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Meeting Report

Rocking cell death

Jan Paul Medema*,[1], Michael Hahne[2], Henning Walczak[3] and Vincenzo De Laurenzi[4]

[1] Department of Immunohematology, Leiden University Medical Center, Albinusdreef 2a, 2333 AA, Leiden, The Netherlands
[2] Institute of Biochemistry, University of Lausanne, Chemin Des Boversesse 155, 1066 Epalinges, Switzerland
[3] Tumor Immunology Program, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
[4] IDI-IRCCS, Biochemistry Laboratory, University of Rome Tor Vergata, Via di Tor Vergata 135, Rome, Italy
* corresponding author: JP Medema, tel: +31 71 5263013; fax: +31 71 5216751 e-mail:medema@rullf2.medfac.leidenuniv.nl
All authors have contributed equally to this report

The First European Workshop on Cell Death: (Death on the Rocks) held at Gran Sasso, Italy, 21–25 October 1998

Abbreviations: cytC, cytochrome C; NO, nitric oxide; PHA, phytohemagglutinin; APRIL, a proliferation inducing ligand; AICD, activation-induced cell death; BCR, B cell receptor

The First European Workshop on Cell Death (Death on the Rocks) was held in the INFN Laboratories in L'Aquila, Italy from 21–25 October at the base of Gran Sasso (Big Rock). The meeting consisted of short talks with ample time for discussions. In this report we will try to give an overview of the advances in cell death that were presented and the subjects that were discussed at the workshop.

Programmed cell death is a physiological mechanism necessary to orderly dispose of unwanted cells in the body. It is essential during embryogenesis, and throughout lifetime for tissue homeostasis as well as elimination of autoreactive T and B cells. Defects in apoptosis have been associated with a number of diseases including cancer and autoimmune disorders. The basic layout of this form of cell death is conserved during evolution and consists of a number of characteristic morphological and biochemical changes.

The caspase family

The intracellular execution of programmed cell death in almost all cases critically depends on the activation of specific cysteine proteases called caspases. Many caspases have already been identified and the number is still growing as was exemplified by the report of a novel caspase at this meeting (Christopher Stroh *et al*, Tübingen, Germany). Using PCR-primers based on the sequences of mu*Casp*12 this group identified a human homolog of this caspase. However, competition in this area is also everlasting since an identical clone, named hu*Casp*14, was recently reported.[1] Despite the rapid expansion of this family, the main questions in this research area remain to be resolved; What are the exact biological functions of the different family members, what are their physiological substrates and is there any redundancy amongst the different members? Several approaches were presented at the meeting to address these issues. Maria van Gurp (Gent, Belgium) reported the usage of the short and long subunit of caspase-1 as bait in a yeast three-hybrid system to screen for novel caspase substrates. Using this method, novel potential substrates were identified, yet their *in vivo* relevance remains to be elucidated. A cellular approach towards the understanding of caspase action was presented by Alexander Stegh (Heidelberg, Germany) who studied the subcellular localization of caspase-8 both before and after CD95 triggering. In MCF7 cells caspase-8 is located primarily at the mitochondria. Upon CD95 triggering, it is processed and the active subunits are released. These subunits translocate to the structural protein plectrin, which was subsequently shown to be a substrate for caspase-8. This is one of the first examples where a caspase can be shown to translocate directly to one of its targets. Whether the cleavage of plectrin is involved in cytoskeletal rearrangements observed during cell death remains to be determined but is a provocative possibility.

