Apoptosis and Response to Radiation: Implications for Radiation Therapy

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Apoptosis is a mode of cell death that is currently of intense research interest in developmental and cancer biology. For more than 40 years, radiobiologists have been aware of cells in irradiated specimens that display the

Introduction

Apoptosis, or programmed cell death, is characterized by specific features that allow it to be distinguished from necrosis in tissues.[1] This distinction is necessary because, whereas apoptosis occurs as part of many normal biologic processes, especially during the development of an organism, it may also play a critical role in certain pathologic conditions, including cancer.[2]

The importance of apoptosis in cancer biology has been generally appreciated only in recent years, and this has led to a surge of reports of its occurrence in various normal and tumor cell systems treated with radiation, drugs, or biologicals. Although these reports clearly demonstrate that apoptosis occurs in model cell systems in response to cytotoxic treatments, the relative impact of apoptotic cell death on tumor response to therapy is essentially unknown. This is due largely to the fact that most studies to date have focused on the assessment of apoptosis in cultured cells. Apoptosis in human tumors following therapy has not been systematically evaluated due to technical and logistical constraints.

Nonetheless, research on radiation-induced apoptosis has uncovered new fundamental concepts relating to the basic processes responsible for cell death following irradiation and, most important, is beginning to reveal the details of the biochemical and molecular pathways that regulate these processes. This knowledge will most likely lead to entirely new strategies for modulating radiation-induced cell killing in tumors or normal tissues for therapeutic advantage. The ability to cure a tumor with radiation may depend on various factors, including tumor cell proliferation kinetics, tumor hypoxia, the number of tumor clonogens, and intrinsic tumor cell radiosensitivity.[3] Apoptosis may play a role in more than one of these factors, but, because apoptosis is known to occur in cells irradiated in vitro, the most obvious role is that apoptotic propensity may dictate intrinsic tumor cell radiosensitivity. Interest in understanding the mechanistic basis for intrinsic cell radiosensitivity has been stimulated by reports that tumor types whose cells display radioresistance in culture are associated with treatment failures.[4] However, the question of what determines a cell's intrinsic radiosensitivity remains a central issue in radiobiology.

As discussed in a recent review, the "classic" view held that the end point relevant to radiation therapy was "reproductive cell death," which results directly from unrepaired DNA strand breaks.[5] Radiobiologists now recognize the need to reevaluate this classic view and consider new concepts, such as effects on cellular membranes, signal transduction pathways, induction of gene expression, cell-cycle regulation, oxidative stress processes, and the influence of growth factors. The need to reevaluate the role of various pathways of cell death in response to radiation has also been recently appreciated. Thus, whereas for many years radiobiologic research focused exclusively on reproductive cell death, much current effort is also being directed toward understanding the role of apoptosis.

This article will briefly review our current understanding of the role of apoptosis in radiation response and will then discuss the implications of this role in radiation therapy.

Model Systems

In Vitro Cell Systems
In vitro cell systems have been used extensively in radiobiology since 1955, when Puck and Marcus[6] developed clonogenic assays for cell survival using cultured mammalian cells. This
important advance provided a model system for the study of the relationship of basic cellular processes that modify radiation response (e.g., DNA damage and repair mechanisms) to loss of cell reproductive capacity. Although the knowledge gained using these cell systems has been critical to our understanding of the effects of radiation on cells, such cell systems necessarily focused investigators' attention on reproductive cell death, to the detriment of research centered on other modes of cell death, such as apoptosis.

The term “apoptosis” was coined by Kerr et al in 1972 to describe a process of programmed cell death distinct from necrosis.[1] Whereas apoptosis has become a “hot” area of research during the last few years, radiobiologists have been aware of it for at more than 40 years. Prior to 1972, what we now refer to as apoptosis was included in a process called “interphase death,” a term that referred to the death of cells before their first postmitotic division. Fundamentals of Radiobiology, a classic textbook written by Bacq and Alexander and published in 1961,[7] clearly distinguishes interphase death from reproductive cell death, which is termed “mitotic death.”

