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MINI-REVIEW

Programmed Cell Death: Historical Notes from Russia

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Abstract—The investigation of cell death mechanisms is one of the fastest growing areas of modern biomedicine. A particular interest in this research topic arose in 1972 after publication of an article by Kerr, Wyllie, and Currie, in which apoptosis, one of the types of cell death, was first considered as a basic biological phenomenon regulating tissue homeostasis. Several Russian groups involved in the investigation of the mechanisms of radiation-induced cell death have drawn attention to the similarity between these two mechanisms. Unfortunately, these studies have been for a long time inaccessible to the international scientific community. These introductory remarks attempt to restore the chain of events that have taken place during the past 50 years.

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K. P. Hanson (1936-2005)

Dedicated to my Teacher.

"What is true for bacteria is also true for an elephant."
Jacques Monod

INTRODUCTION

These historical notes have two purposes. The first is to express my everlasting gratitude to my teacher and friend Professor Kaido Hanson. He has taught me how to treat scientific research, how not to be afraid of difficulties, and how to look critically at the results. It is impossible for me to judge whether I have fully followed his rec-

ommendations, but I can honestly say that I have tried very hard. The second goal is to familiarize the young generation of researchers with the history of the development of such an important biomedical field as the studies of programmed cell death and with the role of Russian researchers in its development. The fact is that even before the publication in 1972 of the fundamentally important research article by Kerr, Wyllie, and Currie [1], several Russian groups had been seriously involved in elucidating the mechanisms of radiation-induced cell death. Most results obtained in these studies were published in Russian journals and, for obvious reasons, had for a long time remained unavailable to the world scientific community. Later, some of these works were translated, published in international journals, and recognized by colleagues outside of Russia. Importantly, our Laboratory of Radiobiology in Leningrad was noted among the world's leading laboratories working in the field of apoptosis in an article written by Andrew Wyllie, one of the pioneers of apoptosis research [2]. Now, almost 50 years later, I want to restore the chain of events of the past. So...

HOW IT WAS?

