiation or as the result of aberrant meiotic recombination, are sensed by several protein complexes that are highly conserved through evolution, and found from yeasts to humans. For example, HUS-1 and MRT-2 are part of the conserved 9-1-1 complex, a heterotrimer thought to be loaded onto sites of DNA damage. Cell cycle progression delay facilitates the repair process. The C. elegans p53 homolog, CEP-1, is responsible for the transcriptional activation of at least one BH3-only domain protein, EGL-1, that leads ultimately to the activation of the apoptotic machinery.

where it recruits CED-3 and activates apoptosis (Chen et al., 2000; Conradt and Horvitz, 1998; del Peso et al., 2000; del Peso et al., 1998; Seshagiri and Miller, 1997; Yang et al., 1998).

Conservation of the genetic pathway of apoptotic cell death between C. elegans and mammals

While the genetic pathways for apoptosis appear quite similar between *C. elegans* and higher metazoans (Figure 2), the biochemical mechanism by which the caspase is activated is, rather surprisingly, quite distinct. While CED-9 and Bcl-2 are both localized to the mitochondria (Chen et al., 2000; Hockenbery et al., 1990), the mammalian CED-4 homologue, APAF1, does not associate with Bcl-2 and is not localized to the mitochondrion; instead, APAF1 has been suggested to exist in a basal complex with Caspase-3 (Hausmann et al., 2000). In worms, transcriptional regulation of the BH3-only protein EGL-1 appears to be the major mechanism of control of developmental cell death, with EGL-1 displacing CED-4 from CED-9, and released CED-4 activating the CED-3 caspase and induction of cell death. By contrast, assembly of the mammalian apoptosome is regulated via controlled release of cytochrome c (cyt c) from the mitochondria – a step that is regulated either directly or indirectly by the Bcl-2 family (Hengartner, 2000; Zou et al., 1997). There is no known role for mitochondrial permeabilization and release of cytochrome c in the activation of CED-4 activity. Significantly, and consistently with this observation, CED-4 differs from APAF1 in that it does not have a cyt c interaction motif. Thus, it appears that while the proteins that mediate cell death have been conserved during evolution, they have also developed new abilities, and the control of cell death in mammals has become more complex.

Programmed cell death in the hermaphrodite germ line

The hermaphrodite gonad in *C. elegans* is a set of two bilaterally symmetrical tubes composed of a syncytium of germ cells surrounded by the somatic sheath cells and joined together through a common

uterus (Sulston, 1988). The gonad of the worm begins to develop in the early larval stages, when the progenitor cells Z(1-4) begin dividing. Z2 and Z3 will become the germ cells, while Z1 and Z4 generate the somatic gonad, which surrounds the germ cells and isolates them from the rest of the worm (Kimble and Hirsh, 1979).

Like the reproductive tissues of higher organisms, including humans, cells in the germ line of *C. elegans* undergo extensive programmed cell death. Unlike the deaths that occur in the soma during development, death in the germ line does not follow a set pattern: there is no fixed lineage specifying which cells will live or die (Gumienny et al., 1999). The cell death machinery is largely conserved between developmental cell death and germ line cell death. The main difference, however, is that there appears to be no requirement for EGL-1 activity during physiological cell death in germ cell death; *egl-1(lf)* mutants show wild type levels of apoptotic death in the hermaphrodite germ line (Gumienny et al., 1999). In addition, a *ced-9(gf)* mutation that affects the CED-9/EGL-1 interaction also has little effect on germ cell death. This would suggest that there must be a novel mechanism for controlling the progression of programmed cell death in the hermaphrodite germ line independent of the BH3-only domain protein EGL-1. Indeed, we and others have been able to identify a number of genes that appear to control physiological germ cell death (Boag et al., 2005; Kritikou et al., 2006; Lettre et al., 2004; Navarro et al., 2001). Intriguingly, many of these genes encode predicted RNA binding proteins. This observation suggests that perhaps apoptosis in the *C. elegans* is regulated via control of mRNA translation and/or metabolism.

Genotoxic stress induces germ cell apoptosis

An effective response to DNA damage is essential for genomic stability and long-term survival of an organism. Defects in genes that mediate response to DNA damage, such as p53, have been implicated in numerous diseases and in predisposition to various cancers. My research group could recently show that *C. elegans* can be used with success to study the molecular pathways that mediate DNA damage-induced

apoptosis (Gartner et al., 2000).