The definitions of Bacq and Alexander[7] do not distinguish between necrosis and apoptosis, however. That distinction is one of the major contributions made by Kerr et al in 1972.[1] One can find numerous reports from the 1950s and early ’60s describing cellular responses to radiation identical to apoptosis. One of these, a set of cinemicrographic observations by Schrek[8] published in 1955, provides unequivocal photographic illustrations of rabbit lymphocytes undergoing apoptosis within 4 hours after 10 Gy of irradiation.

**Apoptosis vs Secondary Apoptosis**—The rather large number of papers published during the last few years reporting apoptosis in irradiated cultured cell systems has possibly led to some misconceptions about the role of the apoptotic process in radiation response. In addition to the occurrence of apoptosis as part of interphase cell death, apoptosis has been reported in cultured cell systems in which the primary mode of death is reproductive cell death. In these latter cases, apoptosis occurs after the first postirradiation mitotic division, and therefore, may have consequences that differ from those occurring when cells die before the first postirradiation division. This issue has been discussed in detail in another review,[9] but some additional clarification may be warranted. Throughout the remainder of this article, I will refer to cell death as "apoptosis" when it occurs before the first postirradiation mitosis. Apoptosis, therefore, occurs rather quickly after irradiation, usually within 4 to 6 hours, and is abundant in those cell systems in which apoptosis is the primary mode of cell death.

Cells with the morphologic features of apoptotic cells are also observed in cultured cell systems in which reproductive cell death is the primary mode of cell demise. These cells appear much later after irradiation, i.e., 24 to 96 hours, and usually with less abundance. Such cells, therefore, may be displaying the features of apoptosis subsequent to reproductive cell death. This process will be referred to as "secondary apoptosis."

**Loss of Tumor Clonogens**—The distinction between apoptosis and secondary apoptosis is not trivial in the context of radiation response, for two reasons. First, cells that die by apoptosis directly contribute to radiation response in appropriate cultured cell systems because, in those cases, there is usually a one-to-one relationship between an apoptotic cell and the loss of cells from the clonogenic pool. Thus, a radiation dose that would induce 50% of the cells to undergo apoptosis would reduce clonogenic survival to 50%.

Secondary apoptosis does not contribute to radiation response in the same way. In cultured cell populations in which secondary apoptosis occurs, cells die primarily by reproductive cell death; this process is quite sufficient, in and of itself, to remove cells from the clonogenic pool, usually by inducing a permanent growth arrest, a senescence-like state. There is no need for apoptosis under these conditions.

Thus, secondary apoptosis probably does not contribute to overall cell killing and radiation response because it most likely occurs in cells that have already permanently lost their reproductive capacity regardless of whether they display the features of apoptosis. In general, there is not a one-to-one relationship between the proportion of cells undergoing secondary apoptosis and cell killing because a much higher proportion of the cells in such a population usually undergo reproductive cell death than display apoptotic-like features.

**Cell Radiosensitivity**—Second, and most important, cell populations for which apoptosis is the primary mode of cell death following irradiation may be more sensitive to radiation than are cells whose primary mode of death is reproductive cell death. In such cultured cell systems, substantial apoptosis is usually induced by doses of 5 Gy or less, and the apoptotic cells are visualized within a few hours of irradiation. In contrast, secondary apoptosis generally requires doses higher than 5 Gy and appears more than 24 hours after exposure. Ian Radford clearly established these principles in a
1991 paper,[10] and they have been verified in subsequent reports.[11,12] Thus, the observation of cells with the features of apoptosis is not necessarily indicative of cell radiosensitivity[12] because the relative sensitivity of the cells in question may depend on whether they are undergoing apoptosis or secondary apoptosis. It should also be pointed out that radiation-induced apoptosis appears to be a feature of cultured cells of hematologic or lymphoid origin, whereas fibroblasts and cultured cells of epithelial origin tend to undergo reproductive cell death and secondary apoptosis following irradiation.[12]

**In Vivo Cell Systems**

Compared to what has been accomplished using cultured cell systems, evaluation of radiation-induced apoptosis in systems irradiated in vivo has been very limited. Nonetheless, several critical evaluations of apoptosis in irradiated normal and tumor tissues have revealed important differences between in vitro and in vivo systems.