Another way to clarify the role of the different caspases involves the use of cell lines lacking a specific caspase. Timothy Zheng (New Haven, USA) studied cells derived from caspase-3$^{-/-}$ animals. His data indicated a clear role for this caspase in CD95-induced membrane blebbing, nuclear and DNA fragmentation as well as cleavage of several substrates like Fodrin, DFF45, Gelsolin and LaminB. Unexpectedly cleavage of PARP, thought to be a typical caspase-3 substrate, appeared unaffected. He also argued that these cells displayed redundancy for this caspase, since cleavage of the typical substrates, except for Fodrin, was only delayed and clearly detected at later timepoints. A similar approach was employed to study the role of caspase-8 in CD95-induced apoptosis. Isolation of mutant Jurkat T cell lines lacking this caspase again revealed its essential and non-redundant role in the induction of apoptosis by this receptor (Peter Juo *et al*. Boston, USA). In contrast to caspases-3 and -8, no clear role in apoptosis induction has so far been assigned to caspase-6. Intriguingly, also the caspase-6-deficient mice do not exhibit an overt phenotype (Timothy Zheng *et al*, New Haven, USA). Therefore, the identification of a specific function for this caspase has to await detailed analysis of these mice.

Beside receptor-induced caspase activation several talks dealt with the role of cytochrome C (cytC) in caspase activation. During the past few years, it has become evident that cytC, next to its role in mitochondrial respiration, is also a major player in the induction of cell death.[2] Using isolated

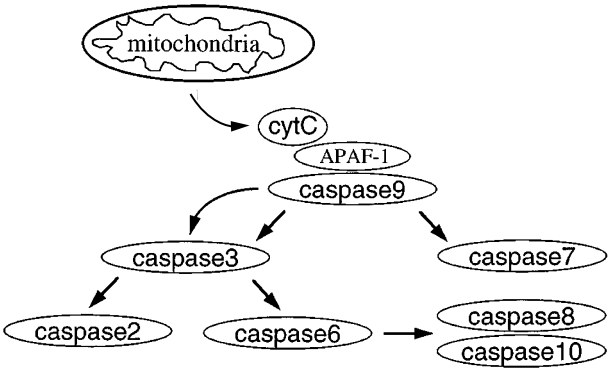

Figure 1 Schematic representation of the caspase activation cascade Courtesy of Seamus J Martin

mitochondria, Juliane Jürgensmeier (Kiel, Germany) presented evidence that mitochondria can perform these two functions at the same time. She showed that exogenously added recombinant Bax releases cytC in sufficient amounts to induce apoptosis, while this does not seem to reduce mitochondrial respiration. Once released, cytC directs a plethora of caspase activations. Cellular extracts depleted of one particular caspase were employed by Seamus Martin (Maynooth, Ireland) who set out to delineate the caspase cascade downstream of the mitochondria by simply adding exogenous cytC to the extracts. A clear hierarchy was established (see Figure 1) and in contrast to the study involving caspase-$3^{-/-}$ cells, no apparent redundancy at the level of caspase-3 or -7 was detected.

Beside death receptor and mitochondrial regulation of caspase-activity the role of the second messengers nitric oxide (NO) in controlling cell death was discussed. Francesca Bernassola (Rome, Italy) showed a protective effect of NO on CD95-mediated and damage-induced apoptosis. Protection is apparently due to the S-nitrosylation of caspase-3-like proteases and the transcription factor AP-1.

Neurons react rather differently to NO; Marcel Leist (Konstanz, Germany) discussed the role of NO in NMDA-mediated neuronal death. In this model system hyperactivation of the NMDA receptor increases cellular NO due to nNOS activation. This leads to the induction of apoptosis, suggesting that NO is an apoptosis mediator. The difference in biological functions of NO in the two model systems exemplifies how the same molecule can exert distinct functions in different cellular settings.

TNF receptor family and FLIP; their role in tumor growth

Several talks covered the actions of cFLIP (casper/I-FLICE/FLAME-1/CASH/CLARP/MRIT/USURPIN)[3,4] in death receptor-induced apoptosis. CFLIP contains two death effector domains and an inactive caspase domain, and can bind to FADD/Mort-1 and caspase-8. Takao Kataoka (Lausanne, Switzerland) characterized the inhibitory effect of cFLIP on a variety of apoptotic pathways. He reported that stable transfectants of cFLIP were protected against CD95-mediated apoptosis, yet cFLIP overexpression failed to inhibit death pathways triggered by perforin/granzyme B, chemotherapeutic drugs or γ-irradiation. In agreement, Carsten Scaffidi (Heidelberg, Germany) reported that cFLIP overexpression renders cells resistant to CD95-induced apoptosis and that this involves recruitment of cFLIP to CD95 as well as subsequent processing of cFLIP to a 43 kDa form.