It is well-known that all biological organisms, either unicellular or multicellular, are mortal. Unfortunately, soon after the creation of the cell theory by Schleiden and Schwann and discovery of cell division and differentiation [3, 4], many researchers have focused their attention on the understanding of the mechanisms of cell proliferation and changes in the cellular phenotype, while almost completely ignoring the processes by which the cells are eliminated. Cart Vogt was the first to describe the process of cell death in the notochord and adjacent cartilage in metamorphic toads [5]. However, this was a single study. It soon became clear that in both vertebrates and invertebrates, a large number of cells die during the development and throughout the lifetime. Moreover, in some cases, entire tissues or organs that had performed their functions are eliminated. There have been rare attempts to classify different types of cell death. Thus, as early as in 1951, Glucksmann proposed to subdivide "natural" cell death into morphogenetic, histogenetic, and phylogenetic in accordance with their biological purpose [6]. Saunders was the first to express the idea that the cell death during embryogenesis and morphogenesis can occur as a result of the genetic program activation triggered by special internal and external signals [7]. Richard Lockshin was the first to experimentally confirm the genetic determinism of cell death and the fact that this process is well-regulated rather than randomly occurring. While studying degeneration of the silkworm intersegmental muscles, he discovered the presence of genetically regulated process of "programmed cell death" and introduced this term into practice [8]. In 1972, Kerr, Wyllie, and Currie [1] published an article that has attracted close attention of many scientists. The authors proposed apoptosis as a genetically programmed form of cell death and an important biological phenomenon that regulates tissue homeostasis. As mentioned above, long before the appearance of this important publication and the sharply increased interest in the apoptosis, there had already been separate papers published on this topic. Back in the 1950s, one of the extensively studied phenomena was radiation-induced cell death. It was customary in radiobiology to distinguish between the two types of cell death: reproductive and interphase. The first one was associated with the mitotic cycle and the loss of the cell's ability to divide. The second, on the contrary, occurred before the cell entrance into mitosis and was not associated with the cell cycle. Therefore, it was called "non-mitotic", "immediate", "interphase", or "death in the absence of division." Okada described three types of the interphase death: (i) death of some types of non-dividing cells or cells with limited replicative capability (lymphocytes, thymocytes, enterocytes, cells of the lens growth zone, etc.) during the first hours after irradiation at the doses from hundredths to several Grays; (ii) death of dividing cells in culture after exposure to the high doses of radiation (tens of Grays); (iii) death of non-dividing or rarely dividing cells (nerve and muscle cells, hepatocytes, cardiomyocytes, etc.) after exposure to ultrahigh doses (tens and hundreds of Grays) [9]. In Russia, this problem had been studied in particular detail. So, in the 1970s, Hanson formulated a hypothesis according to which radiation-induced interphase death of lymphoid cells was considered as an example of programmed cell death, or apoptosis [10]. This hypothesis, which was based on the experimental results of our research group and data published by other laboratories, has led to a surge in the studies of this phenomenon. Hanson looked at the radiation-induced interphase death from a fundamentally new angle. The fact is that ionizing radiation is not the only factor inducing lysis of lymphocytes. Similar morphological and molecular changes can be observed in lymphocytes in response to the treatment with steroid hormones, alkylating agents, and some other chemical compounds. This hypothesis was confirmed by multiple experimental data. First, it was found that regardless of the nature of the initial factor causing the damage, cell death occurs via the same mechanism that involves chromatin condensation and degradation and is accompanied by the morphological changes typical for the death of lymphoid cells. Second, a significant similarity was found between the molecular events occurring during the interphase death of lymphoid cells and the development of the genetic differentiation program, which ends, for example, with the elimination of the cell nucleus. Third, it was found that ultraviolet and gamma radiation in doses causing interphase death is capable of triggering the program of erythroid differentiation of leukemic cells. Fourth, it was known that during immune

differentiation or under stress, a certain population of lymphocytes (cells of the thymus cortex) die after performing their physiological functions in the body. The latter was considered to be an example of programmed cell death. It is important to note that this hypothesis was subjected to a very detailed verification by many laboratories and then commonly recognized by the scientific community [11, 12].