**Early Studies**--Work in this area prior to 1980 was reviewed by Kerr and Searle.[13] They pointed out that Pratt and Sodicoff[14] reported the presence of apoptotic bodies in irradiated salivary glands and that Potten[15] observed apoptosis in the epithelium of intestinal crypts following irradiation. In addition, Kerr and Searle[13] observed apoptosis in a transplantable mouse tumor, a sarcoma, that had received radiation. Thus, this early work established that, in in vivo systems, radiation-induced apoptosis could occur in cells of nonhematologic origin. Through a meticulous quantitative analysis of radiation-induced apoptosis in the intestinal crypt, Potten[15] established two critical points concerning this mechanism of cell death: (1) Apoptotic cells appeared very quickly following treatment, with their numbers peaking between 3 and 4 hours after irradiation. (2) Only some of the cells in the crypt responded to radiation by undergoing apoptosis, but those that did were exquisitely sensitive to this mode of cell death; apoptotic figures were detected following radiation doses as low as .05 Gy.

**M. D. Anderson Studies**--Since these early reports, systematic assessments of radiation-induced apoptosis have been carried out in normal and tumor tissues. Our group’s interest in apoptosis was stimulated by an observation made in the radiation oncology clinic, where irradiation of the salivary glands is often unavoidable in the treatment of head and neck cancer. The response of these glands to radiation is remarkable, in that some patients develop decreased salivary function during the first week of therapy, having received less than 10 Gy.[16] To determine the underlying mechanisms responsible for these troublesome low-dose sequelae, adult female monkeys were irradiated with single radiation doses of 2.5 to 15 Gy.[17] Damage to the salivary gland was assessed from sequential biopsy specimens taken 1 to 72 hours after irradiation. The proportion of apoptotic cells in the tissues was determined by counting the cells displaying the morphologic features of apoptosis. Apoptotic cells could be observed as early as 1 hour after irradiation, and most such cells appeared within 24 hours. Moreover, apoptosis was seen even with the lowest dose used, 2.5 Gy. These findings are similar to those observed for cultured cell systems undergoing apoptosis with respect to dose-responsiveness and kinetics of appearance. They also suggest that, whereas the salivary gland cells are terminally differentiated, cells need not be in the cell cycle or even have reproductive capacity to undergo apoptosis following irradiation.

**Heterogeneity of Apoptotic Propensity**--Examination of apoptosis in model tumor systems has led to the appreciation that apoptosis may play a role in the response of at least some types of tumors to radiation. The intent of our initial studies[18] was simply to determine whether apoptosis was a feature of irradiated tumors. Two transplantable murine tumors were chosen based on their known radiation response: a very radioresistant (TCD50 more than 80 Gy) hepatocarcinoma, HCa-I, and a moderately sensitive (TCD50 = 53 Gy) ovarian adenocarcinoma, OCa-I. (TCD50 refers to the dose required to cure 50% of the tumors.) These tumors were grown in the hind legs of mice and were given radiation doses of 25 Gy or more; histologic sections were prepared from tumors removed at 6, 24, 96, and 144 hours after treatment. This first experiment showed that apoptosis occurred in the OCa-I tumor but not in the HCa-I tumor. Moreover, the apoptotic index was highest at 6 hours following 25 Gy of radiation, declined thereafter, and did not increase with doses over 25 Gy.

A subsequent, more detailed study[19] pinned down the time course and dose response for apoptosis induction in the OCa-I tumor. This study showed that the apoptotic index in the OCa-I tumor peaks at about 30% to 35% of the cells in the histologic sections between 3 and 4 hours after irradiation (Figure 1). Doses of 2.5 Gy induced substantial apoptosis, and the dose response actually leveled off at doses higher than about 7.5 Gy (Figure 2), suggesting that only a subset of cells in the tumor have the propensity for radiation-induced apoptosis. Thus, it became clear from our first two
studies that apoptotic propensity was heterogeneous both among different tumor types and even among cells within a given tumor. These instances of heterogeneity were confirmed in a more elaborate assessment of radiation-induced apoptosis in 14 additional types of murine tumors.[20] This analysis illustrated that some types of tumors, such as adenocarcinomas of the mammary gland and ovaries and lymphomas, have an apoptotic response, whereas other types, such as squamous cell carcinomas, hepatocarcinomas, and fibrosarcomas, do not. Moreover, for tumors that displayed an apoptotic response, their dose responses plateaued at an apoptotic index of 30% at doses higher than 10 Gy. For many of these tumors, we had previously determined their TCD50 and specific growth delay characteristics in response to single doses of radiation. Therefore, we produced correlation plots (Figure 3) of radiation-induced apoptosis vs TCD50 and specific growth delay for the murine tumors included in the analysis described above. These plots showed that tumors that responded to radiation with significant apoptosis tended to have lower TCD50 values (.1 less than P less than .2) and longer specific growth delays (P less than .05).