Although it is evident that overexpression of cFLIP can block death receptor-induced apoptosis, its physiological role is still a matter of debate. Phytohemagglutinin (PHA)-activated peripheral T cells appear to regulate endogenous cFLIP expression in such a way that this could control the apoptosis sensitivity of these cells. It was reported that despite CD95 expression, one day PHA-activated peripheral T cells are resistant to CD95-induced apoptosis, but become sensitive 6 days after activation.[5] A recent report correlated the increased susceptibility of these T cells with decreased levels of cFLIP.[3] With the use of a monoclonal antibody against cFLIP, Carsten Scaffidi (Heidelberg, Germany) demonstrated that cFLIP protein levels are unchanged in day 1 and day 6 activated T cells. Furthermore, only in day 6, but not in resistant activated (day 1) T cells, caspase-8 and cFLIP are recruited to the CD95 DISC, suggesting that another mechanism mediates the resistance of peripheral T cells.

Both human malignant melanoma and melanoma cell lines appear to express high endogenous levels of cFLIP,[5] suggesting that cFLIP can convey apoptosis-resistance in melanomas. This hypothesis is supported by findings of Jan Paul Medema (Leiden, The Netherlands). He described a mouse cell line that forms palpable tumors in wild-type mice. These tumors are rapidly cleared by an efficient T cell response, but upon transfection with cFLIP this cell line becomes highly tumorigenic, indicating that cFLIP may act as an oncogene in overcoming or preventing a CTL response.

A related issue has been the role of receptors and ligands of the TNF-family in tumorigenesis. Michael Hahne (Lausanne, Switzerland) showed that CD95 is downregulated during tumor development. TRAIL on the other hand seems to preferentially affect transformed cell types (Henning Walczak, Heidelberg, Germany). In contrast to recombinant CD95L, which is known to cause severe liver damage, neither human nor murine TRAIL exerted any toxicity in mice. Using this as a starting point, he described that TRAIL exhibits anti-tumor activity, as systemic administration of TRAIL suppressed tumor growth of a TRAIL-sensitive adenocarcinoma cell line in SCID mice. An opposite effect on the regulation of tumor growth was suggested by Michael Hahne (Lausanne, Switzerland) for a novel member of the TNF-family called APRIL (a proliferation inducing ligand). APRIL stimulates proliferation of various tumor cell lines and is upregulated in some types of carcinoma. Apparently, the TNF family is critically involved in the regulation of tumor growth. Clearly, a better understanding of the differential effects exerted by the various family members may in the future prove useful in the control of tumor development in patients.

Non death receptor-induced death

Deciphering the pathway leading to activation-induced cell death (AICD) of B cells was the topic of two talks. Both studies employed B cell receptor (BCR)-induced apoptosis of the human B cell line RAMOS as an *in vitro* model for B cell selection. Susanne Lens (Amsterdam, The Netherlands) first showed that ectopic expression of FADD-DN or viral FLIP in RAMOS cells did not inhibit BCR-induced apoptosis, thus indicating that known death receptors do not seem to be involved in BCR-induced apoptosis. Using zVAD as a caspase-specific inhibitor she then went on to show that during BCR-induced apoptosis caspase-3 is cleaved by a non-caspase protease resulting in its activation. The caspase-3 activation could be inhibited by crosslinking of CD40 up to 12 h after BCR triggering. This suggests that T helper cell signals (CD40L) may govern BCR-induced apoptosis via directly or indirectly inhibiting a non-caspase protease by CD40 ligation on the B cell. Adam Curnock (Oxford, UK) focused on this early event of CD40-mediated inhibition of BCR-induced apoptosis. He found that a selective inhibitor of PI3-kinase (LY294002: LY) was capable of abolishing CD40-mediated inhibition of BCR-induced apoptosis. Yet, LY neither affected CD40-induced Bcl-x_L expression nor CD40-mediated inhibition of caspase-3 activation and subsequent PARP cleavage. Thus, CD40 ligation seems to trigger a PI3-kinase-dependent event capable of inhibiting BCR-induced apoptosis that does not involve Bcl-x_L upregulation and, surprisingly, also does not seem to have an effect on the inhibition of caspase-3 activation.