The studies of programmed cell death included several periods. First, cytological characteristics of physiological cell death were described. Thus, nuclear condensation was recognized as the earliest detectable morphological feature of a cell undergoing cell death. Such changes were described in detail by Kerr et al. [1] as a characteristic feature of apoptosis. Multiple accumulated results have suggested that the cornerstone of the cell death are events taking place in the nucleus. This was the so-called "nuclear period" in the studies of this cellular physiological phenomenon. Indeed, in 1980, Wyllie published an article in *Nature*, in which he showed that the previously described condensation of chromatin typical of apoptosis in the glucocorticoid-treated thymocytes and tumor lymphoid cells is closely associated with the internucleosomal chromatin fragmentation and removal of nucleosomal "beads on a string" structures, apparently through the activation of intracellular (but not lysosomal) endonuclease [13]. It should be emphasized that *in vivo* degradation of chromatin in irradiated lymphoid cells with the formation of regular fragments had been reported long before the publication of Wyllie's article [14]. These data have been confirmed by several research groups [15, 16] and the presence of internucleosomal chromatin fragmentation was recognized as one of the hallmarks of apoptosis. In all fairness, it should be noted that even before the introduction of the term "apoptosis" into the scientific use, it had been shown using simple biochemical approaches that exposure to gamma-radiation, hydrocortisone, and degranol leads to the appearance of low-molecular-weight deoxyribonucleoprotein degradation products in the thyroid gland of rats [17]. Moreover, the protein composition of these products of chromatin endonucleolytic cleavage was determined [18]. Later, it was found that these products are generated during internucleosomal chromatin fragmentation. Further *in vitro* experiments showed that the process of chromatin degradation needs the participation of a nuclease, which requires the presence of calcium and magnesium ions for its activity. Many laboratories have joined the search for this endonuclease and mechanisms for its activation [19]. At the same time, the first data on a possible involvement of proteases in the regulation of apoptosis appeared. Thus, it was shown that the Ced-3 protein, a homologue of the IL1β-converting enzyme, is required for the activation of programmed cell death in the nematode *Caenorhabditis elegans* [20]. It was then found that the IL1β-converting enzyme belongs to a large family of proteins called caspases [21]. It is interesting to note that at the same time, Filippovich et al. [22] published the study in which they established that protease inhibitors can significantly slow down the degradation of nuclear DNA in the thymocytes exposed to radiation or dexamethasone. It is important that neither histones nor proteins of the nuclear matrix of thymocytes underwent proteolysis after such treatments. Moreover, it was shown for the first time that the degradation of chromatin under these conditions was not due to the activation of the Ca/Mg-dependent endonuclease. Next, the work from the Nagata and Wang laboratories demonstrated that the degradation of genetic material is indeed not associated with the Ca/Mg-dependent endonuclease, but depends on the proteolytic cleavage of the endonuclease inhibitor ICAD/DFF45 by caspase-3, leading to the activation of the caspase-dependent endonuclease CAD/DFF40) [23-26]. Subsequent studies of Nagata and Horvitz laboratories have established that DNA of apoptotic cells in both humans and *C. elegans* can be hydrolyzed not only autonomously by CAD/DFF40 in the dying cells, but also by DNase II in the lysosomes of phagocytes after engulfment of apoptotic cells [27, 28].

Along with the accumulation of data on the participation of cytoplasmic proteases in apoptosis, a new concept has formed implying that the main factors regulating the development of apoptotic death are cytoplasmic events, i.e., the research focus has shifted from the nucleus to the cytoplasm. Indeed, the involvement of caspases in the activation of two main apoptotic pathways, receptor- and mitochondria-mediated, supported this assumption, which has been further confirmed by the discovery of the protein targets of caspases. Lazebnik et al. were the first to demonstrate that an active cytoplasmic protease (later found to be caspase-3) is capable of cleaving the nuclear protein PARP, thus disrupting the repair of the damaged DNA and leading to its degradation [29]. Returning to the history of the topic, it should be emphasized that long before the discovery of PARP as the first caspase substrate, Umansky et al. had observed a decrease in the poly(ADP-ribose) polymerase activity 2-3 h after irradiation of thymocytes [30], and this decrease coincided in time with the activation of internucleosomal chromatin degradation in the irradiated cells [31]. Simultaneously, Filippovich and colleagues found that the NAD-poly(ADP-ribose) polymerase system does not trigger the radiation-induced death of thymocytes, but regulates the response of these cells to the radiation damage [32, 33]. Interestingly, this effect was most clearly reproduced in radiosensitive rather than radioresistant tissues. As noted above, the leading role of cytoplasm in apoptosis was also indicated by the fact that caspase-3 cleaves the inhibitor of caspase-dependent endonuclease, which results in the enzyme activation in the nucleus [23-26].

In the mid-1990s, owing to a series of publications by Xiaodong Wang's group, it was suggested that important