For each respective tumor, we also plotted the value for spontaneous apoptosis measured in the nonirradiated tumor vs the value for radiation-induced apoptosis (Figure 4). This correlation was highly significant (P less than .001), suggesting that spontaneous levels of apoptosis predict the apoptotic response to treatment.

**Impact of Dose Fractionation**--The studies described above utilized single doses of radiation, whereas in radiation therapy the dose is given in fractions. The possible influence of dose fractionation was assessed using the OCa-I tumor.[21] Two protocols were tested: (1) two doses of 12.5 Gy separated by various intervals up to 5 days and (2) five daily fractions of 2.5 Gy. These experiments showed that the protocol using two 12.5-Gy doses produced a net total proportion of apoptotic cells of about 45% when the two doses were separated by 5 days (total dose, 25 Gy). The daily 2.5-Gy protocol produced about 50% net apoptotic cells after 5 days (total dose, 12.5 Gy), and a single dose of 25 Gy produced only 36% apoptotic cells. Thus, an apoptotic subpopulation of cells reemerged between doses in the fractionated protocols, with the daily 2.5-Gy protocol being the most effective.

**Role of Apoptosis in Tumor Response**
All of the observations discussed thus far are consistent with the possibility that apoptosis plays a significant role in tumor response to radiation therapy. However, these are only correlations; cause-and-effect relationships are more difficult to establish. Moreover, it is hard to envision how apoptotic indexes of 30% to 35% dictate tumor response in light of a discussion by Dewey et al,[9] which illustrated that killing this proportion of cells in a tumor could reduce the TCD50 by only about 4 to 5 Gy. In contrast, the difference in TCD50 between the sensitive and resistant tumors used in our analysis was 20 to 30 Gy. Therefore, the correlations presented above, while intriguing, cannot directly account for the sensitivity of the tumors analyzed in the study. One possible explanation is that apoptosis indirectly influences tumor response. The spontaneous levels of apoptosis scored in the untreated tumors may be depicting the rates of cell loss in these tumors, and this factor may, in turn, dictate the number of tumor clonogens, a critical parameter governing tumor response to radiation. The number of clonogens in these transplantable tumors can be estimated from the TD50 (a value representing the number of cells required to yield a tumor in 50% of the injection sites in a tumor transplantation assay). In an assessment of the TD50 values for the murine tumors used in our studies,[22] we confirmed that tumors that have a high spontaneous apoptotic index and respond to radiation by undergoing apoptosis tend to have a high TD50, suggesting that their number of tumor clonogens is low compared to those relatively radioresistant tumors that lack apoptosis as a feature.

**Mechanisms of Radiation-Induced Apoptosis**
Radiation-induced apoptosis appears to be a signaled event, rather than simply the passive demise of a lethally damaged cell. As discussed above, some cells that undergo apoptosis in response to radiation do so after very low doses (eg, .05 Gy in the intestinal crypt). Moreover, in many documented cases, the dose-response curve for apoptosis is very steep in the low-dose region.[15,23] These doses are not lethal to cells that die by reproductive cell death. Thus, it does not seem reasonable that the cells have received sufficient macromolecular damage from these low doses to destroy cellular functions critical for survival. Instead, apoptosis in irradiated cells is induced by signals activated in key cellular response pathways in a manner analogous to the well-documented responses involving Fas/APO-1, a member
of the tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor family.[24] Cells that respond through these mechanisms have receptors on the cell surface that recognize the appropriate ligands, in this case, Fas/APO-1 antibody and TNF-alpha. Binding to the receptor induces a cascade of molecular interactions in the cell that is mediated by signal transduction pathways.[25,26] One of these events is the activation of sphingomyelinase to hydrolyze sphingomyelin to ceramide, which, in turn, induces the apoptotic cascade.[27] Interestingly, it has recently been demonstrated that ionizing radiation can also activate sphingomyelinase to produce ceramide and induce apoptosis.[28] In addition, the importance of signal transduction pathways in regulating radiation-induced apoptosis has been illustrated in a number of studies.[29,30] One unresolved issue in this field is whether the signal for apoptosis comes from radiation-induced lesions produced in the cell membrane or in DNA. There is evidence to support either or both of these possibilities.[9] However, in contrast to mechanisms defining reproductive cell death, the role of membrane effects and signaling pathways in apoptosis appears certain, and much current research in apoptosis is focused on examining these pathways.