Elena Ritsou (Heidelberg, Germany) presented a novel type of apoptosis functioning in activated T cells. This form of apoptosis is induced by the triggering of either one of two receptors for HIV on T cells (CD4 or CXCR4). It occurs very rapidly, is both CD95- and caspase-independent, and does not lead to DNA fragmentation. Yet, it retains morphological changes characteristic for apoptosis including cellular shrinkage, chromatin condensation and phosphatidyl serine exposure. Only activated $CD4^+$ T cells appeared sensitive to CD4/CXCR4-mediated apoptosis. Thus, this novel type of apoptosis may contribute to the depletion of $CD4^+$ T cells at the later stages of AIDS, which are characterized by a concomitant increase in the relative numbers of activated T cells that undergo apoptosis.

Point of no return

One of the central discussions at the meeting focused on the assessment of the 'point of no return' after which cells can no longer be rescued. Currently, the general opinion seems to be that activation of downstream caspases represents this point and that a cell cannot survive unless the activation of these proteases is prevented. However, Marja Jäättelä (Copenhagen, Denmark) suggested that cells can actually survive activation of downstream caspases. She showed that cells overexpressing Hsp70 are protected from TNF-, staurosporine- and doxorubicin-induced cell death, despite the fact that disruption of mitochondrial transmembrane potential, cytC release and cleavage of caspase-3-like proteases is observed in the surviving cells.

Further support for this hypothesis came from the results reported by Ann Zeuner and Ruggero De Maria (Rome, Italy). They presented a new physiological role for CD95 and CD95L in erythropoiesis. CD95L produced by mature erythroblasts negatively regulates red blood cell formation by inducing cell death or differentiation arrest in immature erythroblasts. CD95L-induced inhibition of erythropoiesis is antagonized by high levels of erythropoietin, which protects immature erythroblasts from apoptosis and thereby increases erythropoiesis. The anti-differentiative effect of CD95L seems to be specifically due to caspase-mediated cleavage of the transcription factor GATA-1, which is required for erythroid differentiation. Interestingly, when immature erythroblasts express a caspase cleavage site-deficient mutant of GATA-1 they in fact survive and differentiate towards maturity despite the activation of caspases. Obviously, activation of downstream caspases is not necessarily an end stage for a cell.

Death receptor involvement or not?

The involvement of caspases, mainly the more downstream ones, is clear for drug and γ-irradiation-induced apoptosis. However, the reported involvement of death receptors in this induction was confronted by several groups (Annemiek Tepper *et al*, Amsterdam, The Netherlands, Takao Kataoka *et al*, Lausanne, Switzerland). These groups showed that lymphoid cells, rendered resistant to death receptor-induced apoptosis through the overexpression of cFLIP, are fully capable of undergoing DNA-damage-induced apoptosis. In addition, caspase-8 deficient Jurkat T cells only show marginal reduction of apoptosis as compared to the parental line after these stimuli (Peter Juo *et al*, Boston, USA). The marginal difference could be explained by the apparent activation of caspase-8 also downstream of the mitochondria (Seamus Martin *et al*, Maynooth, Ireland, Ingo Engels *et al* and Ania Stepczynska *et al*, Tübingen, Germany) (see Figure 1).