events in the apoptosis development are associated with the mitochondria [34, 35]. First of all, it was shown that, when released to the cytoplasm, the mitochondrial protein cytochrome *c* promotes assembly of the apoptosome complex, which leads to the cascade activation of caspase-9 and caspase-3 [36]. Thus, the "mitochondrial period" in the studies of apoptosis has started. After listening to the Wang's talk at the Gordon Conference and reading his article in the *Cell* journal, I recalled the long-ago radiobiological studies that demonstrated the importance of cytochrome *c* in the development of radiation-induced cell death. As noted above, long before the "era of apoptosis", cell death had been intensely studied in radiation biology, since cell death is an ultimate result of the cell exposure to the ionizing radiation. In the 1950s and 1960s, studies of the radiation-induced changes in cell bioenergetics had been one of the most rapidly developing fields of radiobiology [37-40]. Analysis of mechanisms of early cell death in radiosensitive tissues in the 1950s revealed suppression of oxidative phosphorylation in the mitochondria of the thymus and spleen [38]. In one of these early works, my teacher Kaido Hanson showed that no such phenomenon occurred in the mitochondria isolated from radioresistant tissues [41]. Suppression of oxidative phosphorylation was detected 30-60 min after total X-ray irradiation of rats at relatively low doses (50-100 cGy) [41, 42]. In radiosensitive tissues, suppression of oxidative phosphorylation was accompanied by the formation of pyknotic nuclei (apoptotic bodies) [42]. Also, the rate of the electron transfer between cytochromes *b* and *c* in the thymic mitochondria decreased, which can be explained by a reduction in the content of cytochrome *c* after irradiation [39], presumably, due to the weakening of the cytochrome *c* binding to the inner mitochondrial membrane [43]. Interestingly, addition of exogenous cytochrome *c* stimulated oxygen consumption by the mitochondria isolated from radiosensitive but not radioresistant tissues of irradiated rats [44, 45]. The loss of cytochrome *c* was not caused by its passive release from the mitochondria, because additional washing of the mitochondrial fraction with an isotonic buffer caused no further decrease in the mitochondrial respiration rate [44]. *In vitro* irradiation of isolated mitochondria did not affect the content of cytochrome *c* [44]. Hence, it was suggested that the impairment of the electron transfer in the respiratory chain of the mitochondria of radiosensitive tissues was due to the regulated release of cytochrome *c* from the mitochondria and its appearance in the cytosol [40, 44]. It should be mentioned that the yield of cytochrome *c* was noticeably lower when it was fully reduced vs. partially oxidized or undergoing alternative oxidation-reduction changes. Studies that appeared almost 30 years later supported and clarified these earlier observations on the mechanisms of cytochrome *c* release from the mitochondria in the cells exposed to irradiation [46], and on the importance of the redox state of cytochrome *c* in caspase activation in the cytosolic extracts [47]. Subsequently, many of these data have been confirmed using more accurate technical approaches. Thus, it was found that the appearance of cytochrome *c* in the cytosol is a two-step process. Since cytochrome *c* is located on the outside of the inner mitochondrial membrane, where it forms a complex with cardiolipin, this interaction must first be disrupted to create a soluble pool of the hemoprotein. It was found that the solubilization of cytochrome *c* involves disruption of the electrostatic and/or hydrophobic bonds with cardiolipin that this cytochrome *c* normally maintains [48]. The dissociation of cytochrome *c* is promoted by selective cardiolipin peroxidation [49]. Once cytochrome *c* is solubilized, permeabilization of the outer mitochondrial membrane with Bax/Bak is sufficient to allow extrusion of this protein into the extramitochondrial medium [50]. This clarified how the addition of exogenous cytochrome *c* restores a proper function of the electron transport chain [51]. Finally, using an invasive approach, it was shown that the microinjection of cytochrome *c* into cytosol induced apoptosis in all studied cell types [52]. Interestingly, irradiation caused only a moderate decrease in the ATP level in the thymus, spleen, and some cancer cells [45]. This observation indicated that the loss of the mitochondrial cytochrome *c* was not substantial enough to significantly affect the mitochondrial ATP production, or that this loss could not be compensated by glycolysis. Much later, two studies confirmed the idea that the release of cytochrome *c* together with the maintenance of the vital intracellular pool of ATP are essential for the implementation of the apoptosis program [53, 54]. Reed [55] and Schendel [56] discussed two possible pathways of apoptosis involving cytochrome *c* release from the mitochondria. One of them involves activation of the caspase cascade through the interaction of the released hemoprotein with Apaf-1 and procaspase-9 in the cytosol [36], while the other is associated with a slowdown in the transport of mitochondrial electrons, which leads not only to the disruption of the ATP production, but also to the generation of reactive oxygen species. This second pathway is very similar to the one described by Scaife many years ago [45].