Based on this discussion, a simple model would propose that radiation-induced apoptosis is signaled via interactions of radiation with cell membranes, whereas reproductive cell death and secondary apoptosis are the ultimate consequences of unrepaired or misrepaired DNA damage that leads to chromosomal aberrations and abnormal mitotic divisions. That radiation-induced apoptosis is cell type-dependent, preferentially occurs at relatively low doses of radiation, and happens quickly after irradiation can be explained on the basis that apoptosis occurs only in apoptosis-prone cells. Once triggered, apoptosis proceeds via a cascade of events that is accomplished in a few hours independently of the triggering agent, eg, radiation vs drugs.

**Genes Required for Apoptosis**—The question that remains to be answered is, what determines whether a cell is apoptosis prone? Obviously, all cells contain the genetic information needed to carry out apoptosis, but in some cell types, especially cells of hematologic origin, the apoptosis program is active, functional, and awaiting the signal to begin. In other cell types, the program is inactive or blocked through the pattern of gene expression particular to those cell types. The list of genes that are involved in controlling apoptotic propensity is growing, and this subject has been reviewed with regard to radiation.[9] At least two different types of genes are required: genes encoding proteins that carry out apoptosis, such as proteases and endonucleases, and genes regulating the process, such as the tumor-suppressor gene p53 and members of the bcl-2 family of proto-oncogenes.

A discussion of how these genes function is beyond the scope of this review, but the p53 and bcl-2 genes are especially important for explaining apoptosis-prone cell types. Normal function of the p53 gene is apparently required for radiation-induced apoptosis,[31,32] and abnormal expression of the bcl-2 protein blocks apoptosis.[33,34] Thus, cell types such as fibroblasts and cells of epithelial origin may lack the propensity for apoptosis because they are not expressing certain genes required for apoptosis or because they are expressing genes that block apoptosis. The latter hypothesis is largely based on results from cultured cell systems, but many of the cultured cell lines typically used in radiobiology to examine mechanisms involved in reproductive cell death may lack normal p53 function, eg, HeLa.[35]

Two other genes are worthy of mention, c-myc and Ha-ras, because their influence on apoptosis has been the subject of several reports. These reports claim that c-myc promotes apoptosis and Ha-ras may suppress it.[36-38] In most cases, it appears that expression of these genes modulates secondary apoptosis. In a recent report, Aldridge et al.[12] showed that increasing the susceptibility of fibroblast cell lines to radiation-induced apoptosis by modulating expression of myc or ras did not alter clonogenic survival. The apoptosis observed was manifested mostly between 48 and 72 hours after radiation doses of 12 Gy.

These findings are consistent with the hypotheses presented above, and clearly underscore the importance of knowing whether the apoptosis is primary or secondary before attempting to predict the radiosensitivity of cells on the basis of apoptosis. It is possible, however, that in other cell systems, c-myc and Ha-ras may contribute to radiosensitivity through other mechanisms or may convert the mode of cell death from secondary to primary apoptosis. Even a small shift of this type may have important implications for fractionated radiotherapy.[9]

**Implications for Radiation Therapy**

The foregoing brief review provides a framework for discussing the implications of apoptosis in radiation therapy. As mentioned at the beginning of this article, the success of radiation therapy...
depends on several factors, including the number of tumor clonogens and intrinsic tumor cell radiosensitivity. I have attempted to outline above how tumor cell apoptotic propensity may affect these factors. To reiterate, apoptosis may influence tumor response to radiation by governing the number of tumor clonogens through cell loss mechanisms prior to treatment. It also may influence tumor response by enhancing the intrinsic radiosensitivity of the cells; ie, in cells with apoptotic propensity, death may be triggered by lower doses than are required to produce reproductive cell death.