Genotoxic insults activate two distinct responses in the hermaphrodite germ line. First, mitotic cells arrest in order to repair damage induced by irradiation. Second, meiotic nuclei that are too damaged to survive undergo programmed cell death and are efficiently removed by the engulfment apparatus (Gartner et al., 2000).

The molecular mechanisms that mediate the response to genotoxic insults are just beginning to be dissected in *C. elegans*. Both genetic and reverse genetic approaches have been successful in identifying genes important for these responses. For example, forward genetic screens led to the demonstration that *hus-1* and *mrt-2*, genes encoding subunits of a conserved PCNA-like heterotrimeric clamp, are required for apoptosis in response to double strand DNA (dsDNA) breaks (Ahmed and Hodgkin, 2000; Hofmann et al., 2002). Conversely, reverse genetic experiments showed that CEP-1 (*C. elegans* p53 homologue), the worm homologue of the mammalian p53 tumor suppressor gene, is required for DNA damage-induced apoptosis, but not cell cycle arrest (Derry et al., 2001; Schumacher et al., 2001).

How does CEP-1/p53 promote apoptosis? We found that transcription of *egl-1* is dependent on functional *cep-1*, suggesting that CEP-1 might act as a transcriptional activator of *egl-1* (Hofmann et al., 2002). Interestingly, p53 has been shown to directly regulate transcription of BH3-only domain containing proteins in mammals following genotoxic stress. Thus, the pathway that mediates DNA damage-induced apoptosis is surprisingly similar between nematodes and mammals (Figure 3).

Engulfment and degradation of the apoptotic cell

Engulfment is the process by which cells that have died, either by apoptotic or necrotic means, are cleared, usually by professional phagocytes or bystander cells (Kerr et al., 1972). *C. elegans*, which has no professional phagocytes, uses cells that are immediately adjacent to the apoptotic cell to remove it, usually within a hour of the onset of apop-

tosis (Robertson and Thomson, 1982). Rapid elimination of apoptotic cells has been suggested to be important to prevent autoimmunity. Indeed, in mammals, secondary necrotic lysis of unengulfed apoptotic cells can result in local inflammation and exposure of auto-antigens to immune cells (Mevorach, 1999; Tan, 1994). The ability to remove apoptotic cells and prevent exposure of self-epitopes would thus appear to be essential to averting inappropriate immune responses and diseased states in the animal.

Genetic screens in *C. elegans* identified several genes which, when mutated, result in the accumulation of persistent apoptotic cell corpses (Figure 4). These mutations define two signaling pathways, which appear to act at least partially in parallel to each other, as double mutants defective in both pathways show a significantly stronger persistent cell corpse phenotype (Ellis et al., 1991; Hedgecock et al., 1983). By contrast, double mutants between genes within the same pathway are only as strong as the stronger single mutant (Ellis et al., 1991). Interestingly, mutations between the two pathways do not entirely block engulfment, suggesting that there may be a third salvage pathway that inefficiently removes dead cells throughout the life of the worm.

The first engulfment signaling pathway is comprised of CED-1, CED-6, and CED-7. These three proteins are thought to act early in the activation of a signal transduction cascade (Figure 4). CED-1 is an integral membrane protein, which potentially functions as a receptor that recognizes dying cells. Interestingly, CED-1 is homologous to the SREC (Zhou et al., 2001b) and the CD91/LRP scavenger receptors, which has been previously implicated in the engulfment of apoptotic cells in cell culture systems (Ogden et al., 2001); antibody-mediated cross linking of the CD91/LRP receptor will stimulate uptake of bound erythrocytes (Ogden et al., 2001). Similarly, the only known receptor for engulfment in *Drosophila, croquemort*/CD36, is a scavenger receptor-family molecule (Franc et al., 1996), suggesting a conserved mechanism for the recognition of apoptotic cells by scavenger receptor family members. The ligand that CED-1 recognizes is still unknown; SREC has been implicated in binding acetylated low density lipoproteins (Ac-LDLs) (Adachi et al., 1997), suggesting the CED-1 ligand may be a modified

lipid.