Further studies have shown that not only apoptosis, but other types of cell death as well result from the close interaction of multiple intracellular compartments rather than from the processes occurring in only one of them.

It is important to note that many of the early data obtained by radiobiologists have been later used and confirmed in the studies of the radiation damage caused by the Chernobyl accident, especially in workers, who cleaned the contaminated territory. Thus, analysis of chromatin breakdown in blood cells was used as one of the methods of biological dosimetry. In certain dose ranges, a direct relationship was observed between the radiation dose and the level of accumulation of chromatin degradation fragments.

Unfortunately, many Russian laboratories studying the problem of radiation-induced cell death have stopped their research due to various circumstances. However, new groups have emerged that looked at cell death from a broader perspective. Thus, Vladimir P. Skulachev suggested the use of the term "phenoptosis" for describing the programmed death of an organism by analogy with apoptosis [57]. In contrast to the "acute" phenoptosis, which, according to the author, can be illustrated by the death of pink salmon immediately after the end of spawning, aging may be as an example of "mild", extended in time phenoptosis. It was postulated that the aging program is encoded in the genome as a sequence of lethal biochemical events initiated in the mitochondria by reactive oxygen species (mROS). If so, an antioxidant specifically targeted to the mitochondria could act as an inhibitor of this program. This interesting idea discovered by Skulachev has been confirmed by several groups [58, 59]. Molecular mechanisms of various types of programmed cell death, such as apoptosis, necroptosis, autophagy, ferroptosis, etc. have been largely deciphered. Recently, an attempt has been made to elucidate the molecular mechanisms of phenoptosis. It was found that "moderate" depolarization of the inner mitochondrial membrane is sufficient for complete inhibition of the mROS accumulation, which are one of the most important components in the regulation of aging and phenoptosis [60]. However, detailed deciphering of the molecular mechanisms of phenoptosis requires additional research.

During the last ten years, a renaissance has been happened in Russia in the studies of fundamental aspects of programmed cell death, as well as its role in the pathogenesis of various diseases, primarily cancer. Thanks to a megagrant from the Russian Federation government that has supported the creation of a new laboratory at the Faculty of Basic Medicine, Lomonosov Moscow State University (B. Zhivotovsky). The active work of the laboratories at the Faculty of Biology (G. E. Onishchenko, V. P. Skulachev) and Faculty of Physics (M. A. Panteleev) at the Lomonosov Moscow State University, the laboratory at the Institute of Cytology and Genetics in Novosibirsk (I. N. Lavrik), and laboratories at the Institute of Cytology (N. A. Barlev, M. Piacentini) and the Technological University (G. Melino) in St. Petersburg have significantly advanced the research in the field of programmed cell death. The development of new probes for the study of cell death in the laboratories of S. A. Lukyanov and K. A. Lukyanov has considerably promoted the studies. We hope that this trend will continue, and Russian researchers once again will contribute to the investigations of one of the key medical and biological problems, the programmed cell death.

The importance of the fact that a large number of cells in various tissues self-destruct in their normal development and numerous pathologies is beyond doubt. This issue of *Biochemistry (Moscow)* is dedicated to the 10th anniversary of the establishment of the Laboratory for Investigation of Apoptosis Mechanisms at the Lomonosov Moscow State University, and devoted to the discussion of this phenomenon from various points of view. This volume includes experimental articles and reviews written by young scientists from this laboratory and their colleagues from the above-mentioned laboratories and research centers. I do hope that this issue will interest the readers of *Biochemistry (Moscow)* as well as a wide range of investigators in this field of research.

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