**Decreased Tumor Clonogens**

With respect to the first of these mechanisms, the radiation-induced apoptotic cells may not directly contribute to tumor response, but the spontaneous apoptosis in such tumors may be indicative of high rates of cell loss, which lowers the number of tumor clonogens by offsetting mitosis. When this mechanism is operative, the pretreatment apoptotic index predicts tumor response to therapy. This correlation has been assessed in several different types of human tumors treated with radiation. We reported that a high pretreatment apoptotic index scored from biopsy specimens correlated with patient survival in adenocarcinoma of the cervix[39] and with downstaging after preoperative radiotherapy in muscle-invasive bladder cancer.[40] However, other reports examining different tumor types have indicated that high apoptotic index may be predictive of poor response.[41] Therefore, the impact of this particular mechanism may be tumor type-specific. Nonetheless, at least for some types of tumors, pretreatment apoptotic index may be a useful part of a multiparameter patient assessment prior to therapy, especially considering that this index can be easily determined from the biopsy specimen. Thus, pretreatment apoptotic index values may be used to predict radiation response so that the type or extent of therapy can be tailored accordingly.

**Enhanced Tumor Cell Radiosensitivity**

The second of these two mechanisms, ie, intrinsic tumor cell radiosensitivity dictated by apoptosis propensity, may have far greater implications for radiation therapy over the long term. If apoptosis-prone tumor cell types are more radiosensitive than are tumor cell types for which apoptosis is blocked due to the pattern of gene expression, an obvious treatment strategy would be to sensitize the resistant tumors by restoring their apoptotic propensity. This is well within the realm of possibility, and several such strategies are currently under investigation in a number of laboratories.

A few examples based on the mechanisms described in the previous sections are in order: First, for tumors with mutant p53, restoration of wild-type p53 function via adenovirus-mediated gene therapy has been shown to sensitize human colon carcinoma cells to radiation in vitro and in vivo.[42] In these studies, apoptotic propensity was restored to tumor cells that did not display this mode of cell death prior to transfection. The degree of sensitization observed in these studies was substantial, and a large bystander effect seen in the in vivo experiments suggests that not all cells in the tumor have to be infected with the virus to be affected by the treatment.

In another recent report, a gene therapy approach was used in tumors that overexpress bcl-2. In cells treated with adenovirus-mediated expression of bcl-2xS (a functional inhibitor of bcl-2), radiation response and radiation-induced apoptosis were enhanced.[43] Whereas questions about the ultimate success of these strategies must await the results of clinical trials, they are mentioned here to serve as examples of the possible restoration of apoptotic propensity to radiosensitized human tumor cells. These approaches involve strategies that overcome the blocks to apoptosis by intervention at the molecular level. Various other strategies targeting different pathways in apoptosis are currently under development. These include the use of: (1) inhibitors of specific enzymes in the signal transduction pathways[44] that mediate radiation-induced apoptosis and (2) antibodies to block the activity of growth factor receptors that signal the inhibition of apoptosis.[45]

**Conclusions**

Apoptosis appears to play an important role in the response of tumors to radiation at both the cellular and tissues levels. This may occur through two independent mechanisms. First, the spontaneous levels of apoptosis in untreated tumors may indicate high rates of cell loss. This may counterbalance mitotic activity and reduce the number of tumor clonogens, thereby lowering the total dose of radiation needed to sterilize the tumor.

Perhaps more importantly, mounting evidence suggests that tumor cells with apoptotic propensity may be more radiosensitive than are cells that die exclusively by reproductive cell death following irradiation. Cells in the latter category may be resistant to radiation because of blocks to apoptosis.
pathways mediated by patterns of gene expression. Such blocks may be overcome through a variety of approaches, and this possibility has significant implications for radiation therapy. An exciting array of strategies for restoring apoptosis propensity to radioresistant human tumors is currently under development. These strategies use gene therapy vectors, drugs, and biologicals specifically designed to alter biochemical and molecular pathways in the cell that regulate this mode of cell death. If these strategies work clinically to the extent predicted from the preclinical results, their impact on radiation therapy would be profound.


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