Similarly, the role of CD95/CD95L in c-*myc*-induced apoptosis was discussed. Using a tamoxifen-inducible CD2-*myc* transgenic mouse model Karen Blyth (Glasgow, UK) showed that elevated expression of c-*myc* in thymocytes, results in increased thymocyte apoptosis. This induction is independent of p53 as was determined by backcrossing onto $p53^{-/-}$ animals. However, such data should be interpreted with caution since the role of p53 in DNA-damage-induced apoptosis has become more complicated to analyze due to the recent discovery of p53 analogs (p73, p63 and p51), each of which can exist in different splice variants. In addition to the known p73 splice variants two more were presented by Vincenzo De Laurenzi (Rome, Italy). He reported a γ-form resulting from the splicing of exon 11 and a δ-form resulting from the splicing of exons 11, 12 and 13. All these variants are expressed in various normal tissues and tumor cell lines. However, the biological functions of the different forms in the induction of apoptosis remain to be determined.

Apart from p53 independency, c-*myc*-induced apoptosis of thymocytes seemed independent of the expression of CD95 (*lpr/lpr* background), arguing against an essential role of CD95 in c-*myc*-induced apoptosis as well (Karen Blyth,

Glasgow, UK). Lucy Peltenburg (Leiden, The Netherlands) on her part, generated c-*myc* transfected melanoma lines and intended to analyze CD95L protein expression with the monoclonal antibody clone 33 (Transduction Laboratories) after ionizing radiation. However, analysis at the RNA level and with different antibodies on the protein level, revealed that clone 33 does not recognize CD95L. This observation was supported by several other speakers at the meeting. Apparently clone 33 reacts with an as of yet unidentified secreted protein.

The lack of regulation of c-*myc*-induced apoptosis by CD95 reported by Karen Blyth (Glasgow, UK) was contested by the results of Thomas Brunner (Berne, Switzerland). He partially showed a direct effect of c-*myc* on the CD95L promoter. In addition, blockage of c-*myc*-induced transcription partially prevented activation-induced cell death in T cells. Clearly, much more insight is required to solve these apparent discrepancies and likely new players need to be identified.

Intriguingly, we again may have the possibility to turn to *C. elegans* for the answers. So far apoptosis research performed with *C. elegans* has focused on cell death during development and germ line homeostasis. Studying these two biologically separate forms of cell death, Michael Hengartner (Cold Spring Harbor, USA) came to the conclusion that they are, at least in part, regulated by different molecular players. For instance, a gain of function mutant of *ced-9* blocks developmental but not germ line death. Similarly, *egl-1* is essential for developmental death but dispensable for germ line death.

Although much can be learned about these two physiological forms of cell death studying *C. elegans*, one of the major drawbacks of this organism used to be the incapability to exogenously induce cell death. Indeed used to be, since Michael Hengartner (Cold Spring Harbor, USA) showed that it is feasible to induce cell death in *C. elegans* with DNA damaging agents. Interestingly, this pathway is only operative in the germ line and not in somatic cells, suggesting that this form of cell death is regulated differently. And in fact, neither the gain of function mutant of *ced-9* nor the loss of *egl-1* function affects insult-induced cell death. Likely, genetical screens using this method of apoptosis induction will soon reveal the main players in this form of cell death and will hopefully once again lift mammalian cell death research to a different level.

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References

1. Van de Craen M, Van Loo G, Pype S, Van Criekinge W, Van den Brande I, Molemans F, Fiers W, Declercq W and Vandenabeele P. (1998). Cell Death Differ. 5: 838 – 846.
2. Green DR and Reed JC (1998). Science 281: 1309 – 1312.
3. Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE and Tschopp J (1997) Nature 388: 190 – 195.
4. Rasper DM, Vaillancourt JP, Hadano S, Houtzager VM, Seiden I, Keen SLC, Tawa P, Xanthoudakis S, Nasir J, Martindale D, Koop BF, Peterson EP, Thornberry NA, Huang J, MacPherson DP, Black SC, Hornung F, Lenardo MJ, Hayden MR, Roy S and Nicholson DW (1998). Cell Death Differ. 5: 271 – 288.
5. Klas C, Debatin KM, Jonker RR and Krammer PH (1993). Int. Immunol. 5: 625 – 630.