Domain analysis of CED-6 has identified a PTB (phosphotyrosine binding) domain and a PxxP motif (involved in interactions with SH3 domains), supporting a function as an adapter protein; the interacting partner that utilizes the PxxP motif has yet to be identified. Both CED-1 and CD91/LRP have NPXY motifs that interact with CED-6 and its mouse orthologue GULP, respectively, suggesting that CED-1 uses the CED-6 adaptor protein to transmit a recognition signal into membrane movement. Consistent with this hypothesis, overexpression of CED-6 can partially rescue the engulfment defect of *ced-1* mutants (Liu and Hengartner, 1998). The small adaptor protein CED-6 is thought to homodimerize after activation via a leucine zipper motif to induce engulfment by an as yet uncharacterized mechanism involving the CED-10 Rac GTPase (Su et al., 2000).

CED-7, like CED-1, is a plasma membrane protein. CED-7 is unique among the known engulfment genes in that it is required on both the engulfing as well as the dying cell for function in engulfment (Wu and Horvitz, 1998a). The mammalian CED-7 homologue, ABCA1, was cloned by homology to ABC transporters, and later discovered to be involved in the rearrangement of plasma membrane phospholipids (Hamon et al., 2000). One of the characteristics of mammalian apoptosis is the relocation of phosphatidylserine (PS), a membrane phospholipid normally found only in the inner membrane leaflet, to the outer leaflet of the cell membrane (Fadok and Henson, 1998). ABCA1 has been proposed to promote this PS translocation in both the engulfing and the dying cell, mediating an as yet uncharacterized recognition event. Whether CED-7 performs a similar function remains to be determined.

The second pathway in the worm that functions in engulfment is comprised of three genes: *ced-2*/*crkII, ced-5*/*dock180*, and *ced-12*/*elmo*. These three genes encode a bipartite RacGEF, which we and others have shown acts on *C. elegans* CED-10/Rac1 (Kinchen and Hengartner, 2005). The most upstream member of this pathway is CED-2/CrkII (Reddien and Horvitz, 2000). CrkII was first discovered

in mammalian cells as a viral oncogene, v-Crk, that induces cell migration and increases metastatic potential. CrkII is essentially an adapter molecule comprised of one SH2 domain and two SH3 domains. The N-terminal CED-2/CrkII SH3 domain has been shown to bind to CED-5/Dock180 (Matsuda et al., 1996), through which a signal is transduced to Rac, lead-ing to reorganization of the actin cytoskeleton (Kiyokawa et al., 1998). The order of this pathway has been confirmed in *C. elegans*, with *ced-2*/CrkII and *ced-5*/Dock180 functioning upstream of *ced-10*/Rac1 (Reddien and Horvitz, 2000; Wu and Horvitz, 1998b). Functional conservation of this signaling complex in engulfment in mammalian cells has also been shown (Albert et al., 2000; Gumienny et al., 2001).

CED-5/DOCK180 interacts with Rac1 via a basic region at the C-terminus (Kobayashi et al., 2001), and with CED-12/ELMO via a SH3 domain at the N-terminus (Gumienny et al., 2001; Wu et al., 2001; Zhou et al., 2001a). Recently, a particular domain at the C-terminus of Dock180 has been identified, named the DOCKER domain, which specifically binds to nucleotide free Rac1 and specifically loads GTP in order to activate Rac1 (Brugnera et al., 2002). Indeed, CED-12/ELMO appears to function in the stabilization of the Dock180::nt-free Rac transition state, functioning as a bipartite RacGEF with CED-5/Dock180.

A receptor has yet to be discovered that signals to the CED-2/5/12 signaling complex. In mammalian cells, integrin receptors (α_v,β_3 and α_v,β_5) act upstream of these proteins, with p130Cas bridging the activated integrin receptor and the CrkII adaptor protein (Albert et al., 2000); this does not seem to be the case in *C. elegans*, however, as integrin mutants show little defect in engulfment of apoptotic corpses (Gumienny et al., 2001). Integrin receptor function in engulfment in mammalian tissue culture systems may be a case where increased adhesion to cells also increases the number of cells that are engulfed; further study to ascertain this difference is needed.

We could recently show that the two signaling pathways that I described above converge at the level of CED-10/Rac1 (Kinchen et al., 2005).

Our current data suggest that the two pathways cooperate to recruit CED-10 to the right region on the plasma membrane, and then stimulate GTP loading, leading to a selective rearrangement of the actin cytoskeleton in the direction of the apoptotic cell that needs to be engulfed (Gumienny et al., 2001; Wu et al., 2001; Zhou et al., 2001a). The small GTPase Rac has been implicated in rearrangement of the actin cytoskeleton and remodeling of the plasma membrane in response to extracellular stimuli, known as 'membrane ruffling'. Rac, like other GTPases, cycles between a GDP-bound, inactive conformation and a GTP-bound, active conformation based on molecules that promote GTP exchange (GAPs or GTPase activating proteins), proteins that promote GTP/GDP hydrolysis (GEFs or guanine nucleotide exchange factors), and proteins that keep GDP from dissociating (GDIs or guanine nucleotide dissociation inhibitors). Our data strongly suggest that Dock180 (the CED-5 homologue) is the RacGEF for this process (Brugnera et al., 2002). By contrast, the nature of the GAP (and possibly the GDI)

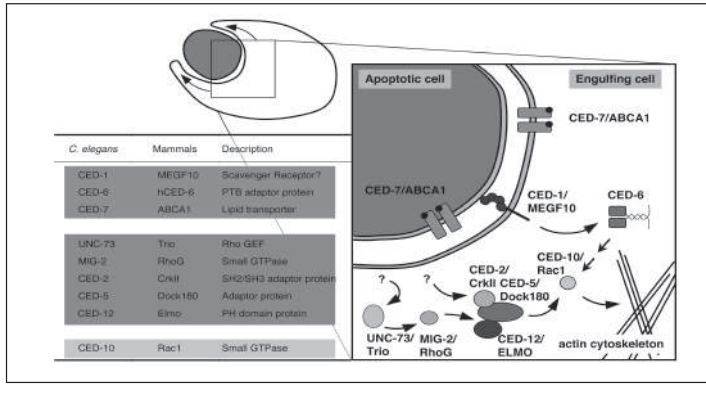

Figure 4
Two pathways converge to mediate the removal of apoptotic cells in C. elegans.
At least two partially redundant signaling pathways mediate engulfment of apoptotic cells in C. elegans. The two pathways cooperate to activate CED-10/Rac, which then promotes actin cytoskeleton rearrangements and «migration» of the engulfing cell around the apoptotic cell corpse. Both signaling pathways are conserved: mammalian homologs of these genes have also been implicated in the removal of apoptotic cells. Many steps in the signaling cascade shown are still unclear (question marks).

involved in phagocytosis is still unknown. Similarly, the Rac effectors that function in engulfment in *C. elegans* remain to be identified.

Suicide vs. Murder – suggestions of phagocyte-mediated cell killing

A picture of engulfment as more than just a janitor has recently begun to emerge. This work comes from many sources and suggests that, in worms, apoptosis is not always determined by the core apoptotic machinery (CED-3, -4, -9, and EGL-1), but rather can be mediated by neighboring cells.

Observations from my laboratory and others have shown that the programmed cell death pathway is not as linear as our models would have us believe (Figure 2). Indeed, in weak *ced-3* mutants, cells that are not visibly apoptotic can be 'murdered' dependent on the engulfment machinery (Hoeppner et al., 2001; Reddien et al., 2001). This close interplay between genes involved in apoptosis and genes involved in engulfment suggests that the linear pathway we conveniently use to think about programmed cell death is not entirely correct, and that there is some overlap between what has previously been thought of as two distinct processes. This could be due to early processing of the 'eat-me' signal by the activated CED-3 caspase, such that a late activation of an caspase inhibitor may be able to stop apoptosis of the cell, but not the exposure of the 'eat-me' signal (Hoeppner et al., 2001). The engulfment machinery would then remove these cells, without the semi-apoptotic cell undergoing the phenotypic stages of apoptosis. Alternately, the 'eat-me' signal may be generated in a caspase-independent manner. Under this scenario, the cell could be efficiently removed even when activation of CED-3 was suboptimal. Further characterization of engulfment mediated cell death should provide an answer.

Conclusion

Genetic studies of apoptosis in *C. elegans* have led, over the last 20 years, to the development of many important concepts about how

apoptosis works. However, it is clear that what we know pales in comparison with what we still ignore. The fact that a large fraction of the genes that control cell death in *C. elegans* are conserved and also regulate apoptosis in mammals suggests that much useful knowledge about ourselves can still be extracted from a dedicated study of this humble crawler. I hope that this concise overview will have given the reader a bird's view of the terrain surveyed so far, and a thirst for more adventuring. Let us learn from the worm